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ORIGINAL PAPER

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Efficient fine mapping of the naked caryopsis gene (*nud*) by HEGS (High Efficiency Genome Scanning)/AFLP in barley

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Abstract The hulled or naked caryopsis character of barley (*Hordeum vulgare* L.) is an important trait for edibility and to follow its domestication process. A single recessive gene, *nud*, controls the naked caryopsis character, and is located on the long arm of chromosome 7H. To develop a fine map around the *nud* locus efficiently, the HEGS (High Efficiency Genome Scanning) electrophoresis system was combined with amplified fragment length polymorphism (AFLP). From bulked segregant analysis of 1,894 primer combinations, 12 AFLP fragments were selected as linked markers. For mapping, an F_2 population of 151 individuals derived from a cross between Kobinkatagi (naked type) and Triumph (hulled type) was used. Seven AFLP markers were localized near the *nud* region. A fine map was developed with one-order higher resolution than before, along with the seven anchor markers. Among the seven linked AFLP markers (KT1–7), KT1, KT2 and KT6 were co-dominant, and the former two were detected for their single-nucleotide polymorphisms (SNPs) in the same length of fragments after electrophoresis with the non-denaturing gels of HEGS. The *nud* locus has co-segregated with KT3 and KT7, and was flanked by KT2 and KT4, at the 0.3-cM proximal and the 1.2-cM distal side, respectively. Four of these AFLP markers were converted into sequence-characterized amplified region (SCAR) markers, one of which was a dominant marker co-segregating with the *nud* gene.

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Introduction

In cultivated barley (*Hordeum vulgare* L. subsp. *vulgare*), hulled vs naked caryopsis is one of the most important agronomic traits because of the direct link to its edibility. Most cultivars have the hulled caryopsis; the husks adhere to the grain, and are called hulled barley. Naked barley has a caryopsis with easily separable husks upon threshing. The naked caryopsis character of seeds is controlled by a single recessive gene *nud*. Hulled barley is mainly used for animal feed and brewing malts, while the naked one can be used as human food. Naked barleys are often found in East Asia, as human staples, but are absent or scarcely found in other parts of the world. Recently, naked barley is attracting attentions as feed and healthy food, because of its high feed-value and abundance of dietary fibers, respectively (Liu et al. 1996). Thus, there will be a prospect for the breeding of naked barley.

The hulled or the naked-caryopsis character can be a suitable trait to follow the origin and domestication process of cultivated barley. All the earliest domesticated barleys, cultivated in the Near East about 8000 B.C., were hulled-type, and the naked-type appeared by about 6500 B.C. (Zohary and Hopf 2000). Accessions of wild barley *H. vulgare* subsp. *spontaneum*, the ancestor of cultivated barley, are all of the hulled type. These facts imply that naked barley appeared after domestication of hulled barley. However, no systematic studies on the origin of naked barley have been conducted, and no suitable fine maps around the *nud* locus have been available. The *nud* gene is located on the long arm of the chromosome 7H (Scholz 1955; Fedak et al. 1972). Linkage maps including the *nud* gene have been constructed based on morphological markers (Franckowiack 1996) and molecular markers (Becker and Heun 1995; Qi et al, 1998; Costa et al. 2001), but the resolution of them was not sufficient

as fine maps due to the low density of the markers used. Therefore, development of a fine map with numerous markers in a large segregating population is indispensable for high-resolution analysis of the *nud* locus. Such markers will be useful not only for breeding but also for studies on the origin of naked barley. We are also considering the positional cloning of the *nud* gene to elucidate the mechanism of caryopsis control.

To construct a fine map, we applied the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) combined with the High Efficiency Genome Scanning (HEGS) system (Kawasaki and Murakami 2000; Kawasaki et al., in press). This high-throughput electrophoresis system enabled efficient development of a fine map around the *nud* locus in this study. Furthermore, four selected AFLP markers near the *nud* locus were converted into sequence-characterized amplified region (SCAR) markers (Paran and Michelmore 1993).

Materials and methods

Plant materials

A segregating F_2 population of 151 individuals derived from the cross Kobinkatagi × Triumph was used for mapping. Kobinkatagi is a Japanese six-rowed naked type cultivar, and Triumph is a European two-rowed hulled type cultivar. Genotypes of hulled F_2 individuals were determined by the progeny test employing F_3 families comprising about 20 individuals. Two sets of wheat-barley whole chromosome addition lines (Islam et al. 1981; Taketa and Takeda 2001) and ditelosomic addition lines for chromosome arms 7HL and 7HS (Islam 1983), were used to confirm chromosomal location of the molecular markers. Barley chromosome nomenclature follows Linde-Laursen et al. (1997).

HEGS/AFLP analysis

Template DNA was extracted from the parents and F_2 individuals according to the method of Komatsuda et al. (1998). To identify the molecular markers closely linked to *nud*, we employed bulked segregant analysis (Michelmore et al. 1991) and the AFLP technique (Vos et al. 1995). To achieve the needed efficiency for electrophoresis, the high-efficiency genome scanning (HEGS) system (Kawasaki and Murakami 2000; Kawasaki et al., in press) was adopted and a set of electrophoresis apparatus equipped with two sets of 24.5×26.5-cm glass plates, each accommodating a gel with 100 lanes, was used. In this system, analysis of 200 samples is practicable in a single run.

For the first screening, the same amounts of DNAs from eight homozygous F_2 individuals were pooled to create hulled (NN) and naked (nn) bulks, respectively. For the second screening, three sets of NN and nn bulks, each pooling DNAs from four homozygous F_2 individuals of the respective genotype. DNA was double-digested with *Eco*RI and *Mse*I. The adapter-ligated DNA was pre-amplified with *Eco*RI and *Mse*I primers which have no selective nucleotide. The selective-amplification was performed with combinations of *Eco*RI and *Mse*I primers having three selective nucleotides at the 3′ end. Amplified products were separated by electrophoresis through 13% non-denaturing polyacrylamide gels and visualized after staining with Vistra Greeen (Amersham Pharmacia Biotech). Gel images were scanned with the FMBIOII Multi-View (HITACHI).

PCR analysis of anchor markers

For anchor markers, we selected five simple-sequence repeat (SSR) markers, Bmag0120, Bmag0341, Bmag0507, EBmac0764 and EBmac0785 (Ramsey et al. 2000), and three cleaved amplified polymorphic sequence (CAPS) or sequence-tagged site (STS) markers derived from RFLP markers CDO673 (Heun et al. 1991), MWG2031 and Ris15 (Künzel et al. 2000), mapped on chromosome 7H. Sequences of the RFLP probes were obtained from the Grain-Genes databases. For CDO673, we designed PCR primers *5′-CTCACGCTTAAGCTCTCGCT-3′* and *5′-CGAGAGGATCCAGTT-CTTG-3′*. For MWG2031, we combined our reverse primer *5′-TACGTCGGCATAATTGGCA-3′* with the forward primer MWG2031T7 developed by Sayed-Tabatabaei et al. (1998). For Ris15, the PCR primers published by Künzel et al. (2000) were used. The PCR products of CDO673 and MWG2031 were digested with *Tsp*509I and *Mwo*I, respectively, for detection of polymorphisms. In Ris15, polymorphism was detected without digestion.

Conversion of HEGS/AFLP markers to SCAR markers

AFLP markers of interest were excised directly from the 1-mm-thick polyacrylamide gels. AFLP fragments were eluted from the gel slices using a Dialysis Membrane (Wako Chemicals) and re-amplified using the corresponding selective primers with the following cycling parameter: 4 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 56°C, 1.2 min at 72°C; 7 min at 72°C; and hold at 4°C. PCR products were electrophoresed on 2% agarose gels. The bands were excised from agarose gels and cloned with the TOPO TA Cloning Kit (Invitrogen). For screening of colonies, PCR was performed on 10–20 white colonies using the corresponding selective primers. These PCR products were digested with four restriction enzymes and resolved on 2% agarose gels. The colonies representing the majority banding pattern were selected for sequencing. Primers were designed by using the software Primer3 (Whitehead Institute, Cambridge, Mass.) on the basis of the sequences of the cloned bands.

Construction of the linkage map

Linkage analysis was performed with the HEGS system using 151 F_2 individuals. Recombination values were calculated by MAPMAKER Version 2.0 (Lander et al. 1987) and a genetic linkage map was constructed based on LOD scores greater than 3.0. Map distances were calculated using the Kosambi function (Kosambi 1944).

Results

Bulked segregant analysis

To identify closely linked markers to the naked caryopsis gene *nud* in high efficiency, bulked segregant analysis was performed using the HEGS/AFLP system (Kawasaki and Murakami 2000; Kawasaki et al., in press). As the first screening, a total of 1,894 primer combinations were examined with an inexpensive apparatus which accommodates 200 samples at a time. The thick (1-mm) gel was made easy to handle the gels and the detection of bands. The large genome size of barley posed no problem for detecting about 35 bands per lane. As shown in Fig. 1, the bands coincident with the types of three bulks of NN or nn were selected as candidates linked to *nud* (Fig. 1A). Then in the second screening, selected bands were analyzed with 24 separated F_2 individuals from the six bulks and other two

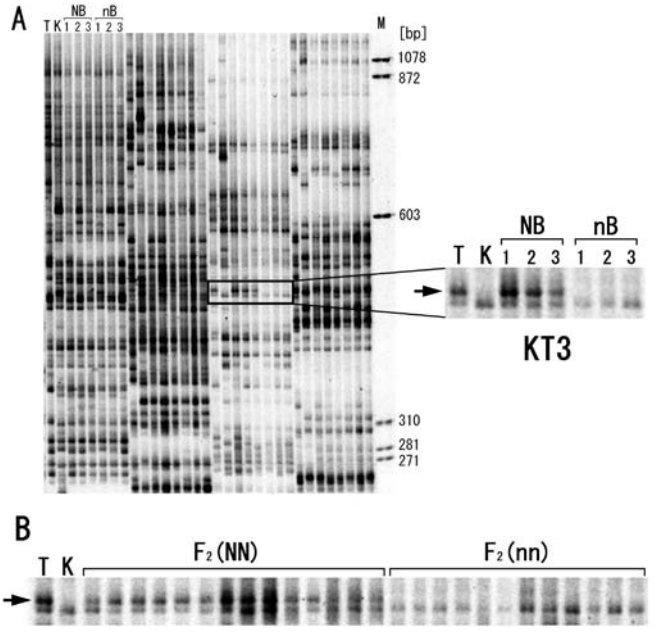

Fig. 1A, B HEGS/AFLP analysis of naked-linked markers with F_2 bulks and individuals. **A** Band patterns of parents Triumph (*T*: hulled) and Kobinkatagi (*K*: naked), and F_2 bulks of hulled (NB1,2,3) and naked (nB1,2,3) homozygous individuals. *M*: size marker of ϕx174*Hae*III digest**. B** An example of naked-linked marker candidate, KT3 (*arrow*: in **A** inlet, **B**) was analyzed for F_2 homozygous individuals

hulled F_2 individuals (Fig. 1B). In this way, a total of 12 AFLP markers were selected. Of these, eight were dominant and four were co-dominant. Of the eight dominant markers, six were derived from the hulled parent, Triumph, and two were from the naked parent, Kobinkatagi.

Construction of the linkage map

Of the 12 AFLP markers selected by the bulk segregant analysis, seven easily distinguishable markers were used for linkage analysis with 151 F_2 individuals, which was also done with the HEGS system. Their primer combinations are shown in Table 1. Three CAPS/STS markers (CDO673, MWG2031 and Ris15) and four SSR markers (Bmag0120, Bmag0341, Bmag0507 and EBmac0764) were included in the analysis as anchor markers. No segregation distortion was detected for *nud* or all markers used for mapping. The resultant linkage map is shown in Fig. 2. Six of the seven AFLP markers were mapped between MWG2031 and EBmac0764, the region where no close markers to the *nud* has been available. The *nud* locus has co-segregated with the AFLP markers KT3 and KT7, and was mapped between two flanking markers KT2 and KT4, at 0.3-cM proximal and at the 1.2-cM distal side to the *nud*, respectively. Thus, more than one-order finer map than before was constructed.

Analysis with wheat-barley ditelosomic addition lines revealed that all four SSR markers in the AFLP map were

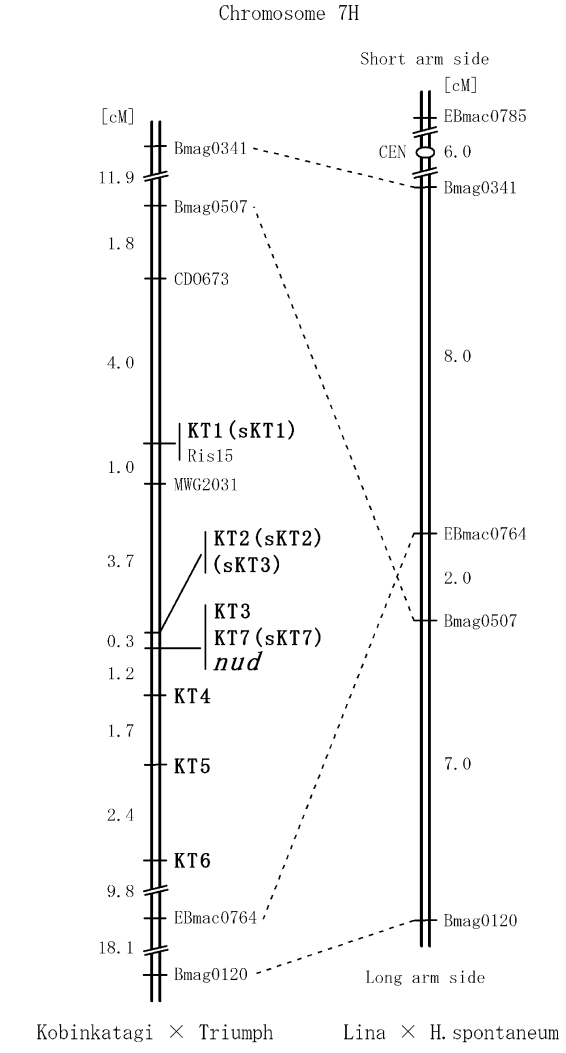

Fig. 2 Comparison of barley chromosome 7H linkage maps around the *nud* locus. Left is the present HEGS/AFLP map and right is the SSR-based one (Ramsey et al. 2000). The newly developed close HEGS/AFLP markers are termed as *KT*, and SCAR markers converted from HEGS/AFLP markers are termed as *sKT* in parentheses. *Broken lines* connect the common SSR markers. *CEN* indicates the approximate centromere position

located in the 7HL arm. Examination of another SSR marker EBmac0785 revealed its location in the 7HS arm. Thus, the centromere position on the SSR-based chromosome 7H map (Ramsay et al. 2000) was estimated to be in the interval flanked by the most proximal 7HL marker Bmag0341 and the 7HS marker EBmac0785.

Conversion of HEGS/AFLP markers to SCAR markers and linkage analysis

Although AFLP markers KT1 and KT2 were both co-dominant, with different mobilities for the parents in the non-denaturing polyacrylamide gel, sequence analysis revealed that their fragment lengths were the same between the parents, 475 bp for KT1 and 350 bp for

Table 1 AFLP markers near the nud locus. KT1 and KT2 lengths are exactly the same for both parents, but are resoluted as co-dominant markers on 13% non-denaturing polyacrylamide gels, reflecting the differences in higher structures in condition. T and K indicate that the AFLP fragments were derived from Triumph (N-associated) and Kobinkatagi (n-associated), respectively

AFLP marker	Primer combination	Marker type[a]	Approximate length of the AFLP fragment (bp)[b]
KT1	E-AGC/M-ATT	Co-dominant	475*
KT2	E-AGG/M-ATA	Co-dominant	350*
KT3	E-AAG/M-CAG	Dominant (N)	497*
KT4	E-AGT/M-ACC	Dominant (N)	860
KT5	E-AGA/M-CGA	Dominant (N)	600
KT6	E-CGG/M-ATT	Co-dominant	630 (T), 615 (K)
KT7	E-AAC/M-CTC	Dominant (n)	665*

[a] (N): N-associated, (n): n-associated
[b] * Marked lengths are determined by sequence analyses

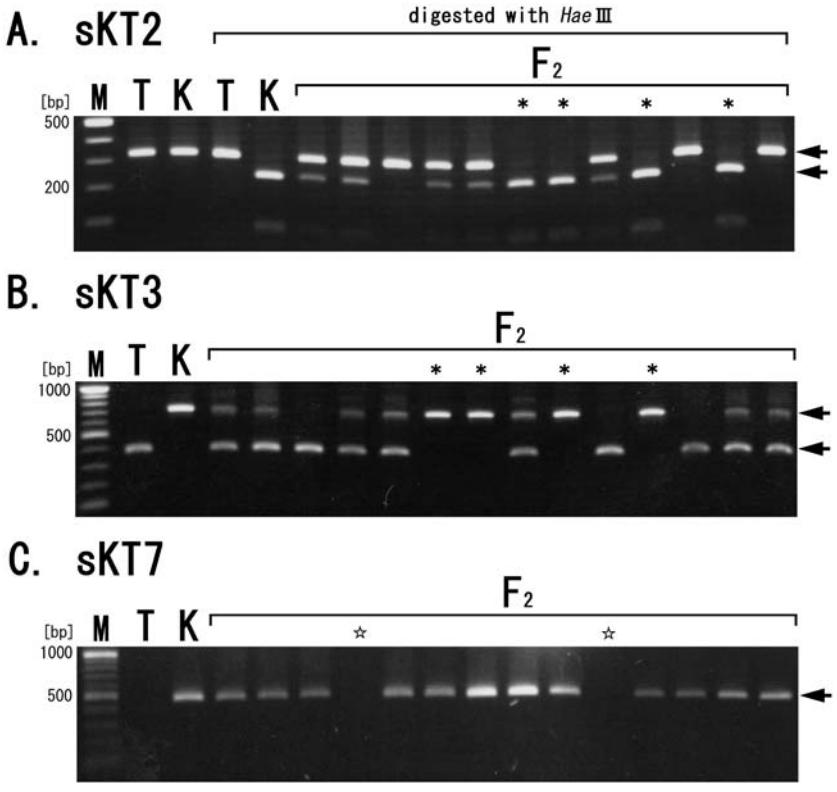

Fig. 3A–C Linkage analysis using SCAR markers sKT2, sKT3 and sKT7. **A** Co-dominant SCAR marker sKT2 (*arrow*) digested with *Hae*III. *T*: Triumph, naked, *K*: Kobinkatagi, hulled, and F_2 individuals of the cross. **B** Polymorphism of co-dominant SCAR marker sKT3 (*arrow*) was obtained without digestion. Recessive homozygous (nn) individuals in *nud* are marked by *. **C** Dominant SCAR marker sKT7 was indicated by an *arrow*. Dominant homozygous (NN) individuals in *nud* are marked by *. *M*: 100 bp ladder-size marker

KT2. Furthermore, in KT1, two fragments derived from the parents had seven single-nucleotide polymorphisms (SNPs), and showed 98.4% homology after removal of adapter sequences. In KT2, both fragments had two SNPs, and showed 99.4% homology after removal of adapter sequences. Thus, HEGS is detecting SNPs through their higher structures, and comparison of their inner sequences of KT1 and KT2 revealed *Hin*fI and *Hae*III sites only in Kobinkatagi, respectively. Therefore, primer pairs flanking the polymorphic restriction sites were designed for both markers to construct SCAR markers sKT1 and sKT2. An sKT2, a single fragment of 326 bp in Triumph, was digested to 238 bp and 88 bp by *Hae*III, in Kobinkatagi (Fig. 3A). SCAR markers sKT1 and sKT2 have co-segregated with their respective original AFLP markers.

AFLP marker KT3 (494 bp) was an N-associated dominant marker, co-segregating with the *nud*. A primer pair designed from the inner sequence of KT3 amplified the expected 372-bp of the fragment from Triumph, but also amplified a 710-bp fragment from Kobinkatagi (Fig. 3B). Sequence analysis of these fragments revealed that the Kobinkatagi fragment had one major insertion of about 300 bp in its middle region, and that the rest of the region, excluding primer sequences, shared 95.5% homology. Thus, these bands were considered allelic and mapped at 0.3-cM proximal to *nud* as the co-dominant SCAR marker sKT3. The slight difference between mapping positions of KT3 and sKT3 is due to the detection of one recombinant plant by sKT3.

AFLP marker KT7 (665 bp) was an n-associated dominant marker, co-segregating with the *nud*. A primer pair designed from the inner sequence amplified the 467 bp of the fragment from Kobinkatagi as expected (Fig. 3C). This band was mapped to the same position as the KT7 and *nud* locus, and named as sKT7.

Table 2 SCAR markers derived from HEGS/AFLP markers

Marker	Marker type	Primer	PCR product length (bp)[a]	Restriction enzyme[b]	After digestion (bp)[c]
sKT1	Co-dominant	*5′-TGGACCTCATAGCAGCCTTT-3′* *5′-GGTGCCACTGAGATTCACCT-3′*	430	*Hinf*I	327+88+15 (T) 296+88+31+15 (K)
sKT2	Co-dominant	*5′-AATTCAGGCCCAAAGGTAGTG-3′* *5′-TAAATATGTAGTCAAGTGTAGACTGG-3′*	326	*Hae*III	326 (T) 238+88 (K)
sKT3	Co-dominant	*5′-TGCGCTAGTCATAGACATCA-3′* *5′-CAGCCTCTAATGGATCAGTC-3′*	372 (T) 710 (K)		
sKT7	Dominant n-associated	*5′-CGATATGCTTGTCCGTGATG-3′* *5′-TGCTCGTACTGCATCGACTC-3′*	467 (K)		

[a, c] T and K indicate the types derived from Triumph (hulled) and Kobinkatagi (naked), respectively
[b] Restriction enzymes used to detect the polymorphisms

Primer sequences for SCAR markers converted from AFLP markers are shown in Table 2.

Discussion

Bulked segregant analysis (Michelmore et al. 1991), combined with the AFLP technique (Vos et al. 1995), has been used as an efficient way to identify molecular markers closely linked to the target genes in barley (Simons et al. 1997), rice (Murai et al. 2001), sorghum (Wen et al. 2002) and potato (Meksem et al. 1995). Therefore, we adopted the same approach to identify AFLP markers closely linked to the *nud* locus of barley in this study. Furthermore, the HEGS electrophoresis system was employed for analyzing a large number of primer combinations efficiently. A total of 1,894 primer combinations were screened and 12 markers were selected. Among them, five close-linked and two co-segregating AFLP markers were mapped around the *nud* locus, a region that is poor in markers. The *nud* gene was mapped within the 1.5-cM interval flanked by two AFLP markers, and with two co-segregating AFLP markers. This proves the high efficiency and effectiveness of the HEGS system in combination with bulked segregant analysis.

Of the seven AFLP markers mapped, three (KT1, KT2 and KT6) were co-dominant markers, showing a different mobility between the parents in the non-denaturing polyacrylamide gel. However, KT1 and KT2 fragment lengths were the same between the parents, although there were seven and two SNPs in their inner sequences, respectively. Thus, the HEGS has the ability to detect some SNPs of the markers through their higher structures in the non-denaturing gels.

The anchor markers included in this mapping allowed comparison of our map with those reported previously. The order of Bmag0507 and EBmac0764 was reversed in our map, in contrast to that reported by Ramsey et al. (2000), and their distance was expanded to 25.9 cM from 2.0 cM (Fig. 2). MWG2031 and Ris15, co-segregating in the map of Graner et al. (1994), were separated to 1.3 cM in our map (Fig. 2). The physical mapping by Künzel et al. (2000) also separated MWG2031 from Ris15 with one translocation breakpoint, but in a reverse way to ours. These discrepancies may be due to either local structural changes of chromosomes or mapping errors. Ramsay et al. (2000) used 86 double-haploids (DHs) of the cross Lina × *H. spontanium* Canada Park, and Graner et al. (1994) used 71 DHs of the cross Igri × Franka.

Becker and Heun (1995) and Qi et al. (1998) mapped the *nud* gene in the cluster of markers near the putative centromere region. However, in our map, *nud* is apparently distant (>23 cM) from the centromeric region (Fig. 2). This is in accordance with Künzel et al. (2000) and Serizawa et al. (2001), who mapped the MWG2031, which is proximal to *nud* in our map, within the distal 60% and 53% regions of the long arm of chromosome 7H, respectively, based on their physical mapping system using translocations and deletions. Therefore, we are rather optimistic about the possibility of cloning the *nud* gene by positional cloning, even in the large barley genome. Probably the cross of Kobinkatagi × Triumph may be one of the very suitable ones for fine mapping of the *nud* gene, because of a rather high recombination rate around the gene.

We could select AFLP markers closely linked to *nud* and could convert some of them into co-dominant or dominant SCAR markers. These markers are very useful for further high-resolution analysis of the *nud* gene, because recombinants between the markers can be easily selected from the large F_2 population at the seedling stages, although the naked caryopsis character is recessive and only distinguishable at the late stage of the ripening period. They should also assist introgression of the *nud* gene into the genetic background of elite hulled barley cultivars through repeated backcrosses. PCR-based selection of plants carrying the *nud* allele at the seedling stage should greatly enhance selection efficiency in such backcross breeding. These markers can be used also for examination of the allelic diversity near the *nud* locus in many barley accessions around the world, to elucidate the origin of naked barley. The nearest SCAR marker sKT7 was a dominant marker associated with the *nud* allele. Because of a higher information content of co-dominant markers, development of co-dominant markers around the *nud* locus is intended by further screening of primer combinations, or through the analysis of the marker-hitting BAC clones.

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