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T. Komatsuda · S. Kawasaki · I. Nakamura
F. Takaiwa · F. Taguchi-Shiobara · S. Oka

Identification of random amplified polymorphic DNA (RAPD) markers linked to the *v* locus in barley, *Hordeum vulgare* L.

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Abstract Recombinant backcross lines of barley were produced from a cross between Kanto Nakate Gold (KNG; two-rowed) and Azumamugi (AZ; six-rowed) after backcrosses of F_1 plants with AZ as the recurrent parent. Each of these lines had an introgressed segment from chromosome 2 of KNG. Two recombinant backcross lines, L1 and M3-13, were used for an initial screening of polymorphism. After screening a total of 888 oligonucleotides as arbitrary primers, we identified eight random amplified polymorphic DNAs (RAPDs) between backcross lines and AZ. Among the RAPD fragments, CMNA-38_{700} was linked to the *v* locus with a recombination frequency of zero, while OPJ-09_{850} and OPP-02_{700} were linked to the *v* locus at a map distance of 1.4 cM. Thus, the three RAPD markers were clustered around the *v* locus since the lengths of introgressed chromosomal segments in the L1 and M3-13 lines were no less than 38 cM. The other five RAPD fragments that we identified were not linked to the *v* locus.

Key words Molecular marker · *v* locus (kernel row type) · *Hordeum vulgare* L. · RAPD · Recombinant backcross line

Introduction

At least 40 loci for genes that control morphology have been mapped on chromosome 2 (2H) of barley (e.g. Franckowiak 1995). Among these loci, the complex *v* locus includes the *v*, *Int-d* and *lr* loci which determine the formation of spikes. Cultivated barleys have triple spikelets at each rachis node. The lateral spikelets are female-sterile in two-rowed barleys, while all spikelets are fertile in six-rowed barleys. The formation of the two- and six-rowed spike is controlled predominantly by the *V* and *v* alleles, respectively (Ubisch 1916; Harlan and Hayes 1920; Griffee 1925). Thus, the *V* allele must suppress a certain step in the formation of female lateral flowers. In wild species of the genus *Hordeum* the triple spikelets consist of one central fertile and two lateral female-sterile flowers, and the triple spikelets function as diaspores (Bothmer et al. 1995). The spike is of particular interest in terms of the phylogeny of barley and wild species of *Hordeum*.

The eventual goal of our research is the map-based cloning of genes that encode the *V*-*v* alleles of barley. First, it is necessary, however, to identify and integrate numerous molecular markers that are linked to the *v* locus. The best molecular markers linked to the *v* locus are the RFLP markers identified in an anther culture-derived doubled-haploid population of Igri (two-rowed) × Franka (six-rowed) (Graner et al. 1991, 1994). In the resultant RFLP map, *v* (*hex-v*) co-segregates with MWG801, MWG865, cMWG699, CDO474c (Graner et al. 1994) and also with MWG2211 (A. Graner, personal communication). In a BC_1F_1 population of Azumamugi (six-rowed, recurrent parent) × Kanto Nakate Gold (two-rowed) cross, containing twice as much segregating progeny as the Igri × Franka population, these RFLP loci were well resolved with the exception of the cMWG699 locus and the *v* locus (Komatsuda et al. 1995). The recombination frequency between the *v* and cMWG699 loci was estimated to be 0.1% when about 1000 chromosomes were analysed. Thus, the MWG801, cMWG699, *v* and MWG865 loci were mapped in that order, from proximal to distal, on the long arm of chromosome 2 (Komatsuda et al. 1996).

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T. Komatsuda (✉) · F. Takaiwa · Taguchi-Shiobara · S. Oka
Department of Biotechnology, National Institute of Agrobiological Resources, Kannondai, Tsukuba, 305 Japan

S. Kawasaki
Department of Plant Physiology, National Institute of Agrobiological Resources, Kannondai, Tsukuba, 305 Japan

I. Nakamura
Iwate Biotechnology Research Center, Kitakami, 024 Japan

Recently, random amplified polymorphic DNA (RAPD) has been demonstrated to be a useful genetic marker (Williams et al. 1990). In barley, RAPD markers linked to the barley yellow mosaic disease-resistance gene *ym4* (Ordon et al. 1995), the stem rust-resistance gene *Rpg1* (Horvath et al. 1995) and resistance to net blotch (James et al. 1996), have been identified. We report here the identification of RAPD markers closely linked to the *v* locus. These molecular markers may also be useful for phylogenic analysis of the genus *Hordeum*.

Materials and methods

Plant materials

BC_5F_1 individuals L1, M1, M2, M3, M4 and M7 were produced previously from a cross between Kanto Nakate Gold (KNG; two-rowed) and Azumamugi (AZ; six-rowed) after backcrosses of F_1 plants to AZ as the recurrent parent with selection for the presence of the *V* allele from KNG (Komatsuda et al. 1995). The M1, M2, M3 and M7 plants were again backcrossed with AZ to produce BC_6F_1 plants, while M4 was self-pollinated to produce BC_5F_2 plants. A BC_3F_1 individual #144 from the same backcross, in which recombination had taken place between cMWG699 and MWG865 (unpublished data), was again backcrossed with AZ to produce BC_4F_1 plants.

Isolation of genomic DNA and analysis of markers

DNA was isolated from leaves as described by Dellaporta et al. (1985). Analysis of RFLP markers for the three loci ABG014, MWG2081 and MWG882 was performed as described previously (Komatsuda et al. 1995). Analysis of sequence-tagged-site (STSs) for cMWG699, MWG503, MWG801 and MWG865 loci was also performed as described by Komatsuda et al. (1996). In addition, leaf esterase isozyme was analyzed as described previously (Komatsuda et al. 1993).

Arbitrary primers

We used a total of 200 12-meric oligonucleotides, designated "Common primers" and consisting of CMNA-00 through CMNA-99 and CMNB-00 through CMNB-99 (Bex, Tokyo), and a total of 688 10-meric oligonucleotides primers, corresponding to OPA through OPZ and OPAA through OPAI (Operon Technologies, Alameda, Calif.), for screening of polymorphisms between backcross lines and AZ. The *T*m (melting temperature) of the 12-meric oligonucleotides varied from 25.4 to 45.8°C, as determined from the nucleotide sequences and application of the formula $Tm = (\text{number of } C + G) \times 3.4 + 15.2$ (°C).

Amplification by PCR

For the 12-meric primers, the mixture for PCR (25 µl) contained 5 ng or 25 ng of template DNA, 300 nM primer, 200 µM each of dATP, dCTP, dGTP and dTTP, and 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100 and 0.625 units of *Taq* DNA polymerase (Promega, Madison, Wis.). PCR with 10-meric oligonucleotide primers was based on the protocol described by Williams et al. (1990) with slight modifications. The reactions (15 µl) contained approximately 25 ng of genomic DNA, 330 nM primer, 100 µM each of dATP, dCTP, dGTP and dTTP, and 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100 and 0.375 units of *Taq* DNA polymerase. After an incubation at 95°C for 5 min, the samples were subjected to 45 cycles of 94°C for 1 min, at the annealing temperature (*T*m) for the specific 12-meric primer, or at 36°C for all the 10-meric primers for 2 min, and at 72°C for 2 min. Then extension of the amplified product was allowed to proceed at 72°C for 7 min. The program Temp Control Systems PC-700 and PC-800 (Astec, Tokyo) were used for these amplifications. Amplified fragments were separated by electrophoresis in 1.8% agarose gels in the case of the products obtained with 12-meric primers and in 1.4% agarose gels in the case of the products obtained with the 10-meric primers, and all gels were prepared in 0.5 × TBE (1 × TBE: 89 mM Tris-borate plus 2 mM EDTA). Approximate sizes of amplification products were determined by reference to the electrophoretic mobilities of fragments of *Hae*III-digested ϕX174 DNA.

Localization of polymorphic DNAs within the *v* region

The 20 recombinant backcross lines were used to determine whether the polymorphic DNAs were located on introgressed chromosomal segments. When a line did not generate the polymorphic DNA fragment, the locus of the fragment was taken as being located outside the introgressed chromosomal segment.

Linkage analysis

The segregation data for RAPD markers were combined with the RFLP mapping data obtained previously from a BC_1F_1 population that contained a total of 144 individuals (Komatsuda et al. 1995), and the RAPD markers were then integrated into the RFLP map.

Results

Recombinant backcross lines

Backcrossed and self-pollinated progeny were generated by use of available markers on the long arm of chromosome 2. Ten types of recombinant in the target region were identified among a total of 20 individuals (Table 1). In Table 1, introgressed segments are indicated by the molecular markers at each end, so that crossing-over might occur distal to the two marker loci. The positions of marker loci are shown in Fig. 1.

Genomic DNA from an L1 plant was used for the screening of 12-meric oligonucleotide primers. Subsequently, M3-13 was self-pollinated and genomic DNA from its seven BC_6F_2 progeny, identified as being homozygous for KNG in the chromosomal region between ABG014 and *Est-11*, were pooled (designated M3-13 bulk DNA) and used for the screening of 10-meric oligonucleotide primers.

Identification of polymorphic DNA fragments

A total of 200 12-meric primers were screened for their ability to generate DNA fragments that were

Table 1 Recombinant backcross lines, introgressed segments of the respective lines, and amplification of the polymorphic DNA fragment

Line[a]	Introgressed segment[b]	CMNA-38_{700}	OPJ-09_{850}	OPP-02_{700}	CMNB-21_{1500}	CMNB-21_{700}	CMNA-33_{900}	OPU-16_{400}	CMNA-58_{650}
M2-4	ABG014–MWG2081	−	−	−	−	−	−	−	−
M3-2	ABG014–MWG081	−	−	−	−	−	−	−	−
#144-2	ABG014–cMWG699, *v*	+	+	+	+	−	−	−	−
M3-9	ABG014–MWG865	+	+	+	−	−	−	−	−
M3-14	ABG014–MWG865	+	+	+	+	+	+	+	−
M3-15	ABG014–MWG865	+	+	+	+	+	+	+	−
M4-5	ABG014–MWG865	+	+	+	−	−	−	−	−
M7-14	ABG014–MWG865	+	+	+	−	−	−	−	−
M2-1	ABG014–*Est-11*	+	+	+	−	−	−	−	−
M3-13	ABG014–*Est-11*	+	+	+	+	+	+	+	−
L1	ABG014–*Est-11*	+	+	+	+	+	+	+	+
M1-4	MWG2081–cMWG699, *v*	+	+	+	+	+	+	+	−
M1-2	MWG2081–MWG865	+	+	+	−	−	−	−	−
M2-2	MWG2081–MWG865	+	+	+	−	−	−	−	−
M1-7	MWG2081–*Est-11*	+	+	+	−	+	+	−	−
M2-3	cMWG699, *v*–*Est-11*	+	−	−	−	−	−	−	−
M3-3	cMWG699, *v*–*Est-11*	+	−	−	−	+	+	+	−
M7-8	cMWG699, *v*–*Est-11*	+	−	−	−	−	−	−	−
M3-7	MWG503–*Est-11*	−	−	−	+	+	+	+	−
M7-9	MWG503–*Est-11*	−	−	−	+	+	+	+	−
AZ	Recurrent parent	−	−	−	−	−	−	−	−
KNG	Chromosome donor	+	+	+	+	+	+	+	+

[a] All the lines were BC_6F_1 individuals with the exception of #144-2 (BC_4F_1), M4-5 (BC_5F_2 in homozygous), L1 (BC_5F_1), and both AZ and KNG (parents)

[b] Identified by molecular and isozyme markers. For marker locations, refer to the genetic map in Fig. 1. Markers on the chromosomal segments distal to *Est-11* were not analyzed in this study so that regions of cross-overs were unknown there

polymorphic between AZ, L1 and KNG. L1 was heterozygous with respect to the *v* region so that we identified polymorphic DNA fragments that were dominant to the alleles of KNG. The screening was performed twice for all the 12-meric primers, using 5 ng and 25 ng of template DNA per reaction, since amplification of DNA that is insensitive to the concentration of template DNA is required for the analysis of numerous samples, such as numbers of segregating populations. CMNA-33 (5′ GACTGCTATACA 3′) generated four or five amplified products, among which a 900-bp fragment was polymorphic. The primer functioned efficiently with 25–50 ng of genomic DNA per 25 µl of reaction mixture, but did not function with 5 ng of genomic DNA. CMNA-38 (5′ ATCTTCTCATCT 3′) generated a discrete polymorphic fragment of 700 bp with 5–50 ng of genomic DNA per reaction, being basically insensitive to the concentration of template DNA. Further analysis revealed that 35–37 cycles were sufficient for amplification with this primer, and the standard 45 cycles resulted in a smeared profile of DNA products or in the disappearance of the 700-bp fragment. An annealing temperature of 27°C resulted in a more-intensive product band than that produced at 29°C, without any loss of specificity. The pattern of amplification with the CMNA38 primer is shown in Fig. 2. CMNA-58 (5′ GTCATGCCTGGA 3′) generated several amplified fragments, among which a 650-bp fragment was polymorphic. The primer operated efficiently with 5–100 ng of genomic DNA, an indication of insensitivity to the concentration of template DNA. CMNB-21 (5′ TATTGGGATTGG 3′) generated fragments of 700 bp and 1500 bp. Amplification with this primer required 100–200 ng of template DNA per tube for the generation of both fragments; when 50 ng of DNA or less was applied, the product bands were less discrete and less reproducible. Thus five polymorphic fragments were identified. These polymorphic fragments were designated CMNA-33_{900}, CMNA-38_{700}, CMNA-58_{650}, CMNB-21_{700} and CMNB-21_{1500} by reference to the convention used by Paran et al. (1991). In addition to these primers, some primers generated DNA fragments that were present in L1 only and absent in AZ and KNG. Such fragments might have been heteroduplexes, as reported by Davis et al. (1995). These primers or DNA products were not included in further studies.

We tested 688 10-meric primers for polymorphisms with the M3-13 bulk DNA and AZ. The M3-13 bulk DNA was homozygous with respect to the *v* region. Thus polymorphic DNA fragments that were dominant with respect to the alleles of either KNG or AZ were identified by the screening. The first screening was performed with all 688 primers, and then screening was replicated three more times with the elimination of primers that generated monomorphic fragments. As a result OPJ-09 (5′ TGAGCCTCAC 3′), OPP-02 (5′ TCGGCACGCA 3′) and OPU-16 (5′ CTGCGCTGGA

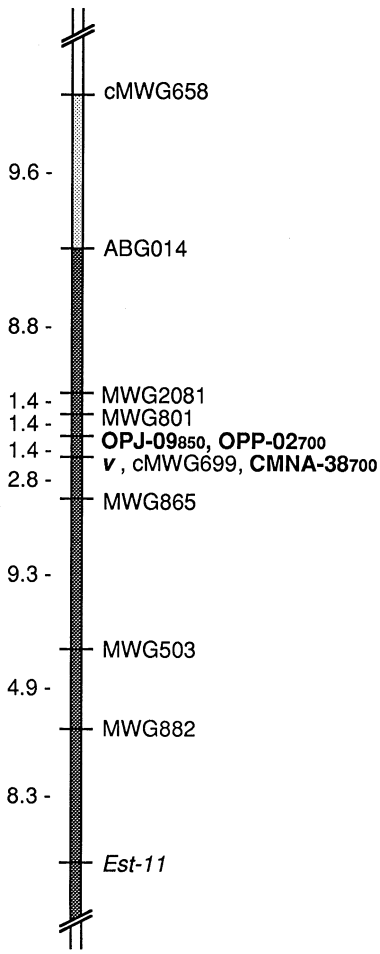

Fig. 1 Genetic map of the *v* region of barley on the long arm of chromosome 2. The upper part is proximal and the lower part is distal. A chromosomal segment introgressed from KNG to L1 and M3-13, which were used for screening of primers, is shown as a *heavily shaded region*, and a segment in which a cross-over occurred is shown as a *lightly shaded region*. Markers on the chromosomal segments distal to *Est-11* were not analyzed in this study so that regions of cross-overs are unknown. RAPD markers identified in this study, namely, CMNA-38_{700}, OPJ-09_{850} and OPP-02_{700}, were mapped close to the *v* locus on the long arm of chromosome 2. A population of 144 BC_1F_1 individuals that had previously been analysed for RFLP mapping (Komatsuda et al. 1995) was used for the mapping of the RAPD markers. Thus, one recombination event was equivalent to 0.7 cM

3′) were identified as primers that generated polymorphic DNA fragments. All the fragments were generated with M3-13 bulk DNA, not with AZ DNA, being dominant to the alleles of KNG. These polymorphic fragments were designated OPJ-09_{850}, OPP-02_{700} and OPU-16_{400}. Patterns of amplification with primers OPJ-09 and OPP-02 are shown in Fig. 2. OPJ-09 generated double bands of fragments of approximately 850 bp with KNG DNA, and the lower band was identical to the band generated with OPJ-09_{850} and M3-13 bulk DNA. No further optimization of conditions for PCR was attempted with these primers.

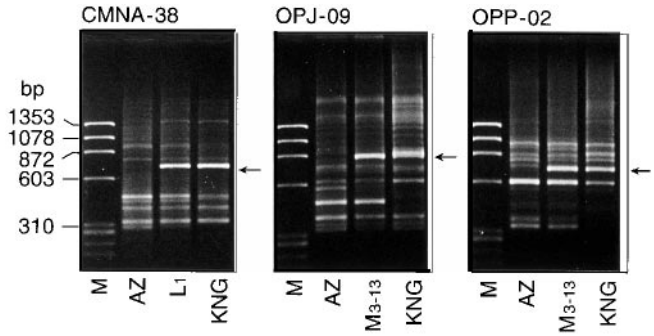

Fig. 2 RAPD markers genetically linked to the *v* locus. Amplification by PCR was performed using a 12-meric oligonucleotide primer (CMNA-38) or a 10-meric oligonucleotide (OPJ-09 and OPP-02). In each panel, *the first lane* (*M*) represents size markers (fragments of ϕX174 digested with *Hae*III), *the second and fourth lanes* represent parental DNA from Azumamugi (*AZ*; six-rowed) and Kanto Nakate Gold (*KNG*; two-rowed), and *the third lane* in each panel represents DNA from L1 for the CMNA-38 primer, or M3-13 bulk DNA for the OPJ-09 and OPP-02 primers. Each RAPD marker exhibits a polymorphism between AZ and KNG, being dominant to the KNG allele (*arrows*). The positions of the three markers on the molecular map are shown in Fig. 1

Localization of RAPD markers on the introgressed chromosomal segments

The genetic localization of polymorphic fragments was performed using the 20 recombinant backcross lines. Some of these lines had identical genotypes for marker loci (e.g. M3-9, M3-14, M3-15, M4-5 and M7-14). However, it was possible that they had different cross-over positions (e.g. between MWG865 and MWG503 for the five lines). Therefore all the lines were used for the analysis. The results are summarized in Table 1. The CMNA-38_{700} fragment was generated with both M1-4 and M2-3 DNA (as well as with M3-3 and M7-8 DNA), so that it was clear that CMNA-38_{700} was located on the chromosomal region between MWG801 and MWG865. This conclusion was consistent with the evidence that the CMNA-38_{700} fragment was generated with DNA from all the other lines that had the chromosomal segment of KNG in this region. The CMNA-38_{700} fragment was not generated with M2-4, M3-2, M3-7 or M7-9 DNA, and each of these lines lacked that segment. From a similar analysis, OPJ-09_{850} and OPP-02_{700} appeared to be located on a chromosomal segment between MWG801 and cMWG699.

Amplifications of CMNB-21_{1500}, CMNB-21_{700}, CMNA-33_{900} and OPU-16_{400} were correlated with one another (Table 1), indicating the possible linkage of these fragments. In particular, the results for the amplification of CMNB-21_{700} and CMNA-33_{900} coincided, indicating that these two polymorphic fragment were located on an identical introgressed segment. However, the four markers were apparently localized outside the region between ABG014 and *Est-11*. CMNA-58_{650} was

not generated in any of the recombinant backcross lines apart from L1, an indication that this polymorphic fragment was genetically independent of any of the RFLP and RAPD markers that we used.

Genetic mapping of the RAPD markers

The polymorphic CMNA-38_{700}, OPJ-09_{850} and OPP-02_{700} fragments were genetically dominant markers. In other words, the alleles of KNG were dominant over the alleles of AZ. Thus, localization of these RAPD markers could be determined with a BC_1F_1 population produced from the cross $AZ \times (AZ \times KNG)$, in which RFLP mapping around the *v* locus had already been performed (Komatsuda et al. 1995). A genetic map, including the RAPD markers, is shown in Fig. 1. The BC_1F_1 progeny did not show any evidence of recombination between CMNA-38_{700} and the *v* locus, indicating that the two loci were closely linked to each other. OPJ-09_{850} and OPP-02_{700} co-segregated, with no recombination, and were localized between MWG801 and the *v* locus. CMNA-33_{900}, CMNB-21_{1500} and CMNB-21_{700} were also tested with the same BC_1F_1 population. Their linkage to the *Est-11* locus was not significant, indicating that the markers were located either on the rather distal part of the long arm of chromosome 2 or on some segments that had introgressed to other chromosomes (data not shown).

Discussion

Screening of recombinant backcross lines for the *v* locus allowed the identification of three RAPD markers. These markers were more closely linked to the *v* locus than any other RFLP markers used in this study with the exception of cMWG699. However, CDO474c, located at the same site on the map of Graner et al. (1994), was not included in the present study. The three RAPD markers, namely CMNA-38_{700}, OPJ-09_{850} and OPP-02_{700}, gave reproducible and discrete results. Thus, they are useful for the high-resolution mapping of the *v* locus. The three RAPD markers were, however, genetically dominant. Thus it would be better to convert them to sequence-characterized amplified regions (SCARs) since alleles of both parents were amplified in some cases (Paran and Michelmore 1993). Co-dominant markers allow a complete classification of segregating F_2 populations, decreasing the standard errors of recombination frequencies (Allard 1956). Co-dominant SCARs might also be useful for phylogenetic analysis of barley and related wild species since the PCR-based STS polymorphisms of cMWG699 were strongly correlated with the kernel row-type in many barley cultivars (Tanno et al. 1996). Such a conversion study is in progress.

AZ is a typical Japanese six-rowed cultivar, produced from a cross between Japanese landraces Tochigi Sekitori 1 (a single isolate from Sekitori) and Torano-o Sai 1 (a single isolate from Torano-o). Thus, AZ has morphological characteristics of typical Japanese landraces, having a six-rowed spike, being semi-brachytic, and having a vernalization requirement. KNG is a two-rowed, spring-type cultivar. Theoretically, 75% of its a genetic background is from a European malting cultivar Golden Melon and 25% is from a Japanese cultivar Shikoku, as deduced from their pedigrees (Nakane et al. 1985). Golden Melon is a classical malting barley cultivar of northern European origin, having two-rowed erectum-type ears and a spring-type habit. It was introduced from Hay-Harper, Leith, Scotland, through the United States, to Japan in 1881 (Takahashi 1980). Shikoku is a Japanese landrace (Takahashi et al. 1983), similar to AZ. KNG retains the *v* region from Golden Melon, so that a relatively high degree of polymorphism could be expected between KNG and AZ in the *v* region. In fact, out of 17 RFLP loci around the *v* locus that were tested for polymorphism between the two cultivars, 13 clones (MWG557, cMWG658, MWG2081, MWG801, cMWG699, MWG865, MWG 503, MWG892, MWG876, MWG581, MWG882, ABC162 and ABG014) revealed polymorphisms with at least one of the five restriction enzymes used, and only four (MWG520, MWG065, MWG2058 and MWG2123) did not (unpublished data). These four loci were rather distant from the *v* locus (Graner et al. 1994). From the RFLP polymorphisms identified in our previous experiments, we expected to identify many RAPD markers around the *v* locus.

The recombinant backcross lines used for screening of the primer, namely, L1 and M3-13, had in common an introgressed segment of 38.3 cM between ABG014 and *Est-11*, as well as additional chromosomal regions distal to *Est-11* (Fig. 1) . Thus, we expected to identify RAPD marker loci that would be distributed throughout the segment. However, such was not the case. Three RAPD markers identified in this study were located within a chromosomal segment of 1.4 cM between the MWG801 and *v* loci, which covers only 4% or less of the total length of the introgressed segment. The three RAPD markers were, thus, clustered around the *v* locus, an indication that, within the introgressed segment, the *v* region contains the DNA sequences that are most divergent between two-rowed and six-rowed barleys. Another possible reason for the clustering of the RAPD markers is the lower frequency of recombination in proximal regions, which is a general feature of barley chromosomes (Laurie et al. 1993; Leitch and Heslop-Harrison 1993; Pedersen et al. 1995). This issue requires clarification by in situ hybridization and large-fragment Southern-blot hybridization, as well as by the use of different cross combinations. However, the lower frequency of recombination in proximal regions is not a phenomenon typical of crosses between two-rowed

and six-rowed lines, because the distance between ABG014 and MWG503 was 25.1 cM for the AZ × KNG cross, which was consistent with the 23.1 cM obtained for the Steptoe (six-rowed) × Morex (six-rowed) cross (Kleinhofs et al. 1994). The recombination frequency around this region was also the same in the Harrington (two-rowed) × TR306 (two-rowed) map (Kasha et al. 1994). However in the Igri (two-rowed) × Franka (six-rowed) map, the *v* (*hex-v*) locus co-segregated with MWG801, MWG865, cMWG699 and CDO474c (Graner et al. 1994) and, in addition, with MWG2211 (A. Graner, personal communication). The reduced frequency of recombination of the molecular markers around the *v* locus might be due to the use of a rather small mapping population, which contained 71 doubled haploid lines, or to factors related to the selection of microspores and/or plantlets through anther culture for production of the lines.

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