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Restriction fragment length polymorphism associated with barley starch granule traits

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SUMMARY

Barley (*Hordeum vulgare*)starch granule traits were mapped using 123 RFLP markers providing an average marker density of 9.6 cM. QTL-STAT was used to analyze data from 150 F_1-derived double haploids from Steptoe x Morex cross, for six starch granule traits. Five significant quantitative trait loci (QTL) (P = 0.001) were found for A granule surface area and volume, proportion of A granules to total starch, and ratio of number of B to A granules. Additional possible QTL effects (P = 0.05) were detected for these four traits plus B granule surface area and volume. Chromosome regions associated with granule surface area coincided with those associated with granule volume. Morex was the source of alleles for increasing A granule surface area and volume, and proportion of A granules to total starch by volume. Steptoe contributed alleles for increasing ratio of number of B to A granules. QTLs for A-granule traits were clustered in three regions on chromosome 2, and one of those chromosomal regions, rbcs-abg2, was associated with all A granule traits. Additional information is desirable on several topics, e.g. role of chromosome 2 in controlling starch granule traits, importance of QTLs with Wald values lower than 10, and influence of environment.

Key words: Molecular markers, starch granules, *Hordeum vulgare*, malting quality, double haploids.

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RESUMEN

Las características de los gránulos de almidón de la cebada (*Hordeum vulgare*) fueron mapeadas utilizándose 123 marcadores moleculares RFLP, proporcionando una densidad de cobertura del genoma de aproximadamente 9,6 cM. El programa QTL-STAT fue utilizado para analizar seis características en datos provenientes de 150 di-haploides del cruzamiento de las variedades Steptoe x Morex Se detectaron 5 regiones cromosómicas (QTLs) (P = 0,001) para área superficial y volúmen de los gránulos A, proporción de gránulos A en relación al almidón total y proporción de número de gránulos B en relación a los gránulos A. Otros posibles QTLs fueron detectados (P = 0,05) para estas cuatro características y para el área superficial y volúmen de los gránulos B La variedad Morex fue la fuente de alelos para aumento del área superficial y volumen de los gránulos A y pro-

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porción de los gránulos A en relación al almidón total. La variedad Steptoe contribuyó con alelos para aumento de proporción del número de gránulos B con relación a los gránulos A. Los QTLs para las características de los gránulos A se concentran en tres regiones del cromosoma número 2, y una de estas regiones, la rbcs-abg2, se mostró asociada a todas las características de los gránulos A. Estudios más detallados en esta área son necesarios para esclarecer la función del cromosoma 2 en el control de las características de los gránulos de almidón, grado de importancia de los QTLs con valores de la estadística Wald inferior a 10 e influencia del ambiente en estas asociaciones.

Palabras clave: Marcadores moleculares, gránulos de almidón, *Hordeum vulgare*, calidad de malta, di-haploide.

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INTRODUCTION

Malting barley (*Hordeum vulgare*) may be subjected to more quality constraints than any other crop and is one of only a few field crops consistently sold on the basis of cultivar. Requirements are specified by commercial maltsters and brewers to ensure a product with consistent high quality.

MacGregor & Ballance (1980), in an *in vitro* study, found that A starch granules are more susceptible to hydrolysis by a-amylase II than B granules at 65 °C, the temperature under which mashing occurs in brewing. The same authors reported that A granules are digested from the inside out, while B granules are digested by surface erosion. These findings suggest that A and B granules may influence malt quality in different ways.

Although no study addressing the quantitative influence of starch granule morphology on malting quality was found in the literature, the 1991 European Brewery Convention suggested as a long-term goal for barley breeding programs development of varieties that have a higher proportion of A starch granules (Pierce, 1991).

In all crops, including barley, most traits of economic importance are commonly controlled by several genes. These so-called quantitative traits exhibit continuous distributions and are often difficult to manipulate. Recently, researchers have suggested that selection for these complex traits might be facilitated by using molecular markers (Quarrie *et al.* 1997, Gebhardt & Salamini, 1992; Paterson *et al.*, 1991). RFLP and AFLP technology offers an approach to genetic dissection and to mapping complex traits. RFLPs have been used to locate quantitative trait loci (QTL) in several crops (Paterson *et al.*, 1991; Parentoni, 1993; Mansur *et al.*, 1993). In barley, the use of RFLPs has made it possible to construct a saturated genetic map with 9.6 cM marker density (Hayes *et al.*, 1993). The availability of the RFLP technology in barley opens a new avenue to locate loci for traits of agronomic and malting quality importance.

Hayes *et al.* (1993) reported a significant association between the *Amy2* locus on chromosome 1 and levels of α-amylase. Protein content and diastatic power were closely associated with the *Amy1* marker on Chromosome 6. A QTL on chromosome 2 was shown to be associated with malt extract level. Therefore such markers can be used to identify lines with improved malting quality in breeding programs.

The research reported in this paper was designed to find associations between molecular markers on the barley genome and starch granule traits.

MATERIALS AND METHODS

The development of the genetic material and the molecular map used in this study are described by Kleinhofs *et al.* (1993). In brief, 150 F1-derived doubled haploid (DH) lines from a Steptoe x Morex cross were developed using the *Hordeum bulbosum* technique, as described by Chen & Hayes (1989). The 150 DH lines and parents were grown at Crookston, MN in summer 1992. The experimental DH lines were grown in single-replicate plots. Increasing number of lines, rather than number of replications, has been demonstrated to maximize the power of tests of hypotheses about QTLs (Knapp & Bridges, 1990) Field plots consisted of four 3.66-m rows spaced 30.5 cm apart. The plots were seeded at a rate of 3.3 g/m in early April. Following harvest, spikes were dried at 37 °C for 24 hours and then stored until required for

starch granule measurements. A sample of 10 g of seed was taken for each DH line and each parents, and ground on a Brinkmann sample mill (Retsch), using a 0.5 mm screen. One-half gram of the resulting flour was diluted in 5 ml sodium dodecyl sulfate (2% w/v) to remove crude protein, and was brought into suspension by vortexing for 60 seconds. The suspension was then subjected to two minutes of ultrasonication (Bransonic 2200). To stain the starch granules, a few drops of iodine solution were added to the suspension and the sample vortexed for 60 seconds. Before each sample was analyzed they were vortexed vigorously for 15 seconds to obtain a uniform homogenate representing the suspension (Bathgate & Palmer, 1972). Next, 50 µl of suspension were removed with the aid of a pipette and placed on a 75 x 25 mm microscope slide and covered with a 18-mm square coverslip. For each experimental unit (subplot) seven to ten microscopic fields in each slide were measured. Thus a minimum of 600 starch granules were measured per experimental unit.

Starch granule size was measured by digital image analysis (DIA), using IBAS 2.0 software on a Kontron image analyzer (Kontron Elektronic Gmbh, Germany), interfaced with a Fischer microscope through a video camera (Sony CCD, model XC-77), for data collection.

Using DIA, starch granule images were captured using a green filter to enhance contrast. The starch granules were stained dark purple, and a sharp focus was obtained for a crisp gray digitized image that was displayed on a high resolution monitor. A 40x objective was used for all readings.

Each particle in the final field image was identified and area, maximum-, minimum- and equivalent diameter, perimeter, and circularity shape factor were measured for each discriminated starch granule. It should be recognized that a two-dimensional image was measured by the DIA, and therefore the dimensions, specially of large granules, refer to granules which were lying flat. From those traits, surface area and volume for large and small granules, ratio of number of small to large granules and proportion of large granules to total starch by volume were derived to more fully characterize the starch granules.

L-granule dimensions were estimated assuming an oblate-spheroid shape and S-granule dimensions were calculated assuming a spherical shape (Oliveira *et al.*, 1994). To calculate L granule surface area and volume, their eccentricity (ε), which is an indication of the sphericity of the granule, was needed. It was calculated as $\varepsilon = 2c/MD_L$, where c is the hypotenuse of a right triangle and MD_L is maximum diameter of the granule (Armstrong, 1992). It was also necessary to estimate granule thickness (T) to compute c. By preparing slides with a large number of granules, sufficient granules were found lying "sideways" which permitted measurement of T. One-hundred minimum diameter values were averaged to obtain the mean thickness (T). Mean thickness obtained from the measurements and used in this study was T = 4.23 µm.

The six starch granule traits reported here were calculated as described below. Component terms are defined following the formulas.

Surface Area of a Granule (S): Outer surface area expressed in μm^2, was calculated as proposed by Armstrong (1992):

L granules: $$SL = \frac{\pi}{2} MD_L^2 + \pi \frac{T^2}{\varepsilon} \ln \frac{1+\varepsilon}{1-\varepsilon}$$

S granules: $$SS = \pi ED^2$$

Granule Volume (V): Granule volume expressed in μm^3 was calculated as:

L granules: $$VL = \frac{\pi T}{3} MD_L^2$$

S granules: $$VS = \frac{\pi ED^3}{6}$$

Proportion of L granules to Total Starch (PV): The cumulative volume of L granules in relation to total volume of granules, expressed in %, was calculated as:

$$PV = 100\, V_L/V_L + (V_S \text{ Ratio S/L})$$

Ratio of Number of S to L Granules (Ratio No. S/L): Ratio of number of S to L granules was calculated as:

$$\text{Ratio No. S/L:} \quad \frac{\text{Number of S}}{\text{Number of L}}$$

In the above equations MD_L is maximum diameter of an L granule, ED is equivalent diameter of a granule, ln is the natural logarithm, V is volume of granule, ε is eccentricity, and T is thickness of the granule, which was found to be on average 4.23 µm.

A 295-point map with an average density of 4 cM, covering 1,250 cM of the barley genome was devel-

oped by the North American Barley Genome Mapping Project (Kleinhofs *et al.*, 1993). Although this map covers more than 90% of the barley genome, two large gaps remain, one on chromosome 7 and one on chromosome 4. Selecting relatively uniformly spaced markers from the 295-point map, Hayes *et al.* (1993) were able to generate a 123-point "skeleton" linkage map resulting in an average marker density of 9.6 cM which was used in this study.

The QTL analyses were done using QTL-STAT, an interval mapping program that uses non-linear models to estimate QTL parameters (Holloway & Knapp, 1994, Tinker & Mather, 1995). The non-linear procedure allows the obtaintion of Wald statistics and QTL genotype means for each interval. A QTL effect was considered significant if it exceeded a Wald statistic value of 10.0, which is approximately equal to P = 0.001. This conservative criteria (P = 0.001) has been used in other mapping efforts (Heun, 1992; Parentoni, 1993). The so-called support interval which is the chromosome segment where the QTL under consideration is located (P = 0.10), was specified by finding the Wald peaks and subtracting 10 from its value. Marker intervals on either side of the peak, with Wald values exceeding the obtained value were included in the support interval.

RESULTS AND DISCUSSION

QTL effects exceeding the Wald threshold value of 10 (P = 0.001) were found for four of the six starch granule traits, and are shown in Table 1. The four traits which showed QTL effects associated (P = 0.001) with molecular markers were A granule surface area and volume, proportion of A granules to total starch and ratio of number of B to A granules. These significant QTL effects (Wald > 10) were grouped on chromosome 2. Wald values, ranging from 0 to 25.56, are presented in graphical format for each trait and chromosome in Fig. 1 to 6.

A Granule Surface Area and Volume

Starch granule surface area and volume are two traits associated with malting quality (Oliveira *et al.* 1994). Volume is a trait directly related to total starch available, while surface area is another important trait once the malting process is result of the action of amylases on the surface of the granules. The correlations among them are affected by granule sphericity (Armstrong, 1992).

The chromosomes and chromosome regions linked with A granule surface area were also linked with A granule volume (Fig. 1 and 2). The Wald statistics were similar for these two traits (Table 1) as expected, given that surface area and volume are positively and highly correlated.

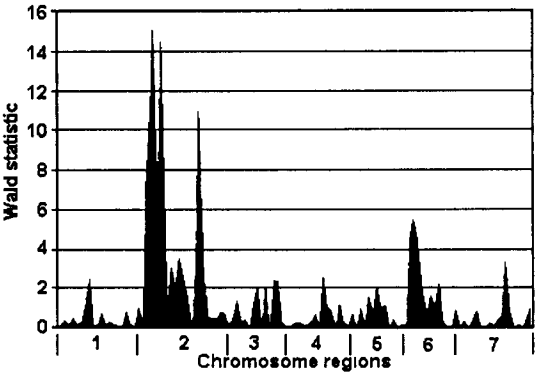

Figure 1. Likelihood plot showing Wald statistic for A-granule surface area for seven barley chromosomes.

The likelihood plot showing Wald statistic for A granule surface area for the seven barley chromosomes is given in Fig. 1. Based on Wald values in Fig. 1 and 2, there are three regions on chromosome 2 associated with A granule surface area and volume. Two of those regions were adjacent (Table 1). The chromosome interval chs1b-abg8 showed the highest Wald statistic peak for these traits. A fourth Wald peak was found in chromosome 6; however, the Wald value of 5.56 (P = 0.020), was well below the threshold value of 10 required for significance. Chromosome 2 has been associated with malting quality traits by other workers (Mather *et al.* 1997, Laurie *et al.* 1993). Readers should refer to Becker *et al.* 1995 for the barley linkage map. The

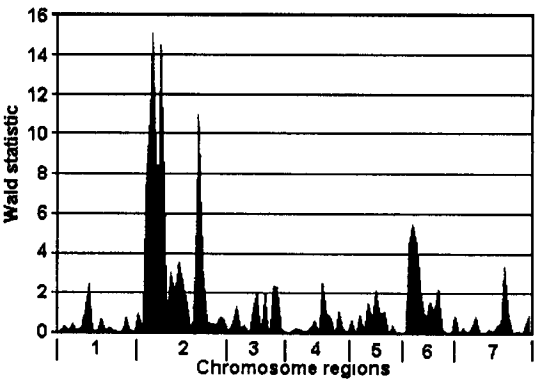

Figure 2. Likelihood plot showing Wald statistic for A-granule volume for seven barley chromosomes.

Table 1. Wald statistics and Wald support intervals (in bold type) for QTL effects for four starch granule traits on chromosome 2, based on 150 double haploid lines evaluated at Crookston, 1992.

Marker interval	Percentage of Recombination[1]	A Granule		Proportion of A to total starch	Ratio No. B/A
		Surface area	Volume		
abg313a-abg703	7.9	**7.83**	**7.80**	**22.00**	**16.77**
abg703-chs1b	11.0	**10.51**	**10.53**	7.70	1.14
chs1b-abg8	7.2	**15.09**	**15.09**	**12.64**	7.64
abg8-rbcs	4.6	**7.21**	**7.27**	**15.82**	**13.69**
rbcs-abg2	11.5	**14.53**	**14.51**	**25.56**	**16.85**
abg2-abg459	9.0	1.38	1.36	**21.91**	**17.43**
abg459-pox	6.8	3.06	3.07	**14.72**	7.55
pox-adh8	5.5	2.26	2.24	**10.12**	4.57
his3c-ksu15	11.9	**3.25**	**3.19**	4.24	4.90
ksu15-crg3a	22.1	**11.10**	**10.97**	7.60	5.20
crg3a-gln2	16.4	**2.87**	**2.88**	2.45	1.90

1. Percentage of recombination between markers.

Wald peak in chromosome 6 which was located between markers nar7 and nir merits further attention in future investigation (Table 1).

B Granule Surface Area and Volume

The likelihood surface for B granule surface area and volume for the seven chromosomes are in Fig. 3 and 4. No significant marker trait associations (P = 0.001) were found on any chromosome. The absence of sites with Wald > 10 for B granule surface area and volume may be due to *i)* absence of genes for B granule surface area and volume with sizeable effects, i.e., effects large enough to result in Wald values larger than 10 or *ii)* size factors in genomic regions not covered by the markers. If a 5% probability level, as suggested by Mansur *et al.* (1993), had been adopted to declare a significant QTL effect, two QTLs would have been found. Here they are being treated as possible QTLs. They are located on chromosome 5 (Wald peak = 4.17, P = 0.043) between markers glb1 and abc160, and on chromosome 6 (Wald peak = 4.85, P = 0.029) between markers nar7 and nir. However, due to the large number of markers

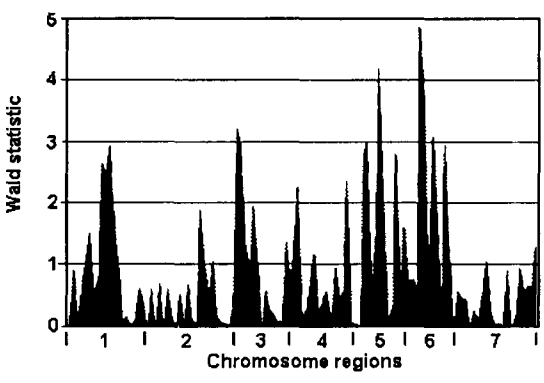

Figure 3. Likelihood plot showing Wald statistic for B-granule surface area for seven barley chromosomes

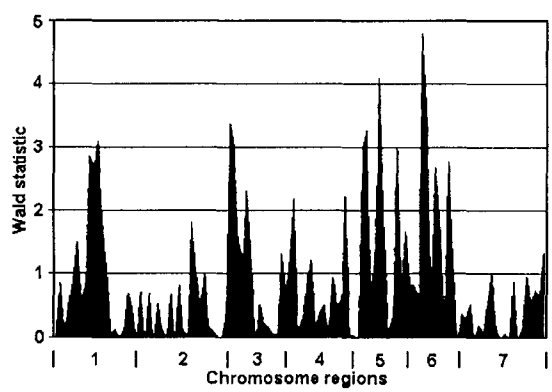

Figure 4. Likelihood plot showing Wald statistic for B-granule volume for seven barley chromosomes

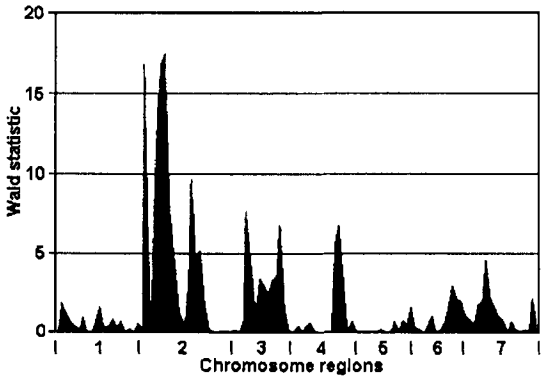

Figure 5. Likelihood plot showing Wald statistic for proportion of A to total starch for seven barley chromosomes

tested, the Wald threshold of 10 was adopted to avoid declaring false positives. Smaller Wald peaks were found at multiple sites throughout the genome. These may represent the effects of minor genes (Heun, 1992). However, their usefulness for molecular marker assisted selection is questionable.

Proportion of A Granules to Total Starch by Volume

QTLs for proportion of A granules to total starch by volume were found on chromosome 2 (Fig. 5). The Wald statistic for the region rbcs-adg2, in chromosome 2, was very large (25.56), indicating the importance of this locus for proportion of A granules to total starch.

Possible QTL effects were found on chromosome 1, between markers abg156d and bg22a (Wald peak = 4.41, P = 0.037); on chromosome 3, between markers abg471 and dor4a (Wald peak = 7.97, P = 0.005); and on chromosome 4, between markers cdo402b and cdo669 (Wald peak = 8.95, P = 0.003). Only one of the three regions on chromosome 2 which were linked to A granule size, interval ksu15-crg3a, did not show linkage with proportion of A granules to total starch by volume.

Ratio of Number of B to A Granules

Three regions on chromosome 2 showed marker-trait association for ratio of number of B to A granules (Fig. 6). The Wald statistic peak in interval abg2-abg459, chromosome 2, was very large, 17.43 (Table 1).

Five possible QTLs were found between markers abg14 and his3c (Wald peak = 9.69, P = 0.002) on chromosome 2; cdo113b and his4b (Wald peak = 7.69, P = 0.006), and abg471 and dor4a (Wald peak = 6.82, P = 0.010) on chromosome 3; abg313b and cdo669 (Wald peak = 6.83, P = 0.010) on chromosome 4; and abc706 and ale (Wald peak = 4.58, P = 0.034) on chromosome 7.

Genetic Contribution of Morex and Steptoe for Starch Granule Traits

The estimated QTL effect shown as the difference between Morex and Steptoe alleles for four starch granule traits is given in Table 2. Morex was the source of alleles for increasing A granules surface area and volume and proportion of A granules to total starch by volume. Steptoe contributed alleles for increasing ratio of number of B to A granules.

The low number of QTLs identified for starch granule traits may relate to low heritabilities for those traits when estimated on a single plot basis. It would be interesting to see if different or more QTLs could be mapped when using data from replicated trials grown in more than one environment, which is known to increase precision of starch granule trait estimates. A second important factor is the conservative criteria that we employed for QTL detection (Wald threshold of 10), which limited the number of declared QTL.

The large effect of chromosome 2 on four starch granule traits raises an intriguing question about whether a single gene or a cluster of genes for starch granule traits is located on that chromosome. The answer to this question requires a detailed study using a more densely saturated map.

Breeding for starch granule traits has been suggested by Pierce (1991). With marker-assisted selection, the introgression of desirable granule traits via selection for chromosomal regions controlling those

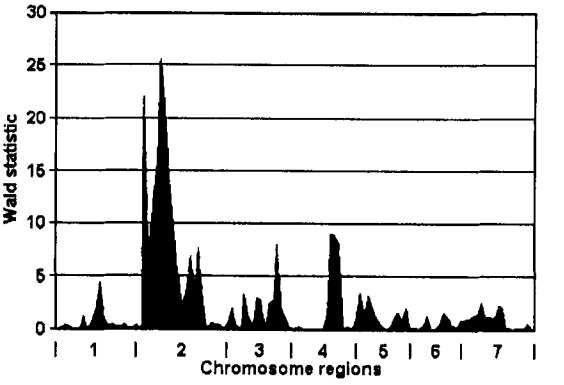

Figure 6. Likelihood plot showing Wald statistic for ratio of number of B to A granules for seven barley chromosomes.

Table 2. QTL genotype difference for starch granule traits where Wald > 10 in chromosome 2. Value in bold type indicates Wald peak. Adjacent values are the support intervals. The letter suffix indicates the parent contributing the larger value allele; S = Steptoe; M = Morex.

Marker interval	Percentage of Recombination[1]	A Granule		Proportion of A to total starch	Ratio No. B/A
		Surface area	Volume		
		(μm^2)	(μm^3)	(%)	
abg313a-abg703	7.9	33.20M	88.49M	**6.52M**	**3.22S**
abg703-chs1b	11.0	38.94M	104.34M		
chs1b-abg8	7.2	**43.14M**	**115.27M**	5.54M	
abg8-rbcs	4.6	30.20M	80.81M	5.81M	3.69S
rbcs-abg2	11.5	**43.33M**	**115.49M**	**7.66M**	4.34S
abg2-abg459	9.0			7.07M	**4.40S**
abg459-pox	6.8			5.74M	
pox-adh8	5.5			4.85M	
his3c-ksu15	11.9	22.67	60.04		
ksu15-crg3a	22.1	**42.95M**	**114.31M**		
crg3a-gln2	16.4	21.53M	57.69M		

1. Percentage of recombination between markers.

traits could be accomplished in less time than would be required in a phenotypically-based breeding program and could be one of the more immediate contributions of molecular marker technology to plant breeding. However, until estimates of QTL effects are verified in molecular-marker-assisted-selection experiments, QTLs should be regarded with the same precaution as traditional estimates of genetic parameters.

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