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Genetic analysis of seed dormancy QTL in barley

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Seed dormancy in wild barley enables drought escape by preventing germination during the hot summer in arid environments. Dormancy in cultivated barley has different effects: it can delay the malting process and/or it can prevent pre-harvest sprouting. Thus, cloning dormancy genes in barley will contribute to understanding the domestication process and it will facilitate optimizing the trait for efficient agronomic and industrial uses. Rates of seed germination were used to evaluate dormancy on physiologically matured grain samples that were dried and stored frozen until use. With this phenotypic scoring procedure, many genetic factors controlling seed dormancy has been reported as quantitative trait loci (QTL). Of these QTL, one at the centrometic region of chromosome 5H (*Qsd1*) has been most frequently identified and shows the largest effect across mapping populations. We also identified this QTL using the EST map based on Haruna Nijo (*H. vulgare* ssp. *vulgare*) crossed with wild barley H602 (*H. vulgare* ssp. *spontaneum*). We have derived both doubled haploid and recombinant chromosome substitution lines (RCSLs) from this cross. At least four QTLs are segregating in this germplasm. RCSLs having only the *Qsd1* segment of wild barley in a Haruna Nijo genetic background were identified and 910 BC_3F_2 plants were scored for dormancy. In these lines, segregation for dormancy fit a mono-factorial ratio. These germplasm resources are appropriate for map based cloning of *Qsd1*. Strategies for cloning *Qsd1* with these resources are discussed.

Key Words: seed dormancy, *Hordeum vulgare*, QTL, cloning, genetic analysis.

Introduction

The wild progenitor of cultivated barley (*Hordeum vulgare* ssp. *spontaneum*) has high levels of seed dormancy after physiological maturity. It usually has several months of seed dormancy (Takeda and Hori 2007). This habit in the wild barley is an adaptation to arid conditions in west Asia where it rains in the winter but is dry and hot in the summer. This high level of dormancy has been modified in domesticated barleys due to cultivation practices that require germinating seed (malting) or growing plants within shorter periods after harvest. Dormancy in cultivated barley has effects on crop functionality: it may dictate the time required in storage prior to malting and/or it may prevent pre-harvest sprouting. Thus, cloning dormancy genes in barley will contribute to understanding the domestication process and it will allow optimization of the trait for efficient industrial and agronomic purposes. A wide range of variation in seed dormancy is reported in barley. Takeda and Hori (2007) evaluated seed dormancy in 4,365 cultivated and 177 wild (ssp. *spontaneum*) barley accessions collected from different regions of the world. Most of the wild barleys showed high levels of dormancy at maturity. Levels of seed dormancy in cultivated barleys differed depending on place of origin. Ethiopian accessions mostly showed non-dormancy, while Japanese, Turkish and North African accessions showed dormancy at maturity.

Seed dormancy in plants shows quantitative inheritance in many species. QTLs were reported in poplar (Frewen *et al.* 2000), *Arabidopsis* (Clerkx *et al.* 2004), rice (Miura *et al.* 2002) and wheat (Mori *et al.* 2005). In barley, Takeda and Hori (2007) used a set of F_1 half diallel crosses by eleven barley accessions with different level of dormancy and estimated that seed dormancy was predominately controlled by additive gene effects. In barley, two major QTLs on chromosome 5H, one near the centromere (SD1) and one in the telomeric region of the long arm (SD2), and multiple minor QTLs on chromosomes 4H (SD3) and 7H (SD4) were identified in the Steptoe/Morex mapping population (Han *et al.* 1996). These QTLs appear to be coincident with QTLs mapped in other studies based on alignment of linkage maps (Romagosa *et al.* 1999, Prada *et al.* 2004, Edney and Mather 2004, Zhang *et al.* 2005). Hori *et al.* (2007) constructed EST-based linkage maps on seven recombinant inbred (RI) populations and one doubled haploid (DH) population derived from crosses involving eleven cultivated barley accessions and one wild barley accession. The parents showed a wide range of seed dormancy levels. Among the eight populations they found QTLs clustered in 11 regions on all chromosomes except 2H. The QTL at the centromeric region of 5H was identified in all the RI and DH populations that showed different degrees of dormancy depth and period. The gene responsible for the QTL may have many alleles

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represented in the parental lines or the QTL may be due to the effects of many tightly linked genes. The largest effect allele was found in ssp. *spontaneum* accession H602, which was used as a parent of a high-resolution transcript mapping population (Sato *et al.* 2009) and a set of recombinant chromosome substitution lines (RCSLs). Cultivar Haruna Nijo was the other parent in the former germplasm and the recurrent parent of the latter germplasm (Sato and Takeda 2009). The segregation of multiple dormancy QTL leads to continuous variation (Hori *et al.* 2007), making it difficult to classify plants by their QTL alleles. The present research aimed to develop the resources for high resolution mapping of the dormancy QTL on the centromeric region chromosome 5H by the phenotypic and genotypic selection of RCSLs and their progeny.

Materials and Methods

Plant material

BC_3F_1 RCSLs derived from the cross between H602 (donor parent) and Haruna Nijo (recurrent parent) were genotyped by Hori *et al.* (2005). According to the QTL analysis of a DH population derived from the same cross (Hori *et al.* 2007), plants heterozygous for a segment on the centromeric region of chromosome 5H containing a dormancy QTL (hereafter referred to as *Qsd1*) but homozygous for recurrent parent (non-dormant) genome at three other dormancy QTL regions (1H, 4H and telomeric regions of 5HL) were selected. Twelve BC_3F_1 plants were identified and the BC_3F_2 seeds derived from these plants were planted in the field at the Research Institute for Bioresources, Okayama University, Kurashiki, Japan (34°35′N and 133°46′E). The nurseries were fall-sown and dormancy was scored on grain samples harvested in the spring of the following year. Plants of each parental accessions and each of the 12 BC_3F_2 populations were grown in single rows. Rows were 90 cm apart. Within-row spacing was 10 cm. Each BC_3F_2 population contained 15 to 152 plants, depending on seed availability.

Seed dormancy scoring

Seed germination percentage was used to score the level of seed dormancy. The protocol for scoring seed dormancy was described by Takeda and Hori (2007). In brief, spikes of each line were harvested at physiological maturity (when all green color was lost from the internode below the spike). After drying two days at 30°C at 10% relative humidity, spikes were hand-threshed and the seed was stored at −20°C. Preliminary monitoring of seed dormancy levels using parents and lines different only at the target QTL region indicated that an after-ripening period of five weeks of 25°C revealed clear differences in seed dormancy. Fifty seeds of each accession were after-ripened for this amount of time and then germinated for four days on moistened filter paper in Petri-dishes at 25°C. Seed dormancy was scored as the percentage of germinated seeds per total seeds. Each germination test was replicated twice.

Confirmation of segmental substitution in recombinant chromosome substitution lines

The parents of RCSLs (Haruna Nijo and H602) were genotyped with 1,448 unigene-derived Single Nucleotide Polymorphisms (SNPs) by Oligonucleotide Pooled Assay (OPA). The detailed methods are described in Sato and Takeda (2009). Briefly, frozen DNA samples were sent to the Southern California Genotyping Consortium, Illumina BeadLab at the University of California – Los Angeles (UCLA) for the OPA-SNP assay with the 1,536-plex detection platform of barley OPA 1 (BOPA1) developed by Dr. Tim Close, University of California. OPA genotyping was performed on Haruna Nijo, H602, 92 DH lines and 99 RCSLs using the Illumina GoldenGate BeadArray. The SNP loci are designated by HarvEST unigene assembly #32 numbers (http://harvest.ucr.edu/). A set of 384 core markers from the high-density EST marker map (Sato *et al.* 2009) was selected to provide uniform genome coverage. Linkage between these framework EST markers and polymorphisms from the OPA analysis was calculated. Of the OPA-SNPs, 732 markers showed polymorphisms. Genotype calls from the RCSLs were placed in map order as determined from the DH lines. The chromosome segments introgressed into Haruna Nijo from wild barley were estimated from the graphical haplotypes (van Berloo 2008) of each BC_3F_5 RCSL.

Results

Segregation of Qsd1 in the BC_3F_2 population

A total of 910 BC_3F_2 plants and their parents were harvested. Dormancy was scored using seed after-ripenined for five weeks at 25°C. Fig. 1 shows the segregation of seed dormancy in parents and all 910 BC_3F_2 plants. The dormant parent H602 showed complete dormancy (0% germination) after 5 weeks of after-ripening treatment at 25°C. The non-dormant parent Haruna Nijo was completely non-dormant (100% germination after the same treatment). The BC_3F_2 plants showed a tri-modal phenotypic frequency distribution, with a clear separation between the dormant group and the intermediate group. The distributions of intermediate group and non-dormant group were continuous. For the purposes of classification, the three groups were defined as follows: dormant homozygous (0–49%), heterozygous (50–89%) and non-dormant homozygous (90–100%).

Then number of plants in each of the 12 BC_3F_2 sub-populations ranged from 15 to 152 depending on the availability of seed from each BC_3F_1 plant (Table 1). From each sub-population, eight plants were randomly selected to check segregation of markers linked to *Qsd1*. As shown in Table 1, segregation in each sub-population approximated, or fit, a 1 : 2 : 1 ration except for sub-population 13-2 ($\chi^2 = 6.39$, $0.01 < P < 0.05$). Based on this result and segregation data of DNA markers (Appendix 1), all the 12 sub-populations were assumed to segregate at *Qsd1* and were thus pooled to estimate the mode of inheritance in the BC_3F_2 population as a whole. The whole population segregated 222 : 472 : 216, which fits a

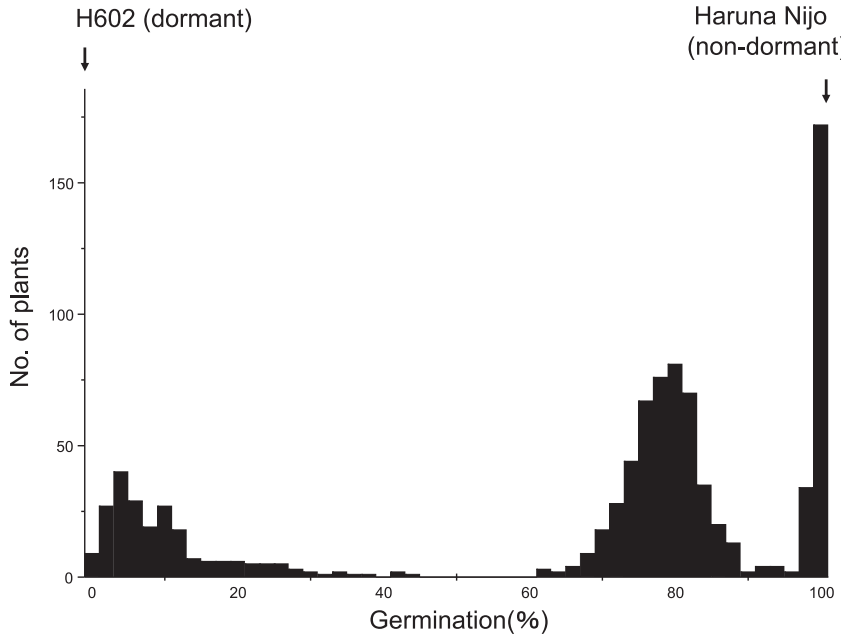

Fig. 1. Frequency distribution of 910 BC_3F_2 plants from the cross between barley cv. Haruna Nijo and H602 wild barley for germination (non-dormancy) after five weeks of after-ripening at 25°C. Dormancy homozygous (0–49%): heterozygous (50–89%): non-dormancy homozygous (90–100%) plants segregated 222 : 472 : 216 and fit a 1 : 2 : 1 ratio ($\chi^2 = 1.35$, $0.05 < P$).

Table 1. Segregation of sub-populations and the pooled total population for seed dormancy (after five weeks of after-ripening at 25°C) in BC_3F_2 plants derived from the cross between cv. Haruna Nijo and H602 wild barley

BC_3F_2 sub-population	Dormant 0–49%	Hetero-zygous 50–89%	Non-dormant 90–100%	Total	χ^2
3-1	14	47	19	80	3.08
3-2	12	18	10	40	0.60
3-3	14	34	12	60	1.20
13-1	3	8	4	15	0.20
13-2	17	27	5	49	6.39*
13-4	11	28	14	53	0.51
13-5	5	16	13	34	3.88
64-1	29	63	26	118	0.69
64-2	29	56	20	105	2.01
64-3	7	31	17	55	4.53
147-2	38	77	37	152	0.04
147-3	43	67	39	149	1.72
Total	222	472	216	910	1.35

* Significantly deviated from the 1 : 2 : 1 at the 5% level.

1 : 2 : 1 mono-genic ratio ($\chi^2 = 1.35$, $0.05 < P$). The average germination scores for each genotype (dormant, heterozygous and non-dormant) were 9.7, 77.8 and 99.0, respectively. The average score of heterozygotes (77.8%) was higher than the average of the two homozygous classes (54.4%), indicating that *Qsd1* shows incomplete dominance with the direction of average dominance toward non-dormancy (higher germination).

Genetic constitution of dormant plants in a BC_3F_5 population

To develop an isogenic line of *Qsd1* in a Haruna Nijo background, single seed descent (SSD) was used from the BC_3F_1 generation until the BC_3F_5 generation. The set of BC_3F_5 RCSLs was genotyped by the whole genome OPA-SNP typing system as described by Sato and Takeda (2009). The coverage of RCSLs was 966.4 cM, which was 81.4% of the total map length as determined by DH lines from the same cross (1187.4 cM). Therefore, some segments may be missing in the RCSLs. One of the RCSLs (64-1), includes a substituted segment of *Qsd1* (Fig. 2) from the wild barley donor parent without having any of the other three seed dormancy QTL alleles identified by Hori *et al.* (2007). This RCSL has the genotype of a dormant homozygote (Fig. 1) and as such it is an isogenic line for *Qsd1* which can be used for functional analysis and isolation of the gene.

Discussion

Population development for monogenic segregation of Qsd1

It is difficult to map a QTL with high resolution if the trait is controlled by multiple loci. The use of chromosome segmental substituted lines (CSSLs) or RCSLs is necessary to develop a high resolution map around the QTL and to find co-segregation between markers and the genetic determinant(s) of the trait. The RCSLs developed by Hori *et al.* (2005) and Hori *et al.* (2007) are the most appropriate materials for placing dormancy QTLs, one at a time, in the genetic background of the recurrent parent (Fig. 2). Subsequently a high resolution QTL mapping population can be developed either by crossing the recurrent parent to a selected RCSL or

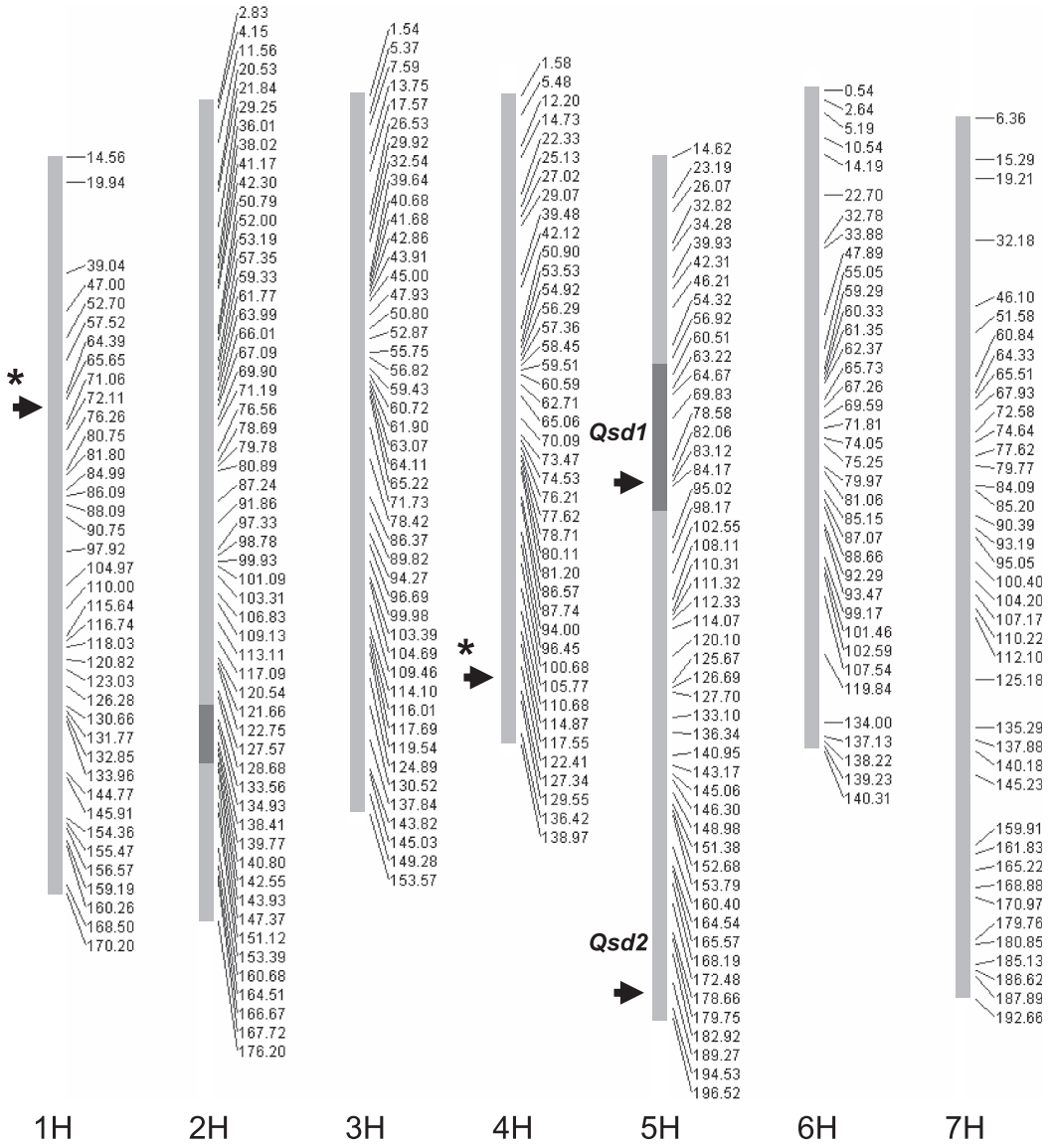

Fig. 2. A graphical genotype of a recombinant chromosome substitution line representing H602 wild barley substituted segments (dark grey) in the background of cv. Haruna Nijo (grey). Each chromosome is oriented with short arms at the top. Map distances are shown in cM. Arrows indicate seed dormancy QTLs identified by Hori *et al*. (2007). Arrows with asterisk are new loci detected by Hori *et al*. (2007).

by extracting and advancing selected lines from any generation, including the BC_3F_1. To minimize the risk of losing the target QTL allele due to poor mapping resolution or incorrect marker order, the QTL-bearing segment in the RCSL should be rather large. This can be accomplished by using markers extending some distance in both directions from the assumed position of the QTL. All the BC_3F_1 plants we selected satisfied this condition. A similar strategy was taken to develop substitution lines from crosses between selected Steptoe/Morex DH lines. By using these sets of lines, Han *et al.* (1999) mapped the SD1 locus to a 4.4 cM marker interval, and Gao *et al.* (2003) mapped the SD2 locus to a 0.8 cM marker interval. As illustrated by these two examples, specific populations are a key resource for developing high resolution maps for cloning QTL.

Segregation at Qsd1

As shown in Table 1, each BC_3F_2 sub-population fit, or nearly fit, the expected monofactorial ratio of 1 : 2 : 1. Therefore, it was appropriate to pool the sub-populations and consider the population as a whole. We confirmed that each sub-population segregated at the *Qsd1* locus and had recurrent parent (Haruna Nijo) alleles of at the other three dormancy QTLs (Fig. 2.) According to Hori *et al.* (2007), *Qsd1* had the largest effect (77.3 and 72.3% of total variance at 5 week after-ripening treatment, in 2003 and 2005, respectively) of four QTLs. Thus, the segregation of *Qsd1* in the BC_3F_2 was clear except for some members of the heterozygote group vs. the non-dormant homozygote group in the germination range of 88–96%. These plants may have other lesser-effect non-dormant alleles at loci other than the four QTLs reported

by Hori *et al.* (2007). Confirmation and understanding the genetic basis of the phenotype in these plants will require progeny testing in later generations.

Gulber *et al.* (2005) explained that seed dormancy, in general, can be caused by a variety of factors arising from either embryonic tissues (embryo-caused dormancy) and/or maternal tissues (coat-based dormancy). This raises the question of whether Fig. 1 shows the segregation in maternal BC_3F_2 tissues or BC_3F_3 embryo tissues. In the case of *Qsd1* this question cannot be unequivocally answered at present. If we assume segregation at the BC_3F_2, the average dominance is 0.53 (incomplete dominance); 2 (heterozygote mean − mid parents)/(non-dormant homozygote mean − dormant homozygote mean) = 2 (77.8 − 54.4)/(99.0 − 9.7). In contrast, if we assume segregation at the BC_3F_3, the average dominance is 1.05 (complete dominance); 4 (heterozygote mean − mid parents)/(non-dormant homozygote mean − dormant homozygote mean) = 4 (77.8 − 54.4)/(99.0 − 9.7)). Both genetic models may explain the segregation in Fig. 1. Since the degree of dominance may provide important clues concerning the tissues responsible for function of *Qsd1*, further segregation analyses with different after-ripening periods are conducted currently to monitor the behavior of heterozygotes.

Hori *et al.* (2007) reported considerable variation in the estimates of QTL parameters (LOD scores, additive variances and estimated additive effects) for *Qsd1*. This may indicate allelic variation at the same locus or the effects of alleles at multiple, tightly linked loci. In either case, this locus (or complex locus) may be a principal determinant of the differences in degree of dormancy observed between wild and cultivated barley, and within cultivated barley.

Strategy and resources for isolating Qsd1

Cloning and characterization of *Qsd1* should prove useful information for understanding and manipulating the genetics of seed dormancy in barley. There are some good examples of map based cloning in barley e.g. virus resistance *rym4/5* (Stein *et al.* 2005), photoperiod sensitivity *Ppd-H1* (Turner *et al.* 2005) and inflorescence morphology *vrs1* (Komatsuda *et al.* 2007). However, map based cloning of QTL is difficult in species without complete genome sequence, like barley. A rare example of successful map based cloning of QTL in barley is that of boron tolerance (Sutton *et al.* 2007). In barley, co-linearity with a related genome i.e. rice gives key information for developing a fine map and estimating the number and function of genes in the target region. We are developing a truly high resolution EST map of the region using the RCSLs (Sato and Takeda 2009) and DH lines (Sato *et al.* 2009) derived from the cross between Haruna Nijo and H602. These mapped ESTs will facilitate marker development for saturating the *Qsd1* region.

A bacterial artificial chromosome (BAC) library was developed from Haruna Nijo consisting of 294,912 clones with an average insert size of 115.2 kb and a genome coverage of about 6.6 genome-equivalents (Saisho *et al.* 2007). A BAC library was also constructed from H602 (Sato unpublished). These two genomic libraries will facilitate identification of the allelic variants of *Qds1* segregating in this germplasm. A collection of 5,006 full length (FL) cDNA sequences from Haruna Nijo (Sato *et al.* 2009) will also provide valuable information for marker generation and mapping genes on the target BAC sequence.

Hori *et al.* (2007) found degrees of variation in the effects of *Qsd1* alleles that could be of immediate practical benefit. These benefits will be greatest when based on an understanding of gene structure and function. The comprehensive genomic resources we have developed will provide a strong platform for connecting with pre-existing genomic and genetic resources to accelerate gene identification in barley.

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Appendix 1. Preliminary genotyping of BC_3F_2 sub-population derived from the cross between cv. Haruna Nijo (A) and H602 wild barley (B). Each sub-population was genotyped by eight plants with two markers (XY) in both directions from the assumed potision of *Qsd1*

BC_3F_2 sub-population	Haruna Nijo homozygote (AA)	Heterozygote (HH)	H602 homozygote (BB)	Recombinant (AB, BA) or score missing
3-1	3	3	2	0
3-2	2	5	1	0
3-3	1	6	1	0
13-1	1	4	2	1
13-2	0	3	3	2
13-4	2	6	0	0
13-5	0	6	0	2
64-1	3	3	2	0
64-2	1	4	3	0
64-3	0	7	1	0
147-2	5	2	1	0
147-3	1	3	2	3