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Y. Mano · T. Komatsuda

Identification of QTLs controlling tissue-culture traits in barley (*Hordeum vulgare* L.)

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Abstract Quantitative trait loci (QTLs) controlling callus growth (CG), subsequent shoot differentiation ratio (SD) and green shoot ratio (GS) in immature embryo culture were identified in barley. A base map was developed from 99 recombinant inbred lines (RILs) of 'Azumamugi' × 'Kanto Nakate Gold'. The tissue-culture traits were evaluated at the F_7 and F_{10} generations of the RILs. The RILs showed wide and continuous variations in each of the three tissue-culture traits. Three QTLs for CG, three QTLs for SD and two QTLs for GS were detected by using composite interval mapping. A QTL for SD on chromosome 3H had a large effect, and 'Kanto Nakate Gold', which has a high differentiation ability, contributed to this QTL. The location of this QTL is identical to, or very close to, the *uzu* locus. We discuss the relationships between tissue-culture loci in 'Azumamugi' × 'Kanto Nakate Gold' and those in other mapping populations.

Keywords Albinism · *Hordeum vulgare* · Quantitative trait loci · Recombinant inbred · Shoot differentiation

Introduction

Suitable culture conditions and the selection of a genotype with high differentiation frequency in in vitro tissue culture are needed for the efficient acquisition of transgenic plants (Ritala et al. 1994; Tingay et al. 1997). Several authors have investigated culture conditions and explant sources, but no culture conditions suitable for all genotypes have been established. Genetic studies of tissue-culture traits, such as callus growth, embryogenesis and differentiation, will make it possible to transfer genes controlling desirable tissue-culture traits into recalcitrant cultivars or species.

Tissue-culture traits are quantitatively controlled, and restriction fragment-length polymorphism (RFLP) markers linked to genes controlling somatic embryogenesis or shoot differentiation ability have been identified in cereal crops such as maize (Armstrong et al. 1992), rice (Taguchi-Shiobara et al. 1997) wheat (Ben Amer et al. 1997) and barley. In barley, quantitative trait loci (QTLs) controlling shoot differentiation ability have already been mapped on several chromosomes by QTL analysis throughout the genome (Mano et al. 1996; Takahashi et al. 1997; Manninen 2000), but major QTLs for this trait have not yet been identified. In order to detect those loci that have major effects on shoot differentiation ability, QTL analysis of a segregating population derived from a cross between parents showing extreme differences in their differentiation abilities is required.

Komatsuda et al. (1989) selected the barley cultivars 'Azumamugi', which has extremely low shoot differentiation ability, and 'Kanto Nakate Gold', which has extremely high differentiation ability. In a BC_1F_1 population of this cross, linkage relationships were observed between a QTL for shoot differentiation ability (*Shd1*) and the *vrs1* locus (row-type) on chromosome 2H, and also between another QTL for shoot differentiation ability and the *uzu* locus (semi-brachytic) on chromosome 3H (Komatsuda et al. 1993). By further analysis of BC_5F_1 plants, the detailed position of *Shd1* was determined in the MWG2081–MWG503 interval (Komatsuda et al. 1995). However, the map position of the QTL associated with the *uzu* locus has not been determined, and the whole barley genome has not yet been analyzed for other QTLs.

Here we report on the identification of loci controlling tissue-culture traits throughout the barley genome by using recombinant inbred lines derived from a cross between 'Azumamugi' and 'Kanto Nakate Gold'. Tis-

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Y. Mano (✉) · T. Komatsuda
National Institute of Agrobiological Sciences,
Tsukuba, Ibaraki 305-8602, Japan
e-mail: mano@affrc.go.jp
Tel.: +81-287-37-7690, Fax: +81-287-36-6629

Present address:
Y. Mano, National Institute of Livestock and Grassland Science,
Nishinasuno, Tochigi 329-2793, Japan

sue-culture traits were evaluated in the F_7 and F_{10} generations.

Materials and methods

Plant materials

We used 99 recombinant inbred lines (RILs) of 'Azumamugi' (AZ) × 'Kanto Nakate Gold' (KNG), which were developed by the single-seed descent method.

Tissue culture

Immature embryos were cultured in three experiments: the F_7 generation (developing seeds on F_7 plants) was evaluated without replication in 1996, and the F_{10} generation was evaluated with two replications (F_{10} rep. 1, F_{10} rep. 2) in 1999. The culture was performed essentially as described by Komatsuda et al. (1989), but we used different culture media.

The culture medium for callus induction was composed of inorganic salts and Fe of MS medium (Murashige and Skoog 1962) with vitamins of the B5 medium (Gamborg et al. 1968), and was supplemented with 30 g/l of maltose, 4 mg/l of 2,4-D, 150 mg/l of glutamine, 30 mg/l of proline and 20 mg/l of asparagine. The medium was adjusted to pH 6.0 with KOH, mixed with 0.8% agar (Wako, Osaka, Japan), and autoclaved at 121 °C for 15 min. The differentiating medium was the same as the callus-induction medium, except that it had a 1/10 concentration of inorganic salts and no hormones. KNG showed the highest frequency of shoot differentiation in these media (T. Komatsuda and M. Komatsuda, unpublished).

Three weeks after callus induction, contaminated or directly germinated embryos were discarded, and then callus growth (CG) was evaluated by measuring mean callus diameter (mm). The calluses were transferred onto the differentiation medium without being subdivided. Six weeks after callus induction, the shoot differentiation ratio (SD) was evaluated by calculating the number of calluses with differentiating shoots (green, albino or both) as a proportion of the total number of calluses. Also, we counted the average number of differentiated shoots per callus (NS) in each RIL, and compared the repeatability of the two indices (SD vs NS). The green-shoot ratio (GS) was evaluated by calculating the number of green shoots as a proportion of the total number of shoots using only highly differentiating RILs (with over 15 differentiated shoots). In each experiment, the total number of calluses analyzed ranged from 15 to 52 (average: 28.6 in the F_7, 33.6 in the F_{10} rep. 1, and 27.1 in the F_{10} rep. 2) for each RIL and from 62 to 100 for the parents.

ANOVA

Analysis of variance (ANOVA) was performed for the tissue-culture traits in the F_{10} generation. Heritabilities (h^2) were calculated from the ANOVA as $h^2 = \sigma^2_G/(\sigma^2_G + \sigma^2_E/r)$, where $\sigma^2_G = [(MS_{genotype} - MS_{Error})/r]$, $\sigma^2_E = MS_{Error}$, and r = number of replications.

QTL analysis

We constructed a linkage map with 272-point markers for the RILs at the F_9 generation. This provided an average density of 6.5 cM/locus, with considerable marker clustering in certain regions (Mano et al. 2001). We selected a subset of 100 markers, to provide a density of 5- to 10-cM intervals without the clustering markers, and reconstructed a "base map" with these markers by using MAPMAKER 3.0 (Lander et al. 1987; Lincoln et al. 1992).

In the F_{10} generation, tissue-culture data from two replications were pooled, and the resulting data set was used for QTL analysis. Since the tissue-culture response was affected by the growing environment of the explant, QTL mapping of tissue-culture traits in the F_7 generation (evaluated in 1996) and the F_{10} generation (evaluated in 1999) were analyzed separately.

QTL mapping for CG, SD and GS (SD and GS were arcsin-transformed) was performed by composite interval mapping (CIM) using the computer program QTL Cartographer Version 1.14 (Basten et al. 2000). CIM was run with the default setting for model 6 in the program (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 log-likelihood (LOD)-score threshold of 3.0 was used to identify regions containing putative loci associated with tissue-culture traits. Since most of the loci (theoretically 98.4%) were fixed to be homozygous in the F_7 generation, the tissue-culture data in the F_7 generation were analyzed by using the F_9 linkage map.

The proportion of the genotypic variance explained by a QTL (Q_G) was calculated as $Q_G = Q_P/h^2$, where Q_P was the proportion of the phenotypic variance explained by the QTL. In the F_7 generation, h^2 and Q_G were not calculated, because there was no replication test.

Results

Phenotypic variations and heritability of traits

CG in the parental varieties measured an average of 6.9 mm for AZ and 7.6 mm for KNG in the environment used for F_{10} culture. CG showed a continuous distribution, with transgressive segregation among the F_{10} RILs (4.5 to 11.3 mm, average 6.9 mm, data not shown). ANOVA of the 72 RILs evaluated in both the F_{10} rep. 1 and the F_{10} rep. 2 showed a significant genotypic effect for CG (Table 1), and h^2 was estimated at 0.615. CG was not measured in the F_7 generation.

The frequencies of SD in the parental varieties differed to an extreme degree, at 2.9% for AZ and 93.8%

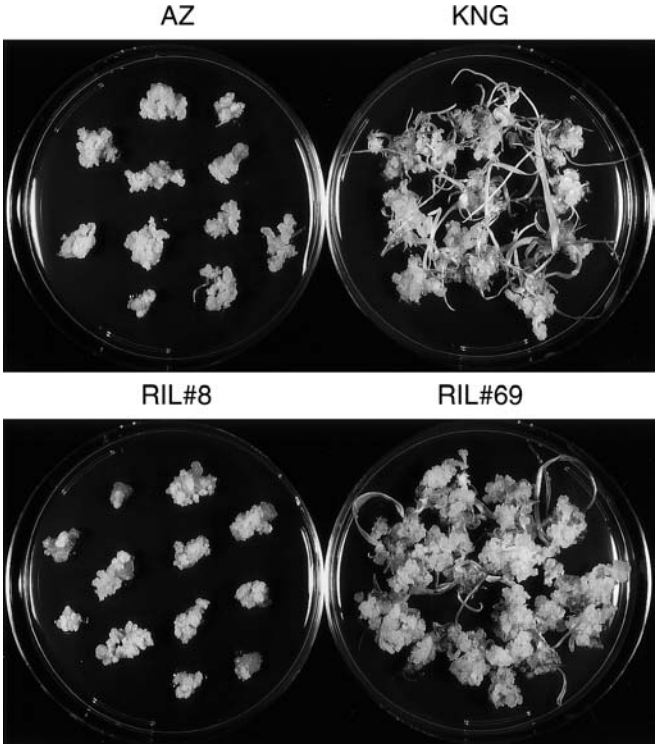

Fig. 1 Callus and shoot differentiation in immature embryo culture of AZ, KNG, RIL#8, and RIL#69

Table 1 Analysis of variance for callus growth (CG), shoot differentiation (SD), and green shoot ratio (GS) in the F_{10} generation

Source	CG		SD		GS	
	df	MS	*df*	MS	*df*	MS
Genotype	71	3.213**	71	882.01**	37	783.70**
Block	1	31.260**	1	139.13	1	2,884.61**
Residual	71	1.238	71	144.59	37	185.90

**: significant at the 1% level

for KNG in the environment used for F_{10} culture (Fig. 1). The RILs also showed an extremely wide and continuous frequency distribution for SD (0% to 97.7% in the F_7 generation and 0% to 100.0% in the F_{10} generation; Fig. 2). ANOVA of the F_{10} data showed a highly significant genotypic effect (Table 1), and h^2 was estimated at 0.836. The correlation coefficient (r) for SD between replications of the F_{10} generation was 0.727 (significant at the 0.1% level). The values of h^2 and r were higher than those for the average number of differentiated shoots per callus (h^2 = 0.804; r = 0.696, significant at the 0.1% level). Therefore, we adopted SD for QTL analysis.

RILs with over 15 differentiated shoots (58 in the F_7 generation and 65 in the F_{10} generation) were selected for the analysis of QTL mapping of GS. The GS showed a wide range among the RILs, from 5.6% to 98.0% in the F_7 generation and from 0% to 100.0% in the F_{10} generation (data not shown). The GS of KNG was moderate, at 43.0% in the environment used for F_{10} culture, whereas that of AZ was unknown owing to this cultivar's poor differentiation ability. ANOVA of the 38 RILs evaluated in both the F_{10} rep. 1 and the F_{10} rep. 2 showed a significant genotypic effect for GS (Table 1), and h^2 was estimated at 0.763.

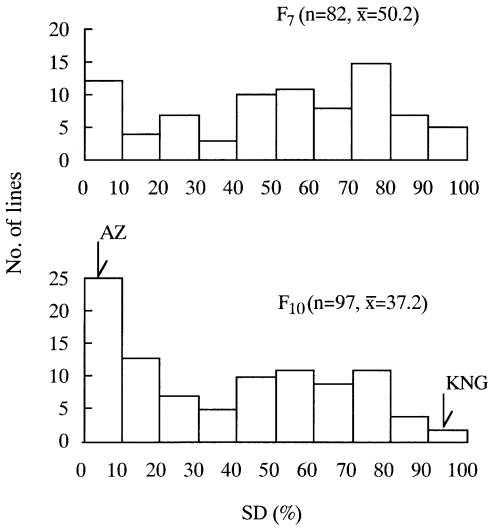

Fig. 2 Frequency distribution for shoot differentiation (*SD*) of the RILs derived from AZ × KNG. The SD of the parents was not evaluated in the environment used for F_7 culture

Mapping QTLs for CG

The CIM analysis detected two loci on chromosome 2H and one locus on 5H controlling CG in the F_{10} generation (Fig. 3). Of these, one QTL linked to the *vrs1* locus on chromosome 2H had a large effect, with a LOD score of 8.64. This QTL accounted for 31.3% of the total phenotypic variance and 50.9% of the genotypic variance (Table 2).

Mapping QTLs for SD

The CIM analysis detected three loci controlling SD on chromosomes 1H, 2H and 3H (Fig. 4), and KNG alleles increased SD in the case of all QTLs (Table 2). A QTL with a large effect was found on chromosome 3H, and this locus was identical with, or very close to, the *uzu* locus. The peak LOD score was 9.64 in the F_7 generation and 17.81 in the F_{10} generation. This QTL accounted for 35.9% of the total phenotypic variance in the F_7 generation, and for 42.8% of the phenotypic, and 51.2% of the genotypic, variance in the F_{10} generation (Table 2), showing a consistent effect across the two growing environments of the explants.

The analysis also found a QTL on chromosome 2H with a LOD score of 5.02. This QTL accounted for

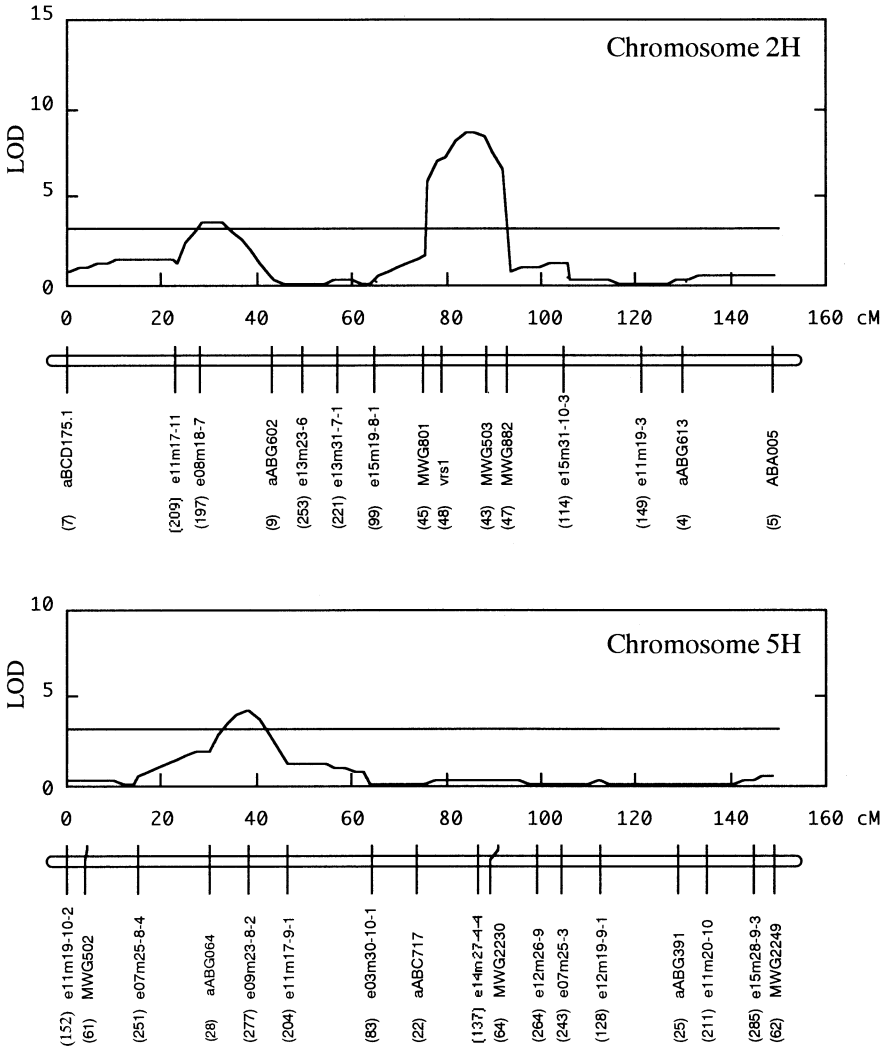

Fig. 3 Chromosome locations of QTLs for callus growth in the RILs of AZ × KNG on chromosomes 2H and 5H. Short arms are on the left. QTL analysis of CG was performed only in the F_{10} generation

Table 2 Chromosome locations and modes of gene action of QTLs for callus growth (CG), shoot differentiation (SD), and green shoot ratio (GS) in the RILs of AZ × KNG

Chromosome	Nearest marker	CG F_{10}	SD		GS	
			F_7	F_{10}	F_7	F_{10}
1H	(169) e06m30-8-2		3.86[a] 7.44[b] K[c] 0.119[d]			
2H	(197) e08m18-7	3.46 0.510 A 0.120 (0.195)[e]				
	(48) *vrs1*	8.64 0.813 K 0.313 (0.509)		5.02 6.73 K 0.100 (0.120)		
3H	(49) *uzu*		9.64 14.00 K 0.359	17.81 14.31 K 0.428 (0.512)		
5H	(277) e09m23-8-2	4.17 0.488 K 0.116 (0.189)				
7H	(66) cMWG704				3.32 7.47 A 0.177	4.84 10.02 A 0.186 (0.244)
	(306) e12m22-10-2					6.81 11.11 A 0.228 (0.299)

[a] LOD score
[b] Additive effect
[c] Letter suffix indicates parent contributing a higher-value allele, where A = 'Azumamugi' and K = 'Kanto Nakate Gold'
[d] Proportion of the phenotypic variance explained
[e] Proportion of the genotypic variance explained. In the F_7 generation, the proportion of the genetic variance explained by the QTL was not calculated, because no replication test was done

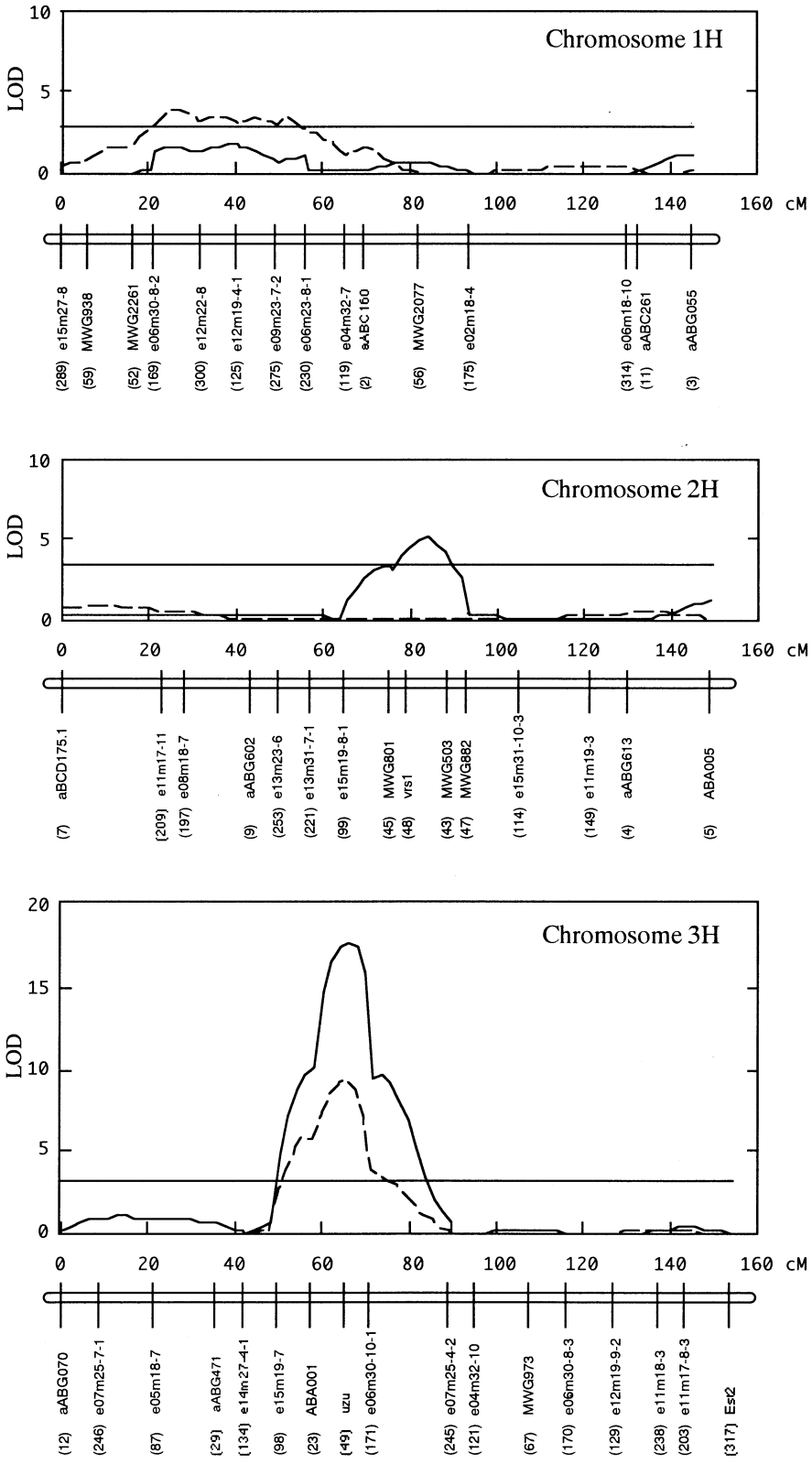

Fig. 4 Chromosome locations of QTLs for shoot differentiation in the RILs of AZ × KNG on chromosomes 1H, 2H, and 3H. Short arms are on the left. *Dotted and solid lines* indicate the F_7 and F_{10} generations, respectively

10.0% of the phenotypic, and 12.0% of the genotypic, variance (Table 2). The position of the QTL was in close agreement with that of *Shd1*, previously reported by Komatsuda et al. (1995). In the region of *Shd1*, the QTL for CG was also identified (Table 2), suggesting that *Shd1* may have a pleiotropic effect in increasing CG. Another QTL, with a LOD score of 3.86 and accounting for 11.9% of the total phenotypic variance, was found on chromosome 1H in the F_7 generation (Table 2).

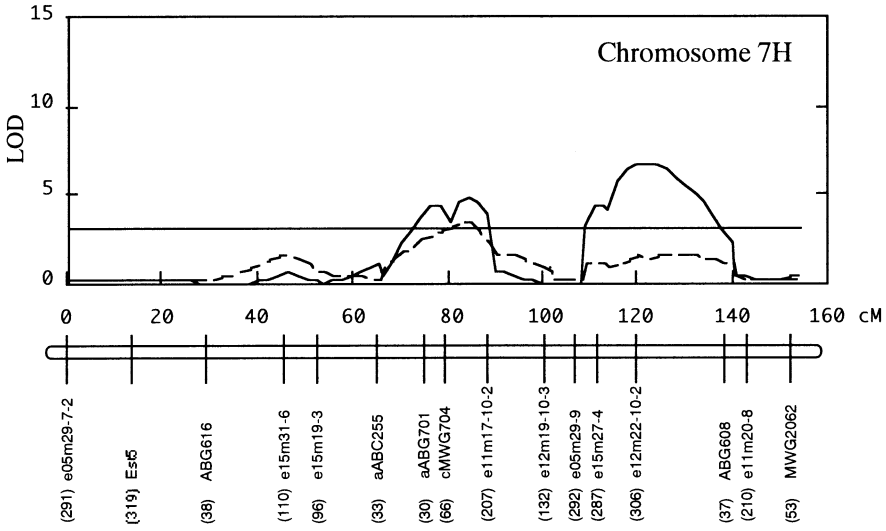

Fig. 5 Chromosome locations of QTLs for green shoot ratio in the RILs of AZ × KNG on chromosome 7H. The short arm is on the left. *Dotted and solid lines* indicate the F_7 and F_{10} generations, respectively

Mapping QTLs for GS

By CIM analysis in the F_{10} generation, two well-dissociated QTLs for GS were identified on chromosome 7H (Fig. 5); they were not resolved by simple interval mapping analysis (data not shown). Of these, one QTL, beside cMWG704, was stable over the different growing environments of the explants, and AZ alleles increased GS. This QTL accounted for 17.7% of the total phenotypic variance in the F_7 generation, and 18.6% of the phenotypic, and 24.4% of the genotypic, variance in the F_{10} generation (Table 2).

Comparison of QTLs across four mapping populations

QTLs for CG in immature embryo culture of barley have already been mapped on chromosomes 1H, 2H and 5H in the Harrington (HA) × TR306 (TR) cross (Takahashi et al. 1997), and on chromosomes 2H and 3H in the Steptoe (ST) × Morex (MO) cross (Mano et al. 1996). Figure 6 shows a comparison of QTLs for CG across AZ × KNG (this study), HA × TR and ST × MO using common STS or RFLP markers. The position of the QTL for CG on the short arm of chromosome 2H in AZ × KNG (nearest marker is e08m18-7) was identical to, or very close to, that of the HA × TR cross (nearest marker is Pox). The locations of other loci for CG were different from each other (data not shown).

QTLs for SD were found on chromosomes 1H, 2H and 3H in the AZ × KNG cross (this study), on chromosomes 2H and 4H in the HA × TR cross (Takahashi et al. 1997), on chromosomes 2H, 3H, 5H and 6H in the ST × MO cross (Mano et al. 1996) in immature embryo culture, and on chromosomes 2H and 3H in the Rolfi (RO) × Botnia (BO) cross in anther culture (Manninen 2000). The position of the QTL on chromosome 2H was identical to, or nearly the same, across the four mapping populations (Fig. 6). In particular, the position of the QTL detected in AZ × KNG (nearest marker is *vrs1*) obviously overlapped with that in the HA × TR cross (nearest marker is MWG865). In addition, the QTL for SD on chromosome 3H was also identified at nearly the same position across the three mapping populations.

Discussion

The mapping population used in this study is suitable for QTL analysis of SD, because the parents of the RILs showed extreme differences in their differentiation ability, and the RILs provided sufficient seeds for replicated experiments. By QTL analysis throughout the whole barley genome, we identified three QTLs that had significant effects in controlling SD (Table 2, Fig. 4). Of these, the QTL on chromosome 2H (*Shd1*) has already been reported by Komatsuda et al. (1993), whereas the one on chromosome 1H was newly identified in this study. Also, we determined a detailed map position for the QTL controlling SD on chromosome 3H, a QTL that has previously been reported to be associated with the *uzu* locus (Komatsuda et al. 1993).

Expression of the QTL for SD associated with the *uzu* locus on chromosome 3H was highly consistent over environments, and the allele of KNG (non-*uzu* type) increased SD (Table 2). In the varietal screening of SD in barley, none of the *uzu* varieties showed a high frequency of SD (Ohkoshi et al. 1991). In wheat, the GA-insensitive dwarfing genes *Rht1*, *Rht2* and *Rht3* acted to decrease the frequency of SD (Mathias and Atkinson 1988), whereas the GA-insensitive dwarfing genes of wheat *Rht8* and rye *ct1* and *ct2* appeared not to influence the tissue-culture traits (Ben Amer et al. 1992). The different actions of the GA-insensitive dwarfing genes to tissue-culture traits may be due to the presence (*Rht1*, *Rht2* and *Rht3*) or absence (*Rht8*, *ct1* and *ct2*) of the pleiotropic effect on decreasing grain size that could be the secondary pleiotropic effect responsible for the decrease in callus size and green spot initiation (Ben Amer et al. 1992). The *uzu* gene has pleiotropic effects on sev-

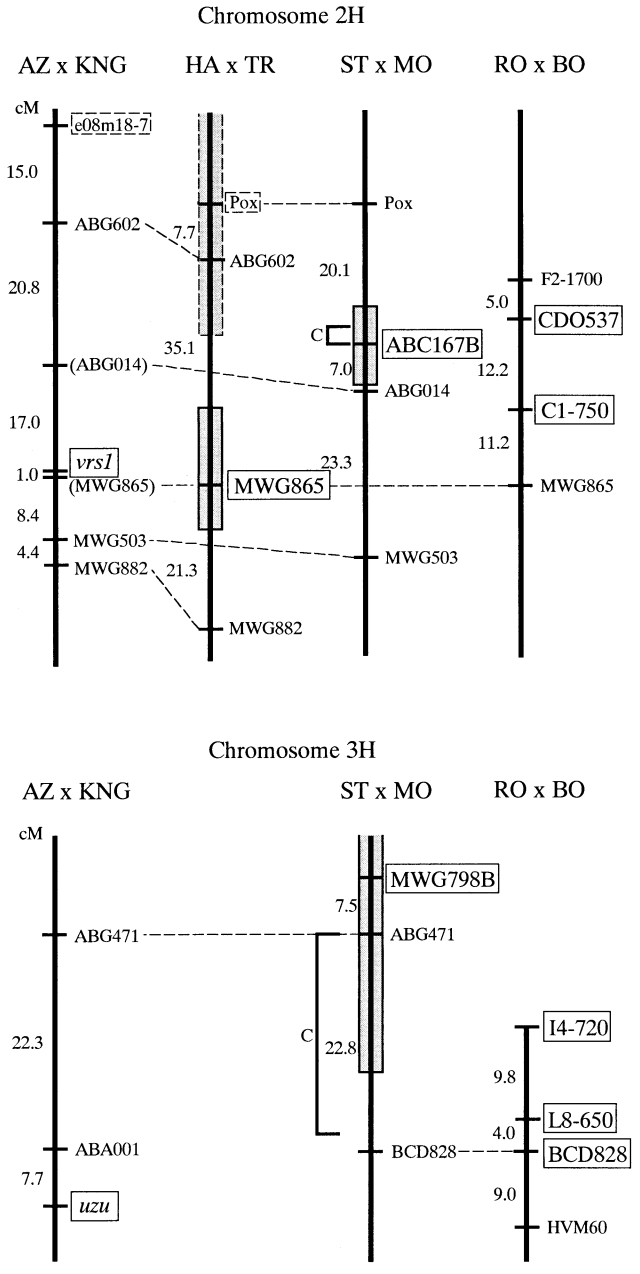

Fig. 6 Comparisons of QTLs for shoot differentiation and callus growth across four mapping populations. QTL maps of AZ × KNG (this study), Harrington (HA) × TR306 (TR) (Takahashi et al. 1997), Steptoe (ST) × Morex (MO) (Mano et al. 1996) and Rolfi (RO) × Botnia (BO) (Manninen 2000) were compared for chromosomes 2H and 3H. Chromosomes are oriented with the short arms on top. "*C*" indicates the position of the centromere. Markers in *solid boxes* indicate the nearest (AZ × KNG, HA × TR, and ST × MO crosses) or significant (RO × BO cross) markers of the QTLs for SD. Markers in *dotted boxes* indicate the nearest markers of the QTLs for CG. The *shaded regions* of chromosomes indicate the confidence intervals of the QTLs of SD or CG. Markers in parentheses are not included in the base map, but were mapped in the study of Mano et al. (1999). The distal marker on chromosome 3H of the RO × BO cross is I4-720

eral agronomic characters including the reduction of grain size (Takahashi and Yamamoto 1951); thus the relationship between SD and small grain caused by *uzu* gene must be investigated.

The *uzu* gene also has a pleiotropic effect in reducing auxin production (Kuraishi 1974), thus possibly affecting the tissue-culture response. By physiological analysis of the *uzu* gene in in vitro tissue culture using near-isogenic lines for the gene (Takahashi et al. 1963), it may be possible to reveal the unique hormonal production of the *uzu* gene and to establish tissue-culture conditions suitable for poorly differentiating cultivars. Another possibility could be that the QTL for SD on chromosome 3H is non-allelic to the *uzu* locus, but is allelic to the QTL detected in the non-*uzu* parent cross, RO × BO (Manninen 2000) (Fig. 6); but further study using common markers is necessary.

Shd1 on chromosome 2H has been consistently identified in the BC_1F_1 and BC_5F_1 generations (Komatsuda et al. 1993, 1995), and in the F_{10} generation (this study). In the F_7 generation, however, *Shd1* was not detected (Fig. 4). Of the 99 RILs, we evaluated 97 for their SD in the F_{10} generation and 82 in the F_7 generation. The 15 missing lines in the F_7 generation may have affected the detection of *Shd1*. When CIM was performed in the F_{10} generation by using the 82 data sets common to the F_7 and F_{10} generations, *Shd1* was also identified (data not shown). Therefore, the absence of *Shd1* in the F_7 generation was not caused by the fact that there were missing lines. It may have been caused by the differences in environmental conditions between the F_7 (evaluated in 1996) and the F_{10} generation (evaluated in 1999).

Larsen et al. (1991) reported genetic factors affecting GS in barley anther culture. In the QTL mapping, however, molecular markers linked to GS were not detected, partly because of a lack of variation in the parents (Manninen 2000). We found two QTLs for GS on chromosome 7H, and the AZ allele of these QTLs increased GS (Table 2, Fig. 5). In the study by Komatsuda et al. (1995), the genotypes of these QTLs in the BC_5F_1 progeny of the same cross were homozygous for the AZ allele and most of the differentiated shoots were green, indicating that these QTLs practically acted in increasing GS. Thus, the QTLs for GS on chromosome 7H could be valuable for increasing GS in anther culture, which has the serious problem of the frequent formation of albino shoots.

By QTL analysis of tissue-culture traits using several mapping populations of different species, it is possible to classify QTLs for tissue-culture traits into QTLs common to a number of species and species-specific QTLs. In this study, common QTLs for CG (chromosome 2H) and SD (chromosomes 2H and 3H) were found across different mapping populations (Fig. 6). In particular, the QTL for SD was located close to the centromere of chromosome 2H across the four mapping populations (Fig. 6). Furthermore, in wheat, the QTL for SD is located at the centromeric region of the same homoeologous group, on chromosome 2B (Ben Amer et al. 1997). Functional analysis of QTLs common to different spe-

cies is the first step toward understanding the differentiation mechanism in the Triticeae.

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