Construction and Characterization of a Bacterial Artificial Chromosome (BAC) Library from the Japanese Malting Barley variety ‘Haruna Nijo’

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Cultivated barley (Hordeum vulgare L.) is well known as one of the most widely cultivated crops in the world and as an extensively studied plant species in the field of genetics. In recent years, despite its very large genome size (ca. 5.000 Mb), the research resources needed for barley genomic studies have become available, including a large number of expressed sequence tags (ESTs). These have been widely used for barley genome analyses, such as DNA marker-generation and the construction of microarrays. However, the availability of a large-insert genomic library, which is also essential for genomic studies, has been relatively limited in the barley research community. We described here the construction and characterization of a barley bacterial artificial chromosome (BAC) library, using the Japanese malting barley variety ‘Haruna Nijo’. The BAC library consisted of 294,912 clones arrayed in 768 384-well microtiter plates. The average size of each cloned insert was estimated to be 115.2 kb, with approximately 0.5% of the clones lacking inserts. Chloroplast DNAs were present in about 1.7% of the library. Thus, the genomic coverage of the ‘Haruna Nijo’ BAC library was estimated to be about 6.6 genome-equivalents. In order to rapidly identify specific BAC clones, we developed a screening scheme that combined PCR analysis of pooled BAC DNAs with colony hybridization. Using this screening scheme, we investigated the genomic coverage of this BAC library, using 13 locus-specific ESTs and a sequence-tagged site marker. By screening the whole library with individual markers, we identified an average of 5.1 clones per marker. This screening scheme also enabled us to rapidly construct a physical contig spanning a region of approx. 190 kb around the HvBRJ1 locus, where the mutation responsible for the semi-dwarf plant type ‘uzu’ is located. These results indicate that the ‘Haruna Nijo’ BAC library will be useful for barley genomic studies.

Key Words: Hordeum vulgare L., ‘Haruna Nijo’, bacterial artificial chromosome (BAC) library, DNA pool.

Introduction

Cultivated barley (Hordeum vulgare L.) is a self-fertilizing cereal crop species that is diploid (2n = 14), with a large haploid genome size of approx. 5,000 Mb (Arunuganathan and Earle 1991). Barley is one of the most important and widely cultivated cereal crops in the world (Pochlman 1985). There is also an extensive history of genetic, mutagenic and cytogenetic studies in barley, resulting in large numbers of mutants and cytogenetic stocks (Ramage 1985). Furthermore, thousands of morphological and quantitative traits and molecular markers have been mapped on the barley genome (Lundqvist et al. 1997, Ramsay et al. 2000, Kleinhofs and Graner 2000, Hays et al. 2003). Since the end of the 20th century, several expressed sequence tag (EST) projects have generated very large amounts of barley EST data (Close et al. 2004). As of May 19, 2006, approximately 437,000 barley ESTs, derived from more than 85 cDNA libraries, were deposited in the “Expressed sequence tags” (dbEST) in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). The HarvEST:Barley project (http://harvest.ucr.edu/) provides the annotation of non-redundant barley ESTs. Conversion of non-redundant ESTs to DNA markers is an effective and useful strategy for marker generation, and several research groups have constructed high-density linkage maps using markers derived from barley ESTs (Kota et al. 2001, Thiel et al. 2003, Sato et al. 2004, Rostoks et al. 2005b). In addition, the Affymetrix 22K barley I GeneChip probe array is available for the biological expression analysis of approx. 22,000 barley genes (Close et al. 2004). Rostoks et al. (2005a) reported on a procedure to identify single-feature polymorphisms (SFPs) using the barley GeneChip, with a high sensitivity (i.e., 67% of known single nucleotide polymorphisms (SNPs) were identified as SFPs). Thus, despite the large genome size of barley, these resources have accelerated the advances of genetic and genomic studies in this crop species.

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In addition to non-redundant and multiple types of genetic markers, the availability of large-insert genomic libraries is also crucial for genome analysis: for physical mapping, map-based gene and QTL cloning, and comparative genomic analyses between homologous chromosomal regions. In the case of barley, several yeast artificial chromosome (YAC) libraries had been constructed, and these contributed to the map-based cloning of three powdery mildew resistance loci: Mlo, Rar1 and Mla (Buschges et al. 1997, Simons et al. 1997, Lahaye et al. 1998, Shirasu et al. 1999, Kleine et al. 1993, 1997). However, it is generally recognized that YAC libraries cannot be easily handled (Baba et al. 2000). In recent years, bacterial artificial chromosome (BAC) libraries have been constructed and used for the genome analysis of many organisms, including crop plants. Several genomics resource centres have been established that distribute resources such as large-insert genomic libraries from model and/or crop plants (e.g. AGI [http://www.genome.arizona.edu/orders/], CUGI [https://www.genome.clemson.edu/], GENEdiner Genomic Resources in TAMU [http://hzb7.tamu.edu/index.htm]). The BAC vector system has become an invaluable tool because of its ability to stably maintain large DNA fragments, and its ease of handling (Shizuya et al. 1992, Woo et al. 1994, Wang et al. 1995). For example, in Arabidopsis (Bevan et al. 1998) and rice (Tao et al. 2001), several BAC libraries have been constructed and used to develop sequence-ready physical maps. In addition to these model species, a number of agronomic and/or biologically important plants have been targeted for the development of infrastructures for genome analysis, including the construction of multiple and/or deep-coverage BAC libraries. These include maize, soybean, cotton (http://hzb7.tamu.edu/home/links/bac_est/bac.htm), other members of the Oryza genus (Ammiraju et al. 2006), diploid ancestors of hexaploid wheat (Lijnveld et al. 1999, Akhunov et al. 2005), tetraploid wheat (Cenci et al. 2003) and common wheat (Liu et al. 2000, Shen et al. 2005, Ling and Chen 2005). In barley, two BAC libraries (Lapitan et al. 1997, Yu et al. 2000) have been constructed using the North American six-rowed malting variety ‘Morex’. Although the library reported by Yu et al. (2000) contained 313,344 gridded clones (6.3 haploid genome-equivalents), the library constructed by Lapitan et al. (1997) consisted of only 10,750 clones with an average insert size of 95 kb, and was estimated to be less than one genome-equivalent. Recently, Isidore et al. (2005) have constructed a barley cv. ‘Cebada capa’ BAC library, and adopted a pooling strategy for rapid, cost-reduced screening to obtain targeted clones. Despite these reports, progress in the construction of BAC libraries has been limited for species with large genome sizes, such as barley. The only barley libraries currently available with complete genome coverage and arrayed individual clones are derived from the variety ‘Morex’, and these are considered to be insufficient for an extensive analysis of the barley genome.

To facilitate further progress in barley genomics, utilizing the accumulated genetic resources now available, we attempted to construct an additional barley BAC library with complete genome coverage and gridded clones. The genetic material used for BAC library construction in the present study was the two-rowed Japanese malting variety ‘Haruna Nijo’, which had been used to generate a large number of ESTs at Okayama University, Japan, and was used as one of the parents to construct a high-density transcript linkage map (Sato et al. 2004). Japanese malting barley varieties, including ‘Haruna Nijo’, have dominant pedigrees derived from old European two-rowed malting landraces (Fischbeck 2003). ‘Haruna Nijo’ was bred to be well-adapted to Japan, with high malting quality profiles (Karakousis et al. 2003). On the other hand, ‘Morex’, the North American six-rowed malting barley variety, was derived from the ‘Manchurian’ type of barley, and was introduced to North America by immigrants from Germany and Russia during the 19th century (Fischbeck 2003). Although both varieties exhibit superior malting quality profiles, their origin is different. Therefore, it is possible that their genomic functions, including adaptation to biotic and abiotic stress conditions, could also be significantly different. The objectives of the present study were as follows: (1) to construct a ‘Haruna Nijo’ BAC library representing at least six haploid genome-equivalents, (2) to evaluate the library for average insert size and chloroplast DNA content, (3) to develop a rapid screening scheme that combines the pooling of BAC DNA with the use of high-density replication membranes (HDRMs) and (4) to evaluate the library by screening it with genetically mapped EST markers and well-characterized genes.

Materials and Methods

Plant material

The Japanese malting barley variety ‘Haruna Nijo’ was selected for the construction of the large-insert genomic library. The cultivar was categorized as Hordeum vulgare ssp. vulgare, with two-rowed spikes and a spring growth habit. ‘Haruna Nijo’ was released from Sapporo Brewery Ltd. in 1981 and has been grown in Japan as a malting cultivar for more than 20 years. Due to its superior malting quality profiles, ‘Haruna Nijo’ has been used as a crossing parent to develop other high-quality malting varieties.

Preparation of protoplasts, and isolation of high molecular weight (HMW) DNA from barley leaves

Protoplasts were prepared from green barley leaves, as described by Cheung and Gale (1990) with slight modifications. Cell walls were digested using an enzyme solution consisting of 0.6 M mannitol, 2% Cellulase Onozuka R10 (Yakult Honsha Co., LTD, Japan) and 0.05% Pectolyase Y23 (Kikkoman Corporation, Japan), for 90 minutes at 32°C. Protoplast density was checked with a hemocytometer and adjusted to 2.5–5.0 x 10^6 ml^-1. Subsequent treatments were performed, as described by Cheung and Gale (1990). The final protoplast pellet was embedded in 1% low-melting-point (LMP) agarose (SeaPlaque GTG, FMC).
Extraction of HMW DNA from the agarose plug was performed, as described by Nakamura et al. (1997). The plug contained a final concentration of 40–50 μg DNA per ml.

Partial digestion of HMW DNA and size fractionation

Plugs (approx. 100 mg) were equilibrated in 1 ml of Hind III digestion buffer without MgCl₂ (10 mM Tris-HCl pH 7.5, 1 mM DTT, 50 mM NaCl and approx. 32 U of Hind III) at 4°C overnight. Then, the Hind III digestion buffer was exchanged for one including MgCl₂ (final concentration 10 mM), and the plugs were incubated at 37°C for 30 min. The reaction was terminated by placing the tubes on ice and immediately adding 100 μl of 0.5 mM EDTA, pH 8.0. Fragmentation of the DNA was first checked using the CHEF DR III electrophoresis system (BIO-RAD, USA) at 5.5 V/cm, with a linearly ramped pulse time of 1-45 sec, for 16h at 10°C, in a 1% agarose gel with 0.5 × TBE (44.5 mM Tris, 44.5 mM boric acid and 1 mM EDTA) buffer. Then, the partially digested DNA was fractionated in a 0.6% LMP agarose gel with 0.5 × TBE buffer, by two-phase CHEF electrophoresis at 5.5 V/cm with a constant pulse time of 90 s for 3 h, followed by a constant pulse time of 6 s for 8 h. DNA fragments, ranging in size from 200-500 kb, were isolated from the unstained gel using the ethidium bromide-stained outer edge of the lane as a guide. Washing, melting and digestion of the gel pieces were performed, as described by Nakamura et al. (1997).

Ligation and transformation

Preparation of the BAC vector, pBAC-Lac (Asakawa et al. 1997), was performed, as described by Nakamura et al. (1997). Competent E. coli DH10B cells were prepared for electroporation, as recommended by the supplier (Invitrogen, USA).

Approx. 200 ng of genomic DNA was ligated into the dephosphorylated BAC vector, at a molar ratio of 1:10 (insert:vector), using 70 U of T4 DNA ligase (Takara, Japan) in the buffer provided by the manufacturer, at 16°C overnight. Droplets of the ligation mixture were then dialyzed against 0.2 × TE for 4 h using VS membranes (pore size 0.025 μm; Millipore, USA).

One microliter of dialyzed solution was used for the electroporation of 20 μl of competent E. coli DH10B cells, in a 0.1 cm-cuvette, using the BIO-RAD MicroPulsertm apparatus, with one pulse of 1.25 kV. Then, 1 ml of SOC medium was immediately added to the cells. The cell suspension was incubated with agitation at 37°C for 1 h, then, plated on LB medium containing 12.5 μg/ml of chloramphenicol (CM), 50 μg/ml of X-gal and 12.5 μg/mL of IPTG. After 24 h of incubation, white colonies were selected and placed into the wells of 384-well microtiter plates (MTP; #264574, Nalge Nunc International K.K., Japan) using the Q-Pix automated colony picker (Genetix, UK). Each well of the MTPs contained 100 μl of LB freezing buffer (Nakamura et al. 1997). The plates were incubated at 37°C for 24 h, then duplicated and stored at −80°C.

Construction of high-density replication membranes (HDMRs)

HDRMs for hybridization-based screening of the library were robotically prepared using the 384-pin colony replicator of the Biomek FX/HDR system (Beckman Coulter, USA). Whole BAC clones from the 384-well MTPs were gripped onto 11.5 cm × 7.3 cm positively charged nylon transfer membranes (GE Healthcare Bio-Science Corp., USA) with a 4 × 4 mini-matrix of each well position. Each mini-matrix contained spots from eight MTPs, duplicate-spotted in a lateral direction, and this gridding pattern enabled the screening of 3,072 BAC clones (8 × 384-well MTPs) per membrane. All the BAC clones in the ‘Haruna Nijo’ library were arrayed on a set of 96 membranes. The HDRMs were processed, as described by Nakamura et al. (1997).

Preparation of BAC DNA pools for PCR-based screening

DNA from the BAC library was pooled for PCR screening. First, the 384 clones arrayed in each MTP were bulked into one pool (primary pool; PP), resulting in 768 PPs (PP #001–#768). BAC DNA from each PP (PP DNA) was isolated by culturing the clone mixtures overnight in liquid cultures (2 ml LB + 12.5 μg/ml CM per well; without shaking), and then DNA was extracted using the NA-2000 robot (Kurabo, Japan). The PP DNAs were arrayed in numerical order in 96-well MTPs. The eight resulting 96-well MTPs, which included all the PP DNAs, were vertically arranged for the three-dimensional pool construction scheme. Super pools of BAC DNA (SP DNA) were made by combining DNA from all the rows (8 rows) in the plates, all the columns (12 columns) and all the wells in each individual plate (8 layers). Thus, a total of 28 super pools were produced. Construction of the SP DNAs was manually performed.

Determination of insert size in the ‘Haruna Nijo’ BAC library

In order to determine the insert size distribution in the BAC library, 191 BAC clones were randomly selected, and DNA was isolated from them automatically using the NA-2000 robot, from small-scale (2 ml) liquid cultures. Not I digestion and CHEF electrophoresis were carried out, as described by Nakamura et al. (1997).

DNA probes and colony hybridization to HDRMs

The barley locus-specific ESTs used for screening the BAC clones, and their corresponding primer sequences, are shown in Table 1. PCR conditions followed those described by Hori et al. (2005). The sequences of the HvBR1I-specific PCR primers, and the conditions for amplification followed those described by Chono et al. (2003). Three Hind III restriction fragments of barley chloroplast DNA (of 9.1, 6.0 and 5.0 kb) were kindly provided by Prof. N. Tsutsuji, University of Tokyo, Japan (Niwa et al. 1996). The PCR products and chloroplast DNA fragments were purified using the MiniElute PCR purification kit (Qiagen, USA).

Probe labelling and colony hybridization were
Table 1. The 13 barley EST clones and HvBRII used for screening the ‘Hauna Nijo’ BAC library

<table>
<thead>
<tr>
<th>Marker</th>
<th>Acc no.</th>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Chr.</th>
<th>Clone no.</th>
<th>Clone ID</th>
</tr>
</thead>
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<tr>
<td>k02301</td>
<td>AV922490</td>
<td>Forward</td>
<td>TGACTGTTCCGCTAGTTGTCCG</td>
<td>1H</td>
<td>7</td>
<td>009A18 199K19 371005 573L04 582305 582N14 647D19</td>
</tr>
<tr>
<td>k02948</td>
<td>BJ66657</td>
<td>Reverse</td>
<td>CCAACGTCCTACAAAGGCTG</td>
<td>1H</td>
<td>5</td>
<td>058A01 065117 233E19 649K42 684H19</td>
</tr>
<tr>
<td>k08122</td>
<td>AV917594</td>
<td>Reverse</td>
<td>CGCAGTGGAGGACACCAACA</td>
<td>2H</td>
<td>7</td>
<td>196809 278005 379M23 470M23 481J07 720802 72G17</td>
</tr>
<tr>
<td>k02080</td>
<td>AV921760</td>
<td>Reverse</td>
<td>AGCCTGACCTACAGTTGAAA</td>
<td>3H</td>
<td>7</td>
<td>008M18 042F18 156L23 446D06 569O17 633M20 661L08</td>
</tr>
<tr>
<td>k02538</td>
<td>AV834075</td>
<td>Reverse</td>
<td>TTTGGAAAGCCTACACAC</td>
<td>3H</td>
<td>6</td>
<td>116E04 218K20 218P01 492L01 565F17 658E23</td>
</tr>
<tr>
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<td>AV911280</td>
<td>Forward</td>
<td>AAATGACGCTAGGATCTTG</td>
<td>4H</td>
<td>3</td>
<td>266A22 576C14 675O6</td>
</tr>
<tr>
<td>k03067</td>
<td>BJ394881</td>
<td>Reverse</td>
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<td>4H</td>
<td>4</td>
<td>151K15 615A14 716H07 718L21</td>
</tr>
<tr>
<td>k07697</td>
<td>BJ463746</td>
<td>Reverse</td>
<td>CCGAGTGAGGACACCTTGAGG</td>
<td>5H</td>
<td>1</td>
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</tr>
<tr>
<td>k03104</td>
<td>BJ468280</td>
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<td>6H</td>
<td>2</td>
<td>105J04 264G14</td>
</tr>
<tr>
<td>k03994</td>
<td>BJ483318</td>
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<td>6H</td>
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<td>478808 703L03</td>
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<tr>
<td>k00889</td>
<td>AV934956</td>
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<td>AGATGCTGGAGAAAGCCTTCA</td>
<td>7H</td>
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<tr>
<td>k07655</td>
<td>AV920110</td>
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<td>ATGGTGTGAGCTTCTTCGTCG</td>
<td>n.d.</td>
<td>11</td>
<td>083N07 099M22 171L01 405M09 409E08 454L18 495A10 551N04 552K13 576C11 668C19</td>
</tr>
</tbody>
</table>

Average 5.1

a) Chromosomal location were previously reported by Sato et al. (2004). ‘n.d.’ means the chromosomal location not determined.

Results

Construction and characterization of the BAC library using the Japanese malting barley variety ‘Haruna Nijo’

The BAC library of the Japanese malting barley variety ‘Haruna Nijo’ consisted of 294,912 clones arrayed in 768 384-well MTPs. HMW DNA was isolated from the protoplasts of green leaves from ‘Haruna Nijo’ seedlings, and DNA with the target size (200–500 kb) was prepared by partial digestion with the restriction enzyme Hind III. After ligation into the pBAC-Lac vector, the transformation efficiency in E. coli DH10B cells was approx. 1.5 × 10^6 cfu/μg dephosphorylated insert DNA. The complete library was gridded onto 96 384-well MTP-sized membranes, with duplication, to produce the HDRMs. Each HDRM consisted of 3,072 BAC clones, arrayed in 384 4×4 mini-matrices, and the 96 HDRMs covered the whole library completely.

In order to investigate the fragment size profile of the ‘Haruna Nijo’ BAC library, 191 BAC clones were randomly selected, and the insert sizes of individual clones were determined after digestion with the restriction enzyme Not I. The results for 36 randomly selected clones are shown in Fig. 1. The 191 clones ranged in size from 22.9 kb to 225.6 kb, and the sizes were normally distributed, as shown in Fig. 2. More than 60% of the clones (117) carried inserts of more than 100 kb. The average insert size was estimated to be 115.2 kb with a standard deviation of 28.8 kb. Among the 191 BAC
clones, the percentage of those lacking inserts was 0.5%.

In order to estimate the level of chloroplast DNA contamination, we carried out colony hybridization using three barley chloroplast genome Hind III-fragments as probes. The fragments were separated in the chloroplast genome by approx. 50 kb, and were derived from the regions: 15.3–24.4 kb, 50.5–56.5 kb and 101.0–106.0 kb (Niwa et al. 1996). Of the 36,864 BAC clones analyzed (12 HDRMs), 636 hybridized using at least one of the three barley chloroplast probes (data not shown), indicating that 1.7% of the BAC clones were contaminated with chloroplast DNA. Based on the average insert size, the percentage of empty clones, the level of chloroplast genome contamination and the haploid genome size of barley (5,000 Mb; Arumuganathan and Earle 1991), we estimated that the coverage of the ‘Haruna Nijo’ BAC library was approx. 6.6 genome-equivalents. This allowed for a probability of more than 99% of recovering any specific sequence from the barley genome (Clarke and Carbon 1976).

Development of a rapid screening scheme that combines PCR analysis of pooled BAC DNA and colony hybridization

To achieve the rapid isolation of targeted BAC clones from the library, we developed a screening strategy consisting of two steps (Fig. 3). The first step involved the PCR analysis of pooled (SP and PP) DNAs, and the second step involves colony hybridization to particular HDRMs. The PCR step enabled to narrow the candidate positive clones down from a total of approx. 300,000 BAC clones to those contained in several 384-well MTPs. The scheme used to produce the primary DNA pools (PPs) and the super pools (SPs) was described in the methods, and is shown in Fig. 3a. PCR analysis of the 28 SPs was followed by the analysis of individual PPs. In this way, it became possible to reduce the number of 384-well MTPs (theoretically, to less than seven) that must be screened in the colony hybridization step. In the example shown in Fig. 3b and Fig. 3c, we screened for barley EST k02080. In the PCR step, we narrowed the number of potential positive clones down to 3,072 (i.e., eight 384-well MTPs) (Fig. 3b). After the PPs that contained the target sequence were identified, the corresponding HDRMs (i.e., the HDRMs that contained colonies represented in the PPs) were selected for colony hybridization, in order to identify the specific BAC clones that carried the target sequence. In the case of the k02080 sequence, eight HDRMs were selected, and colony hybridization was carried out using the k02080-specific probe. The results for HDRM #441–448 and HDRM #569–576 are shown in Fig. 3c. The probe hybridized with the BAC clones 448D06 and 569O17, respectively.

Screening of ‘Haruna Nijo’ BAC clones using barley ESTs

To evaluate the library for genome coverage and quality, we screened the library using locus-specific ESTs (Sato et al. 2004). Thirteen EST markers, and one STS marker, distributed over the whole barley genome, were randomly selected and used to screen the library by the rapid screening method described above. An average of 5.1 positive clones were selected per marker, with the actual numbers ranging from 1 to 11 (Table 1). Yu et al. (2000) constructed a barley BAC library using the variety ‘Morex’ and the restriction enzyme Hind III for cloning. They also evaluated their library coverage by screening with 40 single-copy probes. In the case of the ‘Morex’ library, an average of 6.4 positive clones was identified per probe, and the range was 1–13 BAC clones per probe. Thus, in both studies, wide ranges in the number of positive clones were detected by the EST probe. This tendency could be due to preferential cloning effects associated with the use of the restriction enzyme Hind III (Tomkins et al. 2002).

Development of a BAC contig around the HvBRII gene

To evaluate the usefulness of the barley ‘Haruna Nijo’ BAC library, we constructed a physical contig spanning a particular target locus. For this example, we chose the HvBRII gene, whose mutation is responsible for the barley semi-dwarf plant type ‘uzu’, which is distributed throughout East Asia, including Japan (Chono et al. 2003, Saisho et al. 2004). We identified four BAC clones from the ‘Haruna Nijo’ library, using HvBRII gene-specific primers and the
genomic HvBRII clone (Table 1). The insert sizes of the selected clones (002D19, 566G17, 567B06 and 654J23) were 137.1 kb, 114.8 kb, 104.8 kb and 124.2 kb, respectively, as determined by Not I digestion (data not shown). The four BAC DNAs were digested with Eco RI and Not I and fractionated on a 0.8% agarose gel (Fig. 4a). By manually aligning the clones, we constructed a physical contig around the HvBRII locus (Fig. 4b). All four BAC clones shared a region of approximately 70 kb, which included the HvBRII-specific 1.9 kb Eco R I fragment present in the ‘Haruna Nijo’ variety (Saisho et al. 2004). The contig spanned a region of about 190 kb on chromosome 3H, where the ‘uzu’ gene had been assigned. Thus, we were able to demonstrate the usefulness of the ‘Haruna Nijo’ BAC library, and the screening strategy developed in this study. These resources are presently available for barley genomics research, including genome structure analyses and map-based cloning.

**Discussion**

We reported here the construction of a barley BAC library consisting of 294,912 clones, using the Japanese malt-barley variety ‘Haruna Nijo’. This library consisted of 768 384-well MTPs. We estimated that the genome coverage was about 6.6 genome-equivalents, based on an investigation of the average insert size of randomly selected clones, the level of chloroplast DNA contamination and the number of empty clones. By screening the library using EST-derived genetic markers distributed over the barley genome, we found that the ‘Haruna Nijo’ BAC library provided a genome coverage similar to that of the ‘Morex’ library (Yu et al. 2000). Thus, the ‘Haruna Nijo’ BAC library could be useful as a new resource for molecular genetics and genomics studies in barley. Despite the utilization of different accessions in the course of library construction, more than

![Fig. 3. Development of the two-step BAC library screening method, and identification of BAC clones carrying the barley EST k02080. (a) Schematic representation of the DNA pooling strategy. Primary pool (PP) DNAs were prepared by pooling clones from each original 384-well MTP, and extracting DNA from the pooled clones. The 768 PP DNAs were arranged in 96-well plates, in a three-dimensional stack consisting of 8 rows × 12 columns × 8 plates. The 28 super pool (SP) DNAs were obtained by pooling DNAs from the rows (A–H), columns (1–12) and plates (a–b). After PCR analysis of the 28 SP DNAs, followed by PCR analysis of selected PP DNAs, using specific primers for target loci, the original 384-well MTPs containing the target BAC clones could be identified. (b) PCR screening of the BAC library for DNA pools containing the barley EST marker k02080. PCR reactions were carried out using the 28 SP DNAs. M: molecular size markers. Numbers or letters above the gel pictures indicate the SP DNAs from which specific bands were amplified. Bottom: PCR of selected PP DNAs. Two hundred and ten PP DNAs (6 row-pool DNAs × 7 column-pool DNAs × 5 plate-pool DNAs) were identified as candidates in this experiment, and screened by PCR. Results for 92 PP DNAs are shown. M: molecular size markers. The numbers above the gel pictures indicate the numbers of PP DNAs analyzed in each lane. Three PP DNAs (#401, #448 and #569; shown in bold font) gave bands with the expected size (indicated by the arrows) with the k02080-specific primers. As a result of PCR screening of the SP and PP DNAs, potential clones carrying the k02080 EST sequence were narrowed down to 8 384-well MTPs (#008, #042, #156, #401, #448, #569, #633 and #661). (c) Colony hybridization of HDRMs. The positions of target BAC clone(s) in individual original 384-well MTP(s) can be identified by colony hybridization of HDRMs using locus-specific probes. The pictures show the results for HDRM #441-4448 (left) and #569-4576 (right), after hybridization using the k02080-specific probe. Arrows indicate positive BAC clones (448D06 and 569D17). Low level, background hybridization to the pBAC-Lac vector was used to position the colonies in the blots. In this experiment, we did not identify a positive clone from the candidate 384-well MTP #401 (data not shown).
13 genome-equivalents are presently available to the barley research community in the "Morex" and 'Haruna Nijo' BAC libraries. As mentioned in the review by Meyers et al. (2004), the majority of the published whole-genome physical maps were constructed using BAC clones covering more than 10 genome-equivalents. The approx. 600,000 barley BAC clones generated from 'Morex' (Yu et al. 2000) and 'Haruna Nijo' (the present study) could facilitate the construction of a barley whole-genome physical map.

'Haruna Nijo' which was released by Sapporo Breweries Ltd. in 1981, is a representative Japanese malting variety derived from the old European malting barley, 'Golden Melon', which was introduced as a two-rowed spring variety in 1881 (Takahashi 1980). The 'Haruna Nijo' pedigree also includes a Japanese six-rowed landrace, and the European landraces 'Chevallier' and 'Hanna'. Due to strong selection by Japanese breeders, 'Haruna Nijo' displays several characters for good agronomic performance under Japanese cultivation conditions (such as early maturity), and also high malting quality profiles, including high malt extract levels, high diastatic power and high apparent final attenuation. Karakousis et al. (2003) identified malting and brewing QTLs using a 'Galleon' × 'Haruna Nijo' doubled haploid (DH) population, under Australian growth conditions. Genes controlling these malting quality characters could become important targets for isolation, using the 'Haruna Nijo' BAC library.

We selected 'Haruna Nijo' as one of the materials for the Japanese EST project, and more than 46,000 ESTs from this variety derived from three cDNA libraries, were deposited in the public database (unpublished data). One of these cDNA libraries was derived from germinating shoots, which might express genes controlling malting processes. Sato et al. (2004) and Hori et al. (2005) developed sets of DH lines and recombinant chromosome substitution lines (RCSLs), respectively, using a cross between 'Haruna Nijo' and a wild barley (ssp. spontaneum 'H602'), which is an ancestral form of the cultivated species. They identified a series of major genes and QTLs that control agronomic performance, using

Fig. 4. Physical contig construction around the HvBRII gene. (a) Fingerprint profiles of BAC clones carrying HvBRII-specific sequences, after restriction digestion with EcoR I and Nor I, and electrophoresis on a 1% agarose gel. Numbers above the lanes indicate individual BAC clones: 1: HNB 002D19; 2: HNB 566G17; 3: 567B06; 4: 654J23. M: λ Hind III. The arrow head and the asterisk indicate the vector-specific band (7.0 kb), and HvBRII-specific band (2.0 kb), respectively. (b) Schematic representation of the physical contig around the HvBRII gene, spanning approx. 190 kb.
mainly the above ESTs as molecular markers. Generally, wild barley is considered to be unsuitable for malting, due to the limited availability of seed, and the presence of too many deleterious brewing characters. The RCSLs with ‘Haruna Nijo’ as the recurrent parent could be used to identify genes responsible for malting characters, by comparing ‘Haruna Nijo’ with RCSLs which carry alleles for unfavourable malting and brewing characters (Matus et al. 2003). The RCSLs are useful for identifying malting traits since most of the genomic segments (93.75% in the BC2 generation) are derived from ‘Haruna Nijo’, whose malting process can be easily controlled and evaluated. Thus, using near-isogenic RCSL lines, with markers that are tightly linked to the genes responsible for malting characters, the BAC library could be directly used for the isolation of malting character genes.

Rostoks et al. (2005b) pointed out that the diversity in adaptive and agronomic performance is ultimately based on sequence variations in the barley genome. In contrast to the breeding background of ‘Haruna Nijo’, ‘Morex’ is a six-rowed, Manchurian-type, high-quality malting barley variety developed in Minnesota, USA (Rasmusson and Wilcoxson 1979). ‘Morex’ was also used as a background for the comprehensive detection of the QTL(s) controlling agronomic and malting quality characters, as one of the parents in a DH population derived from a ‘Steepee’ × ‘Morex’ cross (Kleinhofs et al. 1993, Hays et al. 2003). The assessment of allelic variations at the loci controlling agronomic traits could be one of the most significant approaches for the evaluation of superior germplasm accessions for breeding purposes. Recently, intraspecific differences in gene composition have been detected in homologous chromosomal regions by comparative studies between maize inbreds (Fu and Dooner 2002, Song and Messing 2003). Scherrer et al. (2005) observed a rapid and divergent variability in the sequences surrounding the Rph7 leaf rust resistance locus, among 41 cultivated barley haplotypes. These reports highlight the value of sequencing and haplotyping in homologous regions between barley accessions with known performance. In the present study, we developed a rapid BAC screening scheme using a DNA pooling strategy (Fig. 3), and constructed a physical contig around the HvBRII gene, spanning approx. 190 kb (Fig. 4). Using this procedure, it may become possible to compare multiple genomic regions, among three cultivated barley accessions (‘Morex’; Yu et al. 2000, ‘Cebada capa’; Isidore et al. 2005, ‘Haruna Nijo’; the present study). This may enable the cloning and evaluation of allelic variation of particular gene(s), and the haplotyping analysis of defined chromosomal regions surrounding genes of importance, in multiple genomic backgrounds. This parallel genomic sequencing analysis has become more realistic after the recent development of rapid sequencing methods (Metzker 2005). For this analysis, it would be ideal to use several genomic libraries derived from multiple gene pools, including ancestral forms of cultivated barley, in order to broadly evaluate the genetic diversity in barley.

Resource availability information: HDRMs, individual clones and PP/SP DNA samples are available upon request. Please check the information site of National BioResource Project (NBRP; http://www.nbrp.jp/) to request these materials. The scientist in North America may contact Arizona Genomics Institute (AGI; http://www.genome.arizona.edu/orders/) to request the screening filters and clones.

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