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ORIGINAL PAPER

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High-density AFLP map of nonbrittle rachis 1 (*btr1*) and 2 (*btr2*) genes in barley (*Hordeum vulgare* L.)

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Abstract Wild relatives of barley disperse their seeds at maturity by means of their brittle rachis. In cultivated barley, brittleness of the rachis was lost during domestication. Nonbrittle rachis of occidental barley lines is controlled by a single gene (*btr1*) on chromosome 3H. However, nonbrittle rachis of oriental barley lines is controlled by a major gene (*btr2*) on chromosome 3H and two quantitative trait loci on chromosomes 5HL and 7H. This result suggests multiple mutations of the genes involved in the formation of brittle rachis in oriental lines. The *btr1* and *btr2* loci did not recombine in the mapping population analyzed. This result agrees with the theory of tight linkage between the two loci. A high-density amplified fragment-length polymorphism (AFLP) map of the *btr1/btr2* region was constructed, providing an average density of 0.08 cM/locus. A phylogenetic tree based on the AFLPs showed clear separation of occidental and oriental barley lines. Thus, barley consists of at least two lineages as far as revealed by molecular markers linked to nonbrittle rachis genes.

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Introduction

Inflorescences of wild grasses disarticulate or segment at maturity. This character is of great importance for seed dispersal under natural conditions. Plants of the tribe Triticeae form inflorescences called spikes. In the genus *Triticum* (and *Aegilops*), spikes of different species disarticulate above or below each rachis node, giving rise to "wedge-" or "barrel-shaped" individual spikelets, or entirely disarticulate at the base as one unit (Kimber and Feldman 1987, van Slageren 1994). In contrast, in *Hordeum* and *Secale*, spikes disarticulate above each rachis node to form typical wedge-shaped spikelets (Bothmer et al. 1995; Frederiksen and Petersen 1998), although spikes of *Hordeum bogdanii* disarticulate between each glume and floret (Bothmer 1979). Disarticulation scars in wild barley (*H. vulgare* ssp. *spontaneum*—the term "wild barley" means only this taxon in this report) are smooth, whereas in cultivated barley (*H. vulgare* ssp. *vulgare*) threshing produces rough breaking scars on grains detached from rachis segments; this phenomenon serves as principal evidence for barley domestication in archaeological studies (Zohary and Hopf 2000). Anatomically, the rachis nodes are clearly constricted in brittle spikes, but are not constricted in nonbrittle spikes (Ubisch 1915).

Wild barley has complementary genes, *Btr1* and *Btr2*, for the formation of brittle rachis, and cultivated barley carries recessive alleles at either of the loci, resulting in nonbrittle rachis (Ubisch 1915; Schiemann 1921; Johnson and Åberg 1943; Takahashi and Hayashi 1959). The *btr1* and *btr2* loci have been mapped to chromosome 3HS, and the two loci are tightly linked (Takahashi and Hayashi 1964). Interestingly, most occidental cultivars carry the *btr1* allele, and most oriental cultivars carry the *btr2* allele, indicating a clear differentiation pattern of the two groups (Takahashi 1955). We previously identified amplified fragment-length polymorphism (AFLP) loci flanking the *btr1* locus (Komatsuda and Mano 2002), but the position of the *btr2* locus was ambiguous, and we could not clarify the organization of the two loci because the density of

AFLP loci was insufficient for monitoring fine crossover events; each recombinant inbred line (RIL) was crossed with only one of the testers (*btr1* or *btr2*), and a new gene factor, "*d*", independently inherited on chromosome 7H, affected the mapping of the *btr2* locus. Moreover, we did not estimate the genetic mode of action of the modifier gene.

We developed high-density AFLP markers to investigate the organization of both *btr1* and *btr2*. Highly advanced RILs were crossed with two standard testers. Composite interval mapping of quantative trait loci (QTLs) was performed in order (1) to test the hypothesis that the *btr1* and *btr2* loci are tightly linked (Takahashi and Hayashi 1964), (2) to identify the modifier gene *d* previously detected in chromosome 7H (Komatsuda and Mano 2002), and (3) to detect any other QTLs that affect the rachis brittleness of barley. Phylogenetic patterns of cultivated and wild barley were inferred by using AFLP loci linked to the *btr1* and *btr2* loci. We assumed that possession of both the *Btr1* and *Btr2* alleles is a necessary condition for producing brittle rachis in barley (Takahashi and Hayashi 1964). We followed the system of description of barley genes recommended by Franckowiak et al. (1997) and Lundqvist et al. (1997). Barley chromosome designation followed the nomenclature recommended by Linde-Laursen et al. (1997).

Materials and methods

Plant materials

H. vulgare ssp. *vulgare* cvv. 'Azumamugi', 'Kanto Nakate Gold', and 'Soren Oomugi 19329' were obtained from the Barley Breeding Laboratory, National Institute of Crop Science, Tsukuba, Japan; 'Chevalier', 'Golden Promise', and 'Hanna' from the Gene Bank, National Institute of Agrobiological Sciences, Tsukuba, Japan; 'Dissa' from Sapporo Breweries, Nitta, Japan; 'Hayakiso 2', 'Natsudaikon Mugi', and 'Caveda' from the Research Institute for Bioresources, Okayama University, Kurashiki, Japan; 'New Golden' and 'Misato Golden' from Dr. T. Makino, National Institute of Crop Science, Tsukuba, Japan; and 'Bonus' and 'Kristina' from Dr. U. Lundqvist, Svalöf Weibull AB, Svalöv, Sweden. For the genotypes of these cultivars, the studies by Takahashi et al. (1983) and Komatsuda and Mano (2002) were consulted. *H. vulgare* ssp. *vulgare* var. *agriocrithon* is a six-rowed barley with brittle rachis (Bothmer and Jacobsen 1985), and lines OUH786 and OUH802 were obtained from the Research Institute for Bioresources, Okayama University.

Seventeen lines of *H. vulgare* ssp. *spontaneum*, a wild barley, represented by "OUH" numbers, were obtained from the Research Institute for Bioresources, Okayama University. Line H3140A was obtained from Dr. R. von Bothmer, Swedish University of Agricultural Sciences, Alnarp, Sweden. Line PI282597 was obtained from the USDA-ARS National Small Grains Research Facility, Aberdeen, Idaho, USA.

Evaluation of rachis brittleness

'Azumamugi' is a nonbrittle (*Btr1Btr1btr2btr2*), six-rowed cultivar; 'Kanto Nakate Gold' is a nonbrittle (*btr1btr1Btr2Btr2*), two-rowed cultivar (Komatsuda and Mano 2002). Ninety-nine RILs (F_{11}) have been developed from the 'Azumamugi' × 'Kanto Nakate Gold' cross by single-seed descent. A single plant of each of 87 RILs was pollinated with 'Natsudaikon Mugi' (*btr1btr1Btr2Btr2*), and a single plant of each of these and of five other RILs was pollinated with 'Hayakiso 2' (*Btr1Btr1btr2btr2*). The remaining seven RILs were not crossed with the testers because flowering time differed, or were crossed, but insufficient F_1 plants were obtained for genetic analysis. 'Natsudaikon Mugi' is a spring barley (*sgh1/Sgh2/sgh3*) and 'Hayakiso 2' is a winter barley (*Sgh1/sgh2/sgh3*). These two testers were used to test rachis brittleness and spring habit of growth at the Research Institute for Bioresources, Okayama University (Takahashi et al. 1983). The F_1 families were sown in a field at the National Institute of Agrobiological Sciences, Tsukuba, in October 2000 and grown over winter because 'Azumamugi' and 'Hayakiso 2' are winter barleys, and some of the F_1 families of RILs × testers may require vernalization before flowering (Takahashi et al. 1983; Komatsuda et al. 1993). Five to ten plants in each F_1 family and at least two spikes from each plant were evaluated. Awns of opposite rows were gently pulled in an opposite direction. Because some segregating plants showed intermediate brittle rachis, rachis brittleness was evaluated quantitatively as 100% × (number of rachis nodes disarticulated)/(number of rachis nodes in a spike). Brittleness was evaluated twice between 2 and 4 weeks after maturation, and scores of the two times were averaged for each F_1 family.

QTL mapping

Mano et al. (2001) constructed a linkage map for 99 RILs (F_9) from the 'Azumamugi' × 'Kanto Nakate Gold' cross, with 272 point markers, providing an average density of 6.5 cM/locus. Since the linkage map showed a considerable clustering of markers in certain regions, Mano and Komatsuda (2002) constructed a base map eliminating the clustering markers and using the remaining 100 markers to achieve a density of 5–10 cM/locus. On the basis of the map, we performed composite interval mapping of QTLs by using the computer program QTL Cartographer version 1.14 (Basten et al. 2000) on arcsin $\sqrt{p}$-transformed brittleness data (%). The mapping was run with the default setting for model 6 (five background markers and a window size of 10 cM). The inclusion of background markers makes the analysis more precise and permits efficient mapping of QTLs. A LOD score threshold of 3.0 was used to identify regions containing putative loci associated with the trait. The *btr1* and *btr2* loci were mapped as major gene loci on the base map by using MAPMAKER 3.0 (Lander et al. 1987; Lincoln et al. 1993).

AFLP mapping

We carried out DNA restriction by *Eco*RI and *Mse*I, ligation of adapters, nonselective preamplification, selective amplification using primers having three selective nucleotides, and polyacrylamide-gel electrophoresis, as described by Mano et al. (2001), which was a modification of the standard method described by Vos et al. (1995). We made the following additional modifications: We used a slab-gel electrophoresis apparatus (NA-1214A, Nihon Eido, Tokyo, Japan); a gel size of 1 mm thick, 18 cm high, and 18 cm wide; and 33 lanes. The 7% polyacrylamide gel included 0.5× TBE (1× TBE: 89 mM Tris-borate plus 2 mM EDTA) and 8.5 M urea. After polymerization at room temperature, gels were incubated for 30 min at 50°C, and then prerun in 1× TBE for 30 min at 250 V. The sample run was done at 350 V until bromophenol blue migrated to the end of the gel (approximately 2.5 h) at 50°C. After silver staining, polymorphic fragments in the range of 70–1,100 bp were scored. Nomenclature of AFLP bands followed the system described by Mano et al. (2001).

Bulked-segregant analysis was carried out to identify AFLP markers tightly linked to the *btr1* and *btr2* loci. Two bulked DNA were made by combining preamplified DNA of eight RILs of the *btr1btr1* genotype (nos. 2, 5, 7, 10, 15, 22, 29, and 30) and eight RILs of the *Btr1Btr1* genotype (nos.6, 8, 13, 14, 16, 17, 19, and 38) selected from 99 RILs of 'Azumamugi' × 'Kanto Nakate Gold' on the basis of F_9-generation data (Komatsuda and Mano 2002). All 16 lines were nonrecombinants between the *e14m27-4-1* and *e15m19-7* loci, but three (nos.10, 13, and 30) were recombinants between *e15m19-7* and *e05m19-8*—located proximal to *e15m19-7* (Mano et al. 2001). Inclusion of these three RILs allowed us to eliminate AFLP markers proximal to *e15m19-7*, which were less useful in this study. In total, 4,096 combinations of 64 *Eco*RI+3 and 64 *Mse*I+3 primers were analyzed to detect polymorphism between the two bulks. AFLP markers detected by the analysis were tested against 16 individual DNA used in the bulked-segregant analysis to test the linkage with the *btr1* locus. AFLP markers linked to the *btr1* locus were resolved by using 13 RILs that had a recombination between *e14m27-4-1* and *e15m19-7* (Mano et al. 2001).

Phylogenetic analysis

Fourteen cultivars of *H. vulgare* ssp. *vulgare*, two lines of var. *agriocrithon*, and 19 lines of ssp. *spontaneum* were analyzed by using primer combinations that generated AFLP loci linked to the *btr1* and *btr2* loci. We scored presence (1) or absence (0) of 84 AFLP loci, but subjected only 74 of the 84 loci to phylogenetic analysis, because the bands of the remaining ten loci were not clear. As a comparison, we scored another 46 AFLP loci that were independently inherited with the *btr1* and *btr2* loci and used them to construct another phylogenetic tree. Phylogenetic trees were constructed by the minimum evolution method (Nei and Kumar 2000). Each tree was computed with PAUP 4.0b10 (Swofford 1998). Bootstrap analysis with 1,000 replicates was performed to determine the strength of support for individual nodes.

Results

QTL mapping of brittle rachis genes

Figure 1 shows the percentage of brittle rachis nodes in F_1 plants generated by the crosses of 87 RILs × 'Natsudaikon Mugi' (*btr1btr1Btr2Btr2*), and 92 RILs × 'Hayakiso 2' (*Btr1Btr1btr2btr2*). Each of the frequency distributions shows two modes, "tough" and "brittle," but the distributions are continuous. The RILs × 'Natsudaikon Mugi' population was used for mapping the *btr1* locus as a QTL and other QTLs that complement the genotype of 'Natsudaikon Mugi'. Only one major QTL was detected (Fig. 2, top, LOD=27.4). The QTL was flanked by *e14m27-4-1* and *e15m19-7* on chromosome 3H, falling in the same position as the *btr1* locus, mapped previously (Komatsuda and Mano 2002). Therefore, the major QTL is identical to the *btr1* locus. This QTL (*btr1*) explained 72.4% of phenotypic variance (data not shown), and the allele of 'Azumamugi' (*Btr1*) had a positive effect (+19.8°). The result indicates that the *Btr1* allele from 'Azumamugi' was almost sufficient to complement the genotype of 'Natsudaikon Mugi' to produce brittle rachis.

The RILs × 'Hayakiso 2' population was used for mapping the *btr2* locus and other QTLs, which complement the genotype of 'Hayakiso 2'. Three QTLs were detected (Fig. 2, bottom). A major QTL (LOD=27.9) was located at the same position as the *btr1* locus, between *e14m27-4-1* and *e15m19-7* on chromosome 3H. The result indicates that this QTL is identical to the *btr2* locus, because *btr1* and *btr2* are closely linked (Takahashi and Hayashi 1964). This QTL (*btr2*) explained 70.3% of phenotypic variance, and the allele of 'Kanto Nakate Gold' (*Btr2*) had a positive effect (+18.7°). A secondary QTL (LOD=6.9) was located between *cMWG704* and *e11m17-10-2* on chromosome 7H. The region overlapped the *d* (modifier) locus located between *cMWG704* and *e12m22-10-2* (Komatsuda and Mano 2002), indicating that the QTL and *d* are identical. This QTL explained 10.8% of phenotypic variance, and the allele of 'Kanto Nakate Gold' had a positive effect (+7.4°). The third QTL (LOD=3.7) was located between *e07m25-03* and *e12m19-09-1* on chromosome 5HL. This QTL explained 5.2% of phenotypic variance, and the allele of 'Kanto Nakate Gold' had a positive effect (+5.1°). Thus, 'Kanto Nakate Gold' alleles at all three loci were necessary to complement the genotype of 'Hayakiso 2' to produce brittle rachis.

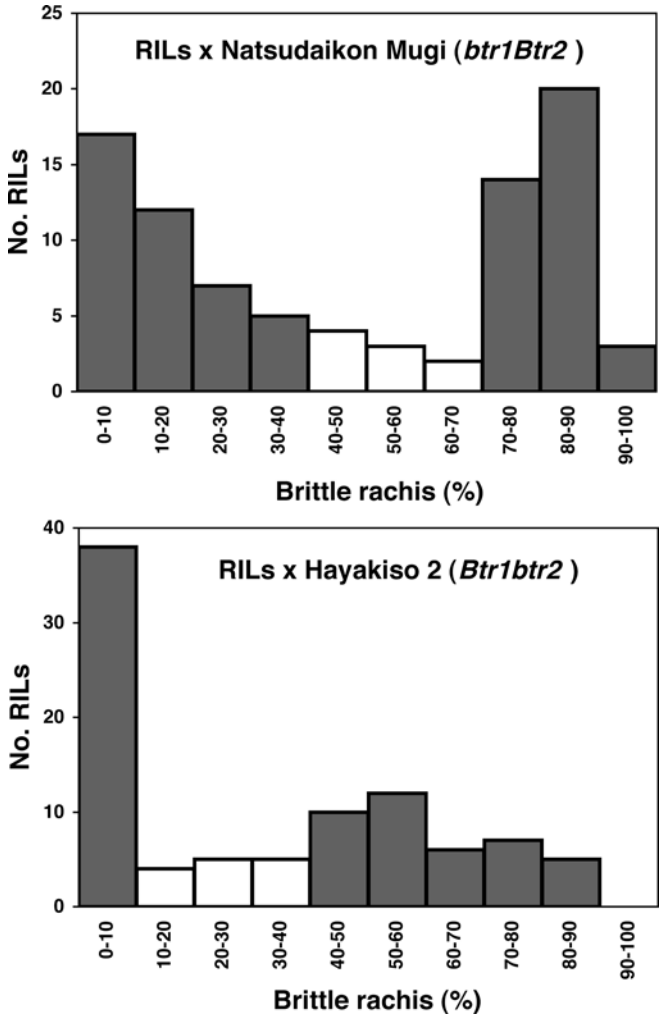

Fig. 1 Percentage of brittle rachis nodes in F_1 families generated by the crosses of barley recombinant inbred lines (RILs) × standard testers. The RILs (F_{11}) were developed from 'Azumamugi' (*Btr1Btr1btr2btr2*) × 'Kanto Nakate Gold' (*btr1btr1Btr2Btr2*), followed by single-seed descent. As testers, 'Natsudaikon Mugi' (*btr1btr1Btr2Btr2*) and 'Hayakiso 2' (*Btr1Btr1btr2btr2*) were crossed to the RILs, and the percentage of brittle rachis nodes was scored for each F_1 family. The sequence of *Btr1_Btr2_* is a necessary condition for the formation of brittle rachis, but modifier genes also have an effect

High-density AFLP map of the *btr1* and *btr2* loci

In the RILs × 'Natsudaikon Mugi' crosses (Fig. 1), we classified 41 F_1 families from 0% to 40% (exclusive) as tough (thus their parental RILs were *btr1btr1*), and 37 F_1 families of 70% and over as brittle (thus their parental RILs were *Btr1Btr1*), and excluded nine ambiguous families between 40% and 70% (exclusive) from the mapping of the *btr1* locus to eliminate misclassification. The *btr1* locus was mapped between *e14m27-4-1* and *e15m19-7* (Fig. 3). In the RILs × 'Hayakiso 2' crosses (Fig. 1), we classified 36 F_1 families from 0% to 10% (exclusive) as tough (thus their parental RILs were *btr2btr2*), and 40 F_1 families from 40% to 90% as brittle (thus their parental RILs were *Btr2Btr2*). We excluded 14 ambiguous F_1 families between 10% and 40% (exclusive) and two families from the class between 0% and 10% (exclusive) because most probably their scores were affected by the QTLs on chromosomes 7H (Electronic Supplementary Material, Fig. 1). The *btr2* locus was mapped in the interval between *e14m27-4-1* and *e15m19-7* (Fig. 3).

A total of 4,096 combinations of 64 *Eco*RI+3 and 64 *Mse*I+3 primers were subjected to the analysis between two bulked DNA, and 438 primer combinations generated 557 polymorphic fragments. The 557 fragments were tested against 16 individual DNA used in the bulked-segregant analysis to eliminate AFLP fragments that exhibited recombination with the *btr1* locus; 148 fragments were retained. These 148 AFLP markers were tested against 13 RILs of 'Azumamugi' × 'Kanto Nakate Gold', which revealed a recombination between *e14m27-4-1* and *e15m19-7* (Mano et al. 2001). As a check, another 22 RILs without recombination between the two AFLP loci were also included. The analysis assigned 64 AFLP loci outside the two AFLP loci and placed the remaining 84 markers on or between the two AFLP loci (Fig. 3). Ten AFLP loci cosegregated with the *btr1* or *btr2* loci. Four AFLP loci were located 0.5 cM distal, and *e37m44-13* was located 2.7 cM proximal to the *btr1/btr2* complex.

An AFLP locus that recombined with flanking AFLP loci on both sides was frequently detected in AFLP mapping by Castiglioni et al. (1998). In this study, RILs nos. 12 and 24 had the alleles of 'Kanto Nakate Gold' at all 84 AFLP loci (Electronic Supplementary Material, Fig. 2), although the F_1 hybrids of these RILs with 'Hayakiso 2' produced tough rachis (<10%, Electronic Supplementary Material, Fig. 1). Since double crossing-over is rare within such a short map distance, the result strongly supports the hypotheses that (1) RILs nos.12 and 24 had the allele of 'Kanto Nakate Gold' (*Btr2Btr2*), and (2) having the allele of 'Azumamugi' (negative effect) at the QTL on chromosome 7H caused the two F_1 hybrids of the RILs with 'Hayakiso 2' to produce tough rachis. Trial inclusion of RILs nos.12 and 24 as *btr2btr2* considerably inflated the map distance of the interval to 12.4 cM, suggesting that the two RILs are not *btr2btr2* (data not shown).

Phylogenetic analysis based on the *btr1/btr2* region

Figure 4 shows an unrooted phylogenetic tree of wild and cultivated barley. The tree was constructed by using 74 AFLP loci assigned in the 6.7-cM region covering the *btr1* and *btr2* loci. The tree shows a clear separation of cultivated barley into two groups. One group consists of "W" (Western)-type barley lines (*btr1btr1Btr2Btr2*). Modern Japanese two-rowed cultivars are included in this group, indicating they have the W-type genes inherited from European barley lines through breeding programs. A Bulgarian cultivar, 'Caveda', and two Moroccan weedy barley lines formed a clade highly supported by bootstrap analysis. A clade of 'Golden Promise', 'Bonus', and 'Kristina' was also highly supported by bootstrap analysis

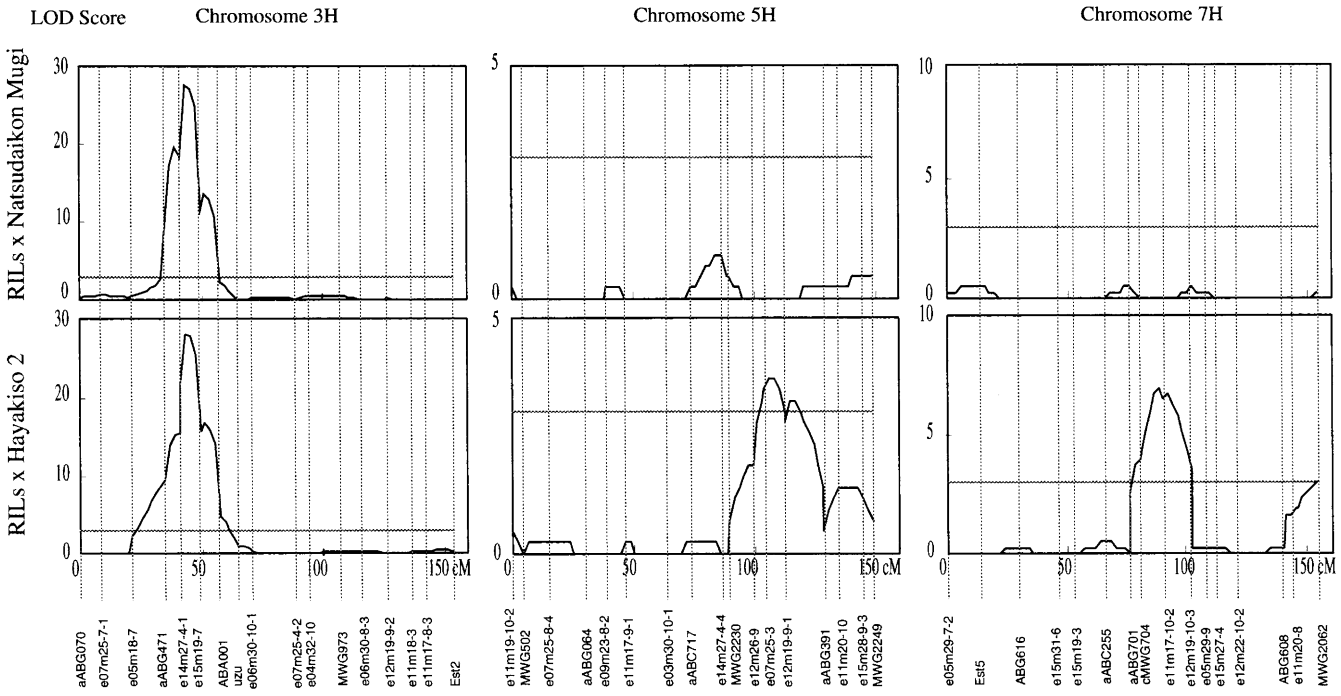

Fig. 2 Detection of quantative trait loci (QTLs) controlling rachis brittleness in barley. RILs of 'Azumamugi' × 'Kanto Nakate Gold' were crossed with 'Natsudaikon Mugi' (*btr1btr1Btr2Btr2, top*) and 'Hayakiso 2' (*Btr1Btr1btr2btr2*, *bottom*). The percentage of brittle rachis was analyzed by composite interval mapping. An LOD score threshold of 3.0 was used to identify regions containing QTLs. QTLs were not detected on chromosomes 5H or 7H in the RILs × 'Natsudaikon Mugi' (*top*) crosses, but LOD graphs are shown to compare with the RILs × 'Hayakiso 2' crosses

and was more or less separated from the other cultivars in the W-type group. A wild barley line from Jordan (OUH638) was included in the W-type group, and two wild barley lines from Iraq (OUH742) and Tibet (OUH825, var. *spontaneum* type) were close neighbors of the W-group. The second group representing "E" (Eastern)-type barley lines (*Btr1Btr1btr2btr2*) included 'Azumamugi', 'Hayakiso 2', and 'Soren Oomugi 19329'. The clade was highly supported by the bootstrap value of 98. Including a wild barley line from Libya (OUH783) and a Tibetan line of var. *agriocrithon* (OUH786) gave the clade the bootstrap value of 100 (Fig. 4). We constructed another tree (Electronic Supplementary Material, Fig. 3) by using 46 AFLP loci inherited independent of the *btr1/btr2* region; the branch length may reveal average distances between accessions based on the whole genome. Wild and cultivated barley lines were separated, and the two var. *agriocrithon* lines were positioned between the wild and cultivated groups. Separation of the E- and W-type cultivars was seen, but not as discretely as shown in Fig. 4. Each pair of wild barley lines originating from the same country formed a clade, indicating their close relationship.

Discussion

Identification of QTLs for nonbrittle rachis

Composite interval mapping revealed that the nonbrittle rachis of 'Azumamugi' is controlled by at least three gene loci: the *btr2* locus and two QTLs on chromosomes 5HL and 7H. Although nonallelic modifier genes have been suggested (Schiemann 1921; Takahashi and Hayashi 1959; Komatsuda and Mano 2002), this study is the first in which modifier genes were precisely identified in the linkage maps. The relatively complicated genetic system for nonbrittle rachis in 'Azumamugi' may represent additional steps of mutation from ancestral barley to landraces or local cultivars. Probably some other oriental cultivars have similar genotypes as 'Azumamugi'. For example, 'Hayakiso 2' did not complement the 'Azumamugi' allele at either of the two QTLs in RILs × 'Hayakiso 2' hybrids, indicating that the two cultivars may have the same alleles at the two QTLs. 'Kanto Nakate Gold' has positive alleles at the two QTLs, and 'Natsudaikon Mugi' probably has the same allele because it complemented the 'Azumamugi' alleles in the F_1 hybrids of RILs × 'Natsudaikon Mugi'.

The QTL on chromosome 7H is probably identical to the dense spike 1 (*dsp1*, formerly *l*) locus, because a major QTL controlling spike internode length (or spike density) was located at the same position in work using the same RILs (Sameri and Komatsuda 2004). The *dsp1* gene occurs naturally in local cultivars from Korea and Japan,

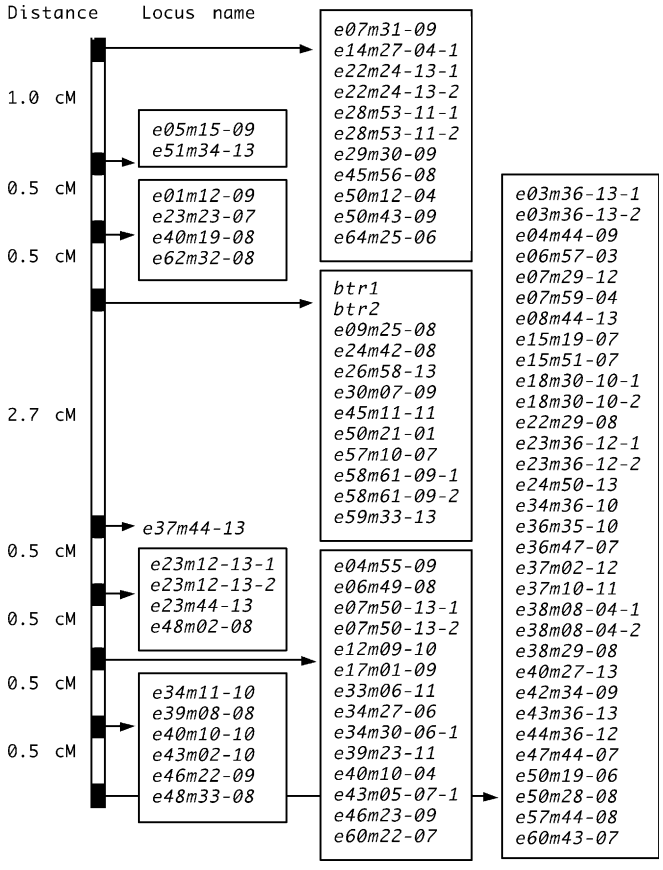

Fig. 3 High-density amplified fragment-length polymorphism (AFLP) map of the *btr1/btr2* complex loci. All 84 AFLP loci between *e14m27-4-1* and *e15m19-7* are included in the map. The *top* is toward the telomere and the *bottom* is toward the centromere of the short arm of chromosome 3H

and both 'Azumamugi' and 'Hayakiso 2' have the *dsp1* allele, whereas 'Natsudaikon Mugi' and probably 'Kanto Nakate Gold' have the normal allele at this locus (Takahashi 1951, 1955; Takahashi et al. 1979, 1983). Segregating F_2 plants with dense spikes tended to show a lower degree of rachis brittleness than those with normal (or lax) spikes (Takahashi and Yamamoto 1949, 1951). Our study clearly demonstrates the effect of spike density on rachis brittleness. In addition to the *dsp1* gene, the *uzu* gene of 'Azumamugi' also reduces the spike internode length. The *uzu* gene occurs in many Japanese and Korean cultivars. It pleiotropically reduces the length of leaves, culms, ears, awns, and glumes (Takahashi 1942), and reduces the degree of rachis brittleness (Takahashi and Yamamoto 1951). No QTL for brittle rachis was detected at the *uzu* locus (Fig. 2) because the recessive *uzu* allele was complemented in F_1 hybrids by normal alleles of the testers. Thus, we do not deny the negative effects of the *uzu* gene or dense spike on rachis brittleness.

Some European two-rowed cultivars have another dense spike gene, which is not allelic to *dsp1* or *uzu* (Takahashi and Yamamoto 1951). 'Kanto Nakate Gold' has a dense spike that is inherited from a European cultivar, 'Golden Melon'. Our recent study detected a QTL for dense spike on chromosome 2H, where an allele of 'Kanto Nakate Gold' reduced spike internode length (Sameri and Komatsuda 2004). No QTL for rachis brittleness was detected on chromosome 2H in this study. The explanation would be that the dense spike allele from 'Kanto Nakate Gold' affects the brittle rachis, but the allele in RILs was complemented by the normal allele of the testers in the F_1 hybrids, or that the dense spike QTL on chromosome 2H has no influence on rachis brittleness.

Some barley cultivars show disarticulation at only one or a few rachis nodes in a spike, resulting in loss of a spike segment. The phenomenon is called "rachis break" or "head shattering" and causes loss of yield. Head shattering QTLs were detected on chromosomes 2H, 3H, and 5HL (Kandemir et al. 2000). Head shattering can be regarded as a special case of brittle rachis, so it is natural that some of the genes or QTLs controlling the two traits are common. The head shattering QTL detected on chromosome 5HL and the brittle rachis QTL that we detected on chromosome 5HL could be identical, but their relative positions remain to be confirmed by the use of common markers. The *btr1/btr2* complex is not allelic to the head shattering QTL on chromosome 3H (Kandemir et al. 2000).

Organization of the *btr1* and *btr2* loci

The *btr1* and *btr2* loci were mapped on chromosome 3HS, and the two loci were linked tightly because no recombinant was obtained from 231 F_2 plants (Takahashi and Hayashi 1964). A question about this gene region is whether it has some structural rearrangements, because E- and W-type barley lines are highly diverged (Takahashi 1955), and the two barley groups show large differentiation in this region. Our high-density map (Fig. 3) includes 84 AFLP loci in the interval of 6.7 cM harboring the *btr1* and *btr2* loci, providing an average density of 0.08 cM/locus. The map shows a fairly even distribution of AFLP loci, except for the clustering detected at one of the flanking markers. Therefore, we did not throw out the hypothesis of even distribution of molecular loci in this region, but insertion/deletion and inversion could not be excluded. Physical mapping is needed to prove this matter.

The results of QTL analysis and the high-density map indicate that the *btr1* and *btr2* loci did not recombine (Fig. 3). In earlier genetic studies of brittle rachis (Takahashi and Yamamoto 1949, 1951), 'Paisha Tayeh 1' (China, *Btr1Btr1btr2btr2*), 'Tammi' (Finland, *btr1btr1Btr2Btr2*), and 'Natsudaikon Mugi' (Korea, *btr1btr1Btr2Btr2*) were used as testers. Later "two kinds of testers" (names unspecified) were used in a series of studies at Okayama University (Takahashi 1955; Takahashi and Hayashi 1964; Takahashi et al. 1983). If one F_1 made by a cross with a W-type tester was tough and another made by a cross with an E-type tester was brittle, the cultivar was classified as W-type. If the relationship was the reverse, it was classified as E-type. Takahashi (1963) reported on the basis of the analysis of 1,267 cultivars from different regions of the world that all cultivars were either E-type or W-type, and none was

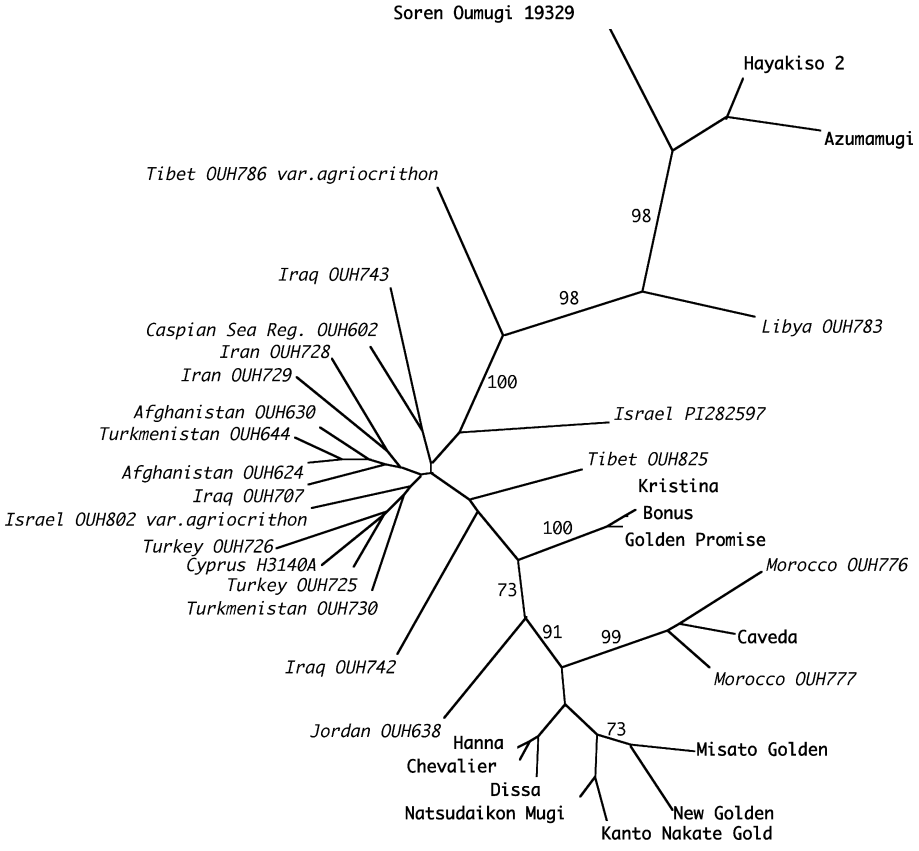

Fig. 4 Evolutionary trees for barley obtained from analyses of 74 AFLP loci linked to the *btr1* and *btr2* loci for brittle rachis. Brittle, mostly wild barley lines are represented by country or regional names, followed by accession numbers in *italics*. The two Moroccan lines are brittle-rachis, two-rowed barleys claimed to be wild ssp. *spontaneum*. (Molina-Cano et al. 1982, 1999). The var. *agriocrithon* lines are brittle-rachis, six-rowed barley classified in the group of *H. vulgare* ssp. *vulgare* (Bothmer and Jacobsen 1985). Nonbrittle barley lines are represented in *boldface*; 'Soren Oumugi 19329', 'Hayakiso 2', and 'Azumamugi' are Eastern type (*btr2*), and the others are Western type (*btr1*). The minimum evolution method (Nei and Kumar 2000) was used. Bootstrap values >60% are shown

btr1btr1btr2btr2 type. Why have double-recessive types not been identified? The most adequate explanation is that the two loci are physically too close to allow the breeding of recombinant lines (Takahashi and Hayashi 1964; this study). However, it is not clear why a double-recessive *btr1btr1btr2btr2* does not occur by spontaneous mutation in cultivated barley. Does it exists in other cultivars but has escaped detection? One explanation is that double-recessive plants are lethal, although this explanation does not account for the fact that double-dominant *Btr1Btr1Btr2Btr2* was not bred in segregating F_2 or RILs (Takahashi and Hayashi 1964; and this study). We maintain the hypothesis of tight linkage between the *btr1* and *btr2* loci. The hypothesis may be tested by simply increasing the size of segregating populations followed by testcrosses. 'Hayakiso 2' may not be the best tester, because it has a *dsp1* gene. It would be favorable to use barley lines with lax spike density in further studies.

Domestication patterns indicated by molecular loci in the *btr1/btr2* region

Wild and cultivated barley are positioned at subspecies level because the two taxa are interfertile (Bothmer et al. 1995). Gene flow between them caused by natural pollinators is possible, so it is difficult to define what is wild barley and what is cultivated barley. Nonbrittle rachis therefore could be used to discriminate cultivated barley from wild barley. The phylogenetic tree based on the AFLP loci linked to the *btr1/btr2* loci (Fig. 4) suggested that these are two completely separate groups of plants and may represent the gene genealogy for the brittle-rachis genes. Analysis of other genes may give other or more complex views, and of course more number of entities as well as DNA sequence data of genes are preferred in further studies. The following discussion is based mainly on the AFLP markers of nonbrittle rachis gene that showed clear separation of barley cultivars into two types.

The tree clearly shows the differentiation of cultivated barley into two clades, E-type and W-type, in good agreement with the differentiation pattern of cultivated barley (Takahashi 1955). The other phylogenetic tree that we constructed is based on AFLP loci randomly distributed in the whole genome (Electronic Supplementary Material, Fig. 3), and accords with the tree produced by Badr et al. (2000) because it makes no special separation within cultivated barley. The two trees (Fig. 4; Electronic Supplementary Material, Fig. 3) show a clear contrast and highlight the contribution of brittle rachis genes to barley domestication. The following discussion focuses on the *btr1/btr2* region (Fig. 4).

What is the origin of var. *agriocrithon* inferred from the analysis? Var. *agriocrithon* is a six-rowed, brittle form of barley. Åberg (1938) first discovered the seeds among seed samples brought from southwest China, naming the taxon *H. agriocrithon* E. Åberg and regarding it to be a wild ancestor of six-rowed barley. Later Freisleben (1943) found seeds of var. *agriocrithon* in seed samples from Tibet and regarded it to be a primitive cultivated form of

six-rowed barley. Bothmer et al. (1995) considered it to be either a secondary mutation or a weedy hybridization segregant. Recently Konishi (2001) proposed the origin of Tibetan var. *agriocrithon* to be natural hybridization between ssp. *spontaneum* and six-rowed cultivated barley. In this theory, var. *agriocrithon* and ssp. *spontaneum* must have a close relationship regarding the *btr1/btr2* region. In the present study, the Tibetan line of var. *agriocrithon* (OUH786) was distinctly separate from the ssp. *spontaneum* group, being much more distantly separated from the Tibetan line of ssp. *spontaneum* (OUH825). Therefore, the result was not consistent with the theory of Konishi (2001). Two other hypotheses (Bothmer et al. 1995) are that var. *agriocrithon* originated from hybridization between six-rowed cultivated barley lines of E- and W-types followed by recombination to breed *Btr1Btr1Btr2Btr2*, and that reverse mutations from non-brittle cultivars to brittle forms have been maintained in Tibetan fields. In each case, the Tibetan line of var. *agriocrithon* is expected to be closely related to cultivated barley lines. However, it lies intermediate between the group of E-type cultivated lines and the central cluster of ssp. *spontaneum* in the phylogenetic tree (Fig. 4). Therefore, our result does not support the hypotheses. var. *agriocrithon* has been found frequently in Israel, Cyprus, and Libya (for review see Bothmer and Jacobsen 1985). In this case our results agree with the hypothesis of hybrid origin between ssp. *spontaneum* and six-rowed cultivated barley (Bothmer et al. 1995), because the var. *agriocrithon* from Israel (OUH802) was included in the group of ssp. *spontaneum* (Fig. 4). Probably OUH802 has a *btr1/btr2* gene region obtained from sympatric populations of ssp. *spontaneum*. The var. *agriocrithon* lines from Tibet (or China) and Israel may have different origins.

It seems surprising that a Libyan wild barley line (OUH783) has a strong connection with the E-type cultivars. However, more than 90% of North African barley cultivars carry E-type gene sequences for brittle rachis (Takahashi et al. 1983), in clear contrast to the fact that more than 95% of Abyssinian barley cultivars carry W-type gene sequences (Takahashi 1963; Takahashi et al. 1983). Our phylogenetic analysis suggests that the E-type barley lines of Asia and North Africa have the same lineage.

The W-type group shows a strong connection with a wild barley line from Jordan (Fig. 4). This result agrees in part with the connection between monophyletic cultivated barley and Israeli–Jordanian wild barley lines found in the study of Badr et al. (2000). However, in the present study the E-type cultivars were not connected with the wild barley line from Jordan (Fig. 4). Two Moroccan weedy lines and 'Caveda' formed a highly supported clade. These three accessions have a common haplotype (D-type) for a DNA sequence tightly linked to the *vrs1* locus (row type, the other trait associated with domestication of barley, Tanno 1999; Tanno et al. 1999). The D-haplotype was sporadically found in a small fraction of barley germplasm from southern Europe and North Africa (Tanno 1999; Tanno et al. 2002). The two Moroccan lines had two-rowed, brittle spikes, were collected as weeds in common six-rowed barley fields, and were assumed to be of wild origin, ssp. *spontaneum* (Molina-Cano et al. 1982, 1999). The tree shows that the Moroccan lines are unique and separated from the majority of wild barley lines. The origin of the Moroccan wild lines and their connection to the D-type barley lines remains to be further studied with respect to *btr1/btr2* by using weedy lines from North Africa and cultivated barley lines from Europe.

As in barley (*Hordeum*), brittle-rachis genes are located on homeologous group 3 chromosomes in *Triticum*, *Aegilops*, *Dasypyrum*, and *Thinopyrum* (Watanabe and Ikebata 2000 and references cited therein). This coincidence suggests that the brittle-rachis genes are orthologous in the tribe Triticeae, although comparative mapping has not been constructed. Barley could be a representative of the Triticeae in the challenge to clone the brittle-rachis genes because it is diploid and rich in genetic diversity, and molecular resources such as high-density linkage maps, YAC and BAC libraries and enormous EST databases have been developed. In rice (*Oryza* spp.), seed shattering is controlled by multiple genes or QTLs (Eiguchi and Sano 1990; Fukuta et al. 1996; Cai and Morishima 2000), and a single gene controls nonshattering in the special case of isogenic lines and mutants (Oba et al. 1990; Fukuta 1995; Sanchez et al. 2002). The major shattering QTL on rice chromosome 1 (Fukuta et al. 1996) would be a candidate as an orthologue of the nonbrittle rachis loci on barley chromosome 3, because the two chromosomes show synteny. This matter may be clarified by rice and barley in silico mapping. In wild rice (*Zizania* spp.), two complemental genes or three QTLs control seed dispersal (Elliott and Perlinger 1977; Kennard et al. 2002). Furthermore, ten QTLs have been identified in maize (Paterson et al. 1995). On the other hand, sorghum shows an exceptional pattern, where nonshattering mapped to a single locus accounts for ~100% of phenotypic variance (Paterson et al. 1995). These studies suggest that seed dispersal is under the control of a set of multiple epistatic genes and reveal various mutation processes in the domestication of cereal crops.

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