Theor Appl Genet (2008) 116:991–1002
DOI 10.1007/s00122-008-0730-6

ORIGINAL PAPER

Inheritance of the number and thickness of cell layers in barley aleurone tissue (*Hordeum vulgare* L.): an approach using F2–F3 progeny

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Received: 22 May 2007 / Accepted: 9 February 2008 / Published online: 29 February 2008

Abstract The aleurone tissue of cereal grains, nutritionally rich in minerals and vitamins, is an important target for the improvement of cereals. Inheritance of the thickness and the number of cell layers in barley aleurone was studied on the F2–F3 progeny of an Erhard Frederichen × Criolla Negra cross in which the parental lines have three or two aleurone layers, respectively. F3 grain was sampled from each F2 plant and 96.8% of the entire F3 grain population was classified as being either the 2- or 3-layer type. Using microsatellite, single nucleotide polymorphism (SNP) and morphological markers on 190 F2 plants, a linkage map was built. Three quantitative trait loci (QTLs) affecting aleurone traits were revealed on chromosome 5H (max. LOD = 5.83) and chromosome 7H (max. LOD = 4.45) by interval mapping, and on chromosome 2H by marker analysis with an unmapped marker. These QTLs were consistent with genetic sub-models involving either 2-cell type dominance for 7H and 2H, or putative partial dominance for 5H where 2-cell-layer dominance and additivity gave similar LODs. The number of aleurone cell layers and aleurone thickness were strongly correlated and QTL results for these traits were alike. An SNP marker of *sal1*, an orthologue of the maize multilayer aleurone gene was mapped to the 7HL chromosome arm. However, the 7H QTL did not co-locate with the barley *sal1* SNP, suggesting that an additional gene is involved in determining aleurone traits. These new mapping data allow comparisons to be made with related studies.

Communicated by A. Graner.

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Introduction

The barley (*Hordeum vulgare* L.) aleurone layer has been studied extensively for its role in the mobilization of kernel reserves at germination. It is used to study embryo–aleurone interactions with commercial applications in the barley malting process and breeding. The aleurone layer has also been studied in maize (*Zea mays* L.), and more recently in *Arabidopsis*, in relation to the ontogeny of kernel tissues (Brown et al. 1994; Olsen 2004). In addition, the biochemical composition of the aleurone layer in barley is of considerable interest nutritionally as the aleurone region is especially rich in high quality proteins, lipids, minerals and micronutrients (Antoine et al. 2002). Recently, there has been renewed interest in increasing aleurone layer thickness in the genetic improvement of cereals (Chanliaud et al. 2005).

In most *Poaceae*, wild types have one aleurone cell layer. In oats (*Avena sativa* L.) and rice (*Oryza sativa* L.), "one or two" layers have been reported (Bonnett 1961; Chung and Wu 1983). However, there may be up to five layers in rice aleurone (Del Rosario et al. 1968). Maize usually has a single-layer aleurone, but the South American landrace Coroico has multiple layers (Wolf et al. 1972).

Over the past 50 years, most publications on the morphology of the barley aleurone layer deal with the ontogeny and differentiation of either wild type or shrunken endosperm mutants (Felker et al. 1985; Bosnes et al. 1987; 1992; Brown et al. 1994; Olsen 2004). Generally barley has

three aleurone cell layers, differing from the single layer common in other cereals (Olsen 2004). Harz (1885) reported there being two to four aleurone layers in barley. Several authors (1903–1935), reviewed by Sawicki (1950), extended these results with respect to the protein content and taxonomy. From 1937 to 1950, Sawicki (1950) studied the structure of the barley aleurone layer in 103 world accessions. The highly reproducible genotypic range was from 1.7 to 3.0 cell layers; the genotype, "site/year" and residual error effects accounted for 85–90%, 0.5–0.8% and 9–14% of the total variance, respectively, indicating the high heritability of this trait. However, no true inheritance study on segregating progenies was made.

Here we aimed to investigate how the "number of aleurone cell layers" trait in barley is inherited. Progeny from a cross of two parental genotypes differing in this trait were used to locate possible QTLs and the relative position of a homologue of the multiple-aleurone-layer maize gene, *sal1* (Shen et al. 2003).

Material and methods

Plant material and DNA

A cross between two barley accessions, Erhard Frederichen and Criolla Negra, was made in Spring 2003. Erhard Frederichen (Accession Nr: PL 43199 from IHAR Genebank, Radzikow, PL) is an old six-row Danish spring barley with on average 2.93 aleurone cell layers in the grain. Criolla Negra (Accession Nr: K19982 from VIR Genebank, St-Petersburg, RUS) is a two-row Ethiopian landrace with on average 2.11 aleurone cell layers. Criolla Negra belongs to the botanical variety *deficiens* (STEUD) and forma *copticum* (VAV) with reduced lateral spikelets and it may be related to *H. copticum* VAV types used by Sawicki (1950).

F1 plants were grown in a greenhouse at INRA, Clermont-Ferrand, France (August 2003–January 2004), as were F2 plants (January–May 2004), from which 190 randomly tagged plants were harvested. The F3 generation was grown in a field nursery (February–July 2005).

For DNA extraction, 150 mg of green leaves per F2 plant or parent were collected at the 3-tiller stage, placed in 2-ml screw-top tubes on ice and stored at −80°C. DNA was extracted using a metabisulfite protocol (Cheung et al. 1993) and diluted to 1 μg μl^{-1} (in 0.1× TE buffer).

Polymorphisms in sal1

To look for possible SNPs, all the gene sequences of *sal1* homologues from different species available in Genbank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) were aligned using ClustalX (Thompson et al. 1997). PCR primers were designed to match conserved regions of this alignment using OLIGO® 6 software (Rychlik and Rhoads 1989). Left and right primer sequences to amplify *sal1* were 5′-GCAACCCGGAGAAGCTGAT-3′ and 5′-TCGACCTTCTCCTTCTCCTTGG-3′, respectively. PCRs with these primers on genomic DNA from parental lines were as described in Ravel et al. (2006). The PCR products obtained from the DNA of both parents were double-strand sequenced by Genome Express, Meylan, France. The partial sequences of *sal1* obtained were assembled to find polymorphisms.

Genotyping the F2 generation

Three types of genetic markers were used: simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), and morphological markers.

A total of 452 SSR markers, amplifying a single locus whenever possible, were tested for amplification and polymorphism on the parent accessions. They included barley SSRs from sequences published by Ramsay et al. (2000), Pillen et al. (2000), Karakousis et al. (2003), and Michalek et al, (2002), and the GBMS and GBM series provided by IPK Gatersleben under a material transfer agreement (Li et al. 2003; Thiel et al. 2003). In addition, 102 SSRs derived from wheat ESTs (Zhang et al. 2005) were tested. Forward PCR primers included a 5′ M13 tail.

The F2 population was genotyped in a protocol using Applied Biosystems® FAM fluorochrome. Using 384-well plates, the parents plus four F2 plants were tested for polymorphisms alongside the remaining 186 F2 progeny that were genotyped with eight markers per multiplex plate (with four fluorochromes). The PCR mix (6.5 μl per well) contained 25 ng of DNA, 2.345 μl of dH_2O, 2.3 mM of $MgCl_2$ buffer, 830 μM of dNTPs, 0.031 U/μl *Qiagen*® Taq, 50 nM of forward primer with M13 tail, 500 nM of reverse primer, and 1 μM of labeled M13 primer. The standard PCR program for amplifying barley DNA was a denaturation step at 95°C for 5 min; 7 cycles of 95°C for 30 s, 62°C for 30 s (with a decrease of 1°C per cycle), 72°C for 30 s; 20 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min.

After electrophoresis on an ABIPRISM3100 sequencer, the data were pre-analyzed using *Applied Biosystem*® Genscan software, and then analyzed with Genotyper software to give the final curve results.

SNPs were used in genotyping with specific primers designed for each allele.

Four robust Mendelian morphological markers were used: black lemma and pericarp (*Blp* gene), number of ear rows (*vrs1* gene), wide elongated outer glume (*eog* gene) and short rachilla hairs (*srh* gene) (Franckowiak 1997). The F2 and F3 generations were genotyped to assess F2 heterozygosis of dominant traits.

Building a genetic linkage map and search for QTLs

The genetic map of the (Erhard Frederichen × Criolla Negra) F2 population was constructed using MAPMAKER/EXP 3.0 software (Lander and Green 1987) with a LOD threshold of 3 and the Kosambi mapping function with a maximal distance of 40 cM. Linkage groups were assigned to chromosomes or segments named 1H–7H relating to existing barley consensus maps, e.g. by Ramsay et al. (2000), Karakousis et al. (2003), Li et al. (2003), and Thiel et al. (2003). Markers giving a χ^2 value significant at the $\alpha = 0.01$ level for distortion from a 1:2:1 segregation were included on the map in a second step. After correction, MultiQTL® Version 2.4 (Korol et al. 1996) was used to search for possible QTLs. Unlinked markers were investigated for correlation with the phenotypic traits. Resulting potential QTLs were tested, using *t* tests to compare the three genotypic classes. The independence of QTL effects could be studied for the QTLs determined by interval mapping only. For this purpose, the sub-population of genotypes with alleles of the two flanking markers coming from the same parent, at each QTL locus, was considered. It was thus assumed that double crossing-overs would have occurred with a quite low frequency in these 10–12 cM segments, and can be neglected. ANOVA tests were used to test for epistasis between QTLs.

Phenotyping the F2-F3 population for aleurone layer traits

Preliminary observations were made of the aleurone of ripe grains from the two parent lines. Then eight F3 kernels were sampled from each of the 190 F2 plants genotyped. The grains were harvested from both the sides of the spike rachis from the middle part of the spikes, whenever possible from up to four spikes. Caryopses were moistened overnight before observing them with a light microscope at 275× magnification. Using a cryogenic Reichert microtome, 30–50 µm transverse sections of caryopses were collected in a Lugol iodine reagent and fixed in lactic acid onto a microscope slide (1 section/coverslip, 2 coverslips/slide). Two fields of view per section, one from each side of the dorsal kernel rib, were observed as described below.

To estimate the number of aleurone cell layers, fields of 50–70 cell stacks along the pericarp were scanned visually and the proportion of 2-cell stacks per field was scored according to the scale shown in Table 1. For each field of view, the thickness of the aleurone layer between the pericarp border adjacent to the aleurone and the border of the Lugol-stained starchy endosperm was measured using a micrometric ocular lens. One measurement was taken per field of view. Thus a total of 32 series of aleurone layer observations per F2 plant/F3 grain progeny were obtained.

F3 grains generally have stacks of two or three cell layers, but rarely there are grains with intermediate proportions of stack types or heterogeneous fields. Therefore, an additional variable "the proportion of 2-cell types (D), 3-cell types (T) and heterogeneous (H) grains, over the total of eight F3 kernels observed per F2" was considered. Most rarely, 4-cell stacks were found, particularly in the 3-cell type grains like the Erhard Frederichen parent; these were included with the 3-cell type in the estimations.

Table 1 Scoring scale (11 classes) used to record the number of aleurone cell layers in each microscope field of view according to the proportion of 2-cell stacks estimated visually

Observed % 2-cell stacks in microscope field	Analyzed corresponding number of aleurone layers
0–5	3.10–2.95
5–10	2.95–2.90
10–20	2.90–2.80
20–30	2.80–2.70
30–40	2.70–2.60
40–60	2.60–2.40
60–70	2.40–2.30
70–80	2.30–2.20
80–90	2.20–2.10
90–95	2.10–2.05
95–100	2.05–2.00

Results

SNPs in *sal1*

As a first step in studying the inheritance of aleurone layer traits, primers specific to *sal1*, the maize multilayer aleurone gene, were used to amplify fragments from two parental barley lines, Erhard Frederichen and Criolla Negra. The sequences of the 562 bp fragments were aligned with known *sal1* sequences and neighbor-joining tree analysis (not shown) indicates clearly that the new sequences are homologous to the maize and rice *sal1* genes. Accession numbers assigned by EMBL database are: AM931535 for Erhard Frederichen sequence and AM931536 for Criolla Negra. Two polymorphisms were detected: A/C and T/G changes at position 38 and 234 of the consensus sequence, respectively. The T/G change leads to an amino acid substitution. The TCC (in Erhard Frederichen) and GCC (in Criolla Negra) codons specify serine and alanine, respectively. This SNP was used for genetic mapping. Two 17-base (excluding the M13 tail) forward primers were designed with a dimorphic nucleotide at the 3′ end specific to each parental SNP variant. The forward primer sequences, including the 19-base M13 tail, were 5′-CACGACGTTGT AAAACGACCCTCGACGCCGTCGTCG-3′ for Criolla

Negra and 5′-CACGACGTTGTAAAACGACCCTCGA CGCCGTCGTCT-3′ for Erhard Frederichen. The reverse primer used was that designed in the conserved region (described above). As expected, DNA from the specific parent amplified well with these primer pairs but not that from the other line. Of the 190 F2 plants, 6 (3.2%) gave inconsistent results due to non-amplification.

Genetic linkage map of the F2 (Erhard Frederichen × Criolla Negra)

A map was built of the F2 progeny of a cross between Erhard Frederichen and Criolla Negra using 137 markers (Fig. 1). In total, the map covered 845 cM in seven linkage groups, plus two unlinked markers, Hvm36 and EBmac708 on chromosome 2H and 3H, respectively. Four morphological traits (genes *Blp*, *vrs1*, *eog*, and *srh*) with no segregation distortion were mapped in their expected positions (Franckowiak 1997).

sal1 was mapped on chromosome 7H, between the markers EBmag757 and the Hvm49–GBM1065 pair. This sequence corresponds to a single copy. The orthologous gene in rice was located on a syntenic segment of chromosome 6. Primer sequences of five wheat SSRs were used: Xcfe2, Xcfe87, Xcfe175, Xcfe214 and Xcfe220 (see Graingenes database).

The map had an overall missing coverage of 28% with reference to the SSR barley map of Ramsay et al. (2000), that is, 12, 46, 73, 17, 22, 2 and 5% for chromosomes 1H–7H, respectively. Thirteen markers showed a significant segregation distortion, including a cluster of five markers near the 7H chromosome centromere. All markers but three, Hvm30, AWBMS22 and GBMS88, on this map have the same position as in previously published barley maps.

Phenotype of aleurone layer traits of F3 grains and hypotheses on how traits are inherited

For Erhard Frederichen and Criolla Negra, 4 microscope fields per grain for 26 grains per parent line were observed and scored (Table 2). The 104 observations on each parent gave consistent results, although the homozygous material showed some variation within single plants, within spikes, and even within kernels. Erhard Frederichen aleurone generally has 3-cell stacks, some 2-cell stacks, and more rarely 4-cell stacks (Fig. 2a). Criolla Negra aleurone has mainly 2-cell stacks with some 3-cell stacks (Fig.2b). The plants of parental genotypes seemed homogeneous as pure

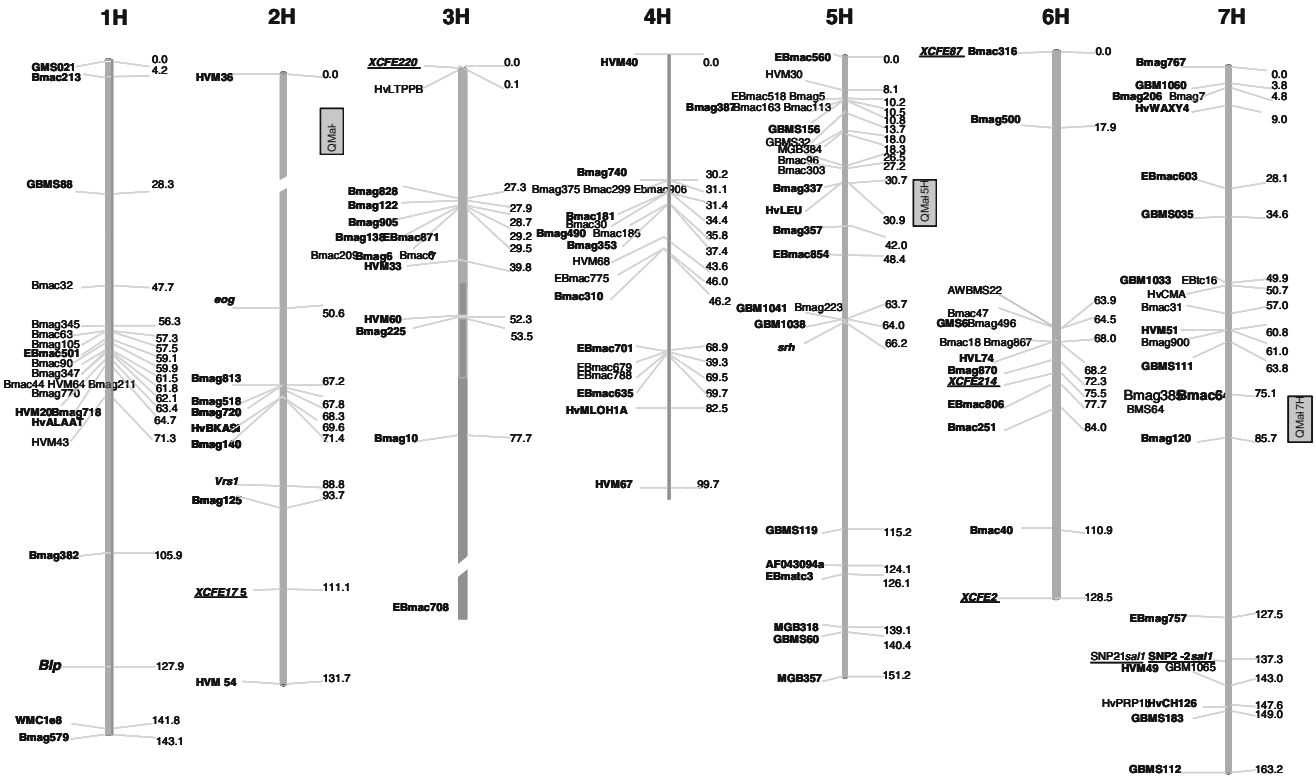

Fig. 1 Genetic linkage map of F2 barley population (Erhard Frederichen × Criolla Negra). SSR markers unlinked by Mapmaker are assigned as on consensus maps. SSR markers have all been previously reported except for five wheat-EST-derived SSRs (*Xcfe2, Xcfe87, Xcfe175, Xcfe214, XcfeE220*), and novel SNPs in the *sal1* gene. Kernel or spike morphological markers are in *bold italics* (***Blp, vrs1, eog, srh***). Markers used for aleurone trait QTL detection and mapping are in *bold*. QTL maximum LOD interval is shown in *rectangles* along chromosome 2H (QTL "QMal-2H"), 5H (QTL "QMa1–5H") and 7H (QTL "QMal-7H")

Table 2 Aleurone layer trait data of Erhard Frederichen and Criolla Negra: means and confidence intervals at the (1–2α) = 0.95 level

Parent	Aleurone trait		Sample origin			
			Genebank[a]	Glasshouse[b]	Parent plants[c]	Total
		Nr grains observed	6	10	10	26
Erhard Frederichen	Number of layers	Mean	3.07	2.98	2.91	2.99
		Conf. interval	3.03–3.11	2.94–3.02	2.82–2.99	2.93–3.05
	Thickness (μm)	Mean	81.7	84.7	81.7	82.7
		Conf. interval	76.9–86.5	80.9–88.5	76.5–86.9	77.9–87.5
Criolla Negra	Number of layers	Mean	2.11	2.13	2.10	2.11
		Conf. interval	2.03–2.19	2.05–2.21	2.02–2.18	2.07–2.15
	Thickness (μm)	Mean	54.8	47.3	57.5	53
		Conf. interval	51.8–57.8	43.6–51.0	53.9–61.1	50.4–55.6

[a] Seed from IHAR (PL) for Erhard Frederichen, and VIR (RUS) for Criolla Negra

[b] Glasshouse, INRA Clermont-Ferrand, 2003

[c] Spikes of parental plants in (Erhard Frederichen × Criolla Negra) cross, INRA Clermont-Ferrand, polythene greenhouse, 2003

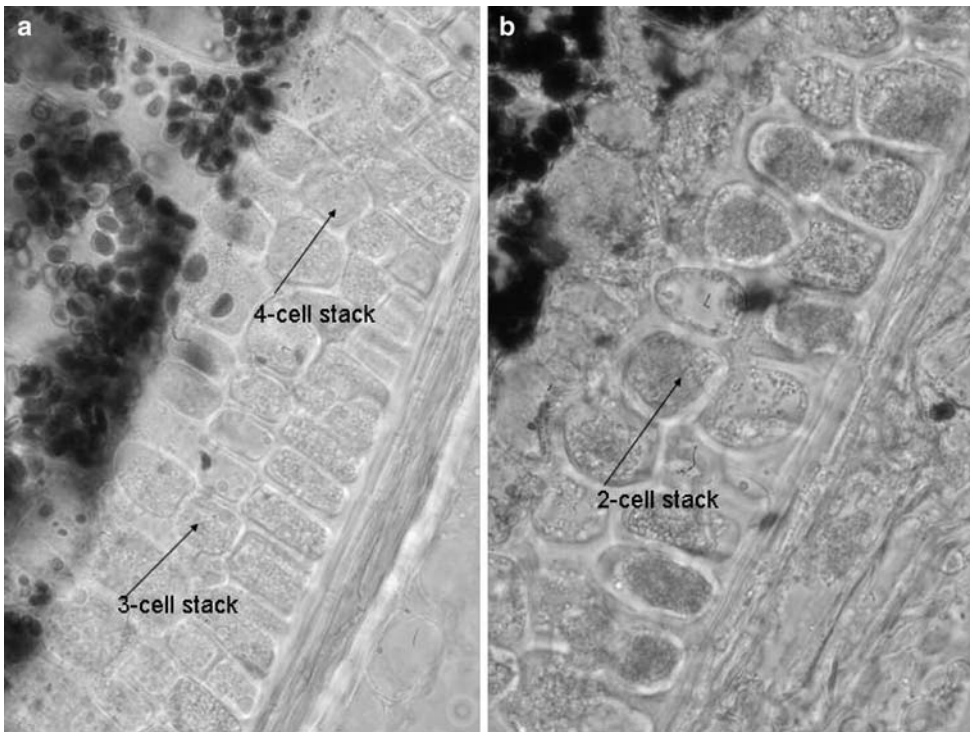

Fig. 2 Sections of **a** Erhard Frederichen and **b** Criolla Negra grains showing the aleurone layer with *3-cell* and *2-cell stacks*, respectively. A *4-cell-stack* is also seen in **a**. On *the left*, starchy endosperm amyloplasts stained dark with Lugol reagent. On the *right*, peripheral tissues (crushed nucellus epidermis, grain integuments and part of the pericarp)

lines grown in the greenhouse and in the field over the 3 years from 2003 to 2005.

The F2-derived F3 progeny from the cross between Erhard Frederichen and Criolla Negra were phenotyped. A striking feature was that most of the 1,520 F3 kernels had an aleurone layer similar to that of one or other parent, and seldom had intermediate proportions of 2-cell versus 3-cell stacks and/or heterogeneous values. Very few grains were classed as having a 40–60% 2-cell-layer aleurone (Fig. 3a). Generally, for each grain, all observations gave the same pattern estimated as being either 2-cell (=D) or 3-cell (=T). However, there were some exceptions. Out of 1,520 kernels, 48 (3.16%) were classified as heterogeneous (H), where results differed between the two sections of the same grain or between two fields of the same section. A small proportion (0–10%) of atypical stacks within a microscope field or between fields of the same grain was not considered as heterogeneity as similar variations also exist in the homozygous parents (Table 2). Overall 840 F3 grains were classified as "D" (55.26%) and 632 F3 grains as "T" (=41.58%). This difference between "D" and "T" frequencies is highly significant ($\chi^2 = 29.4$; $P = 5.9 \times 10^{-8}$ with

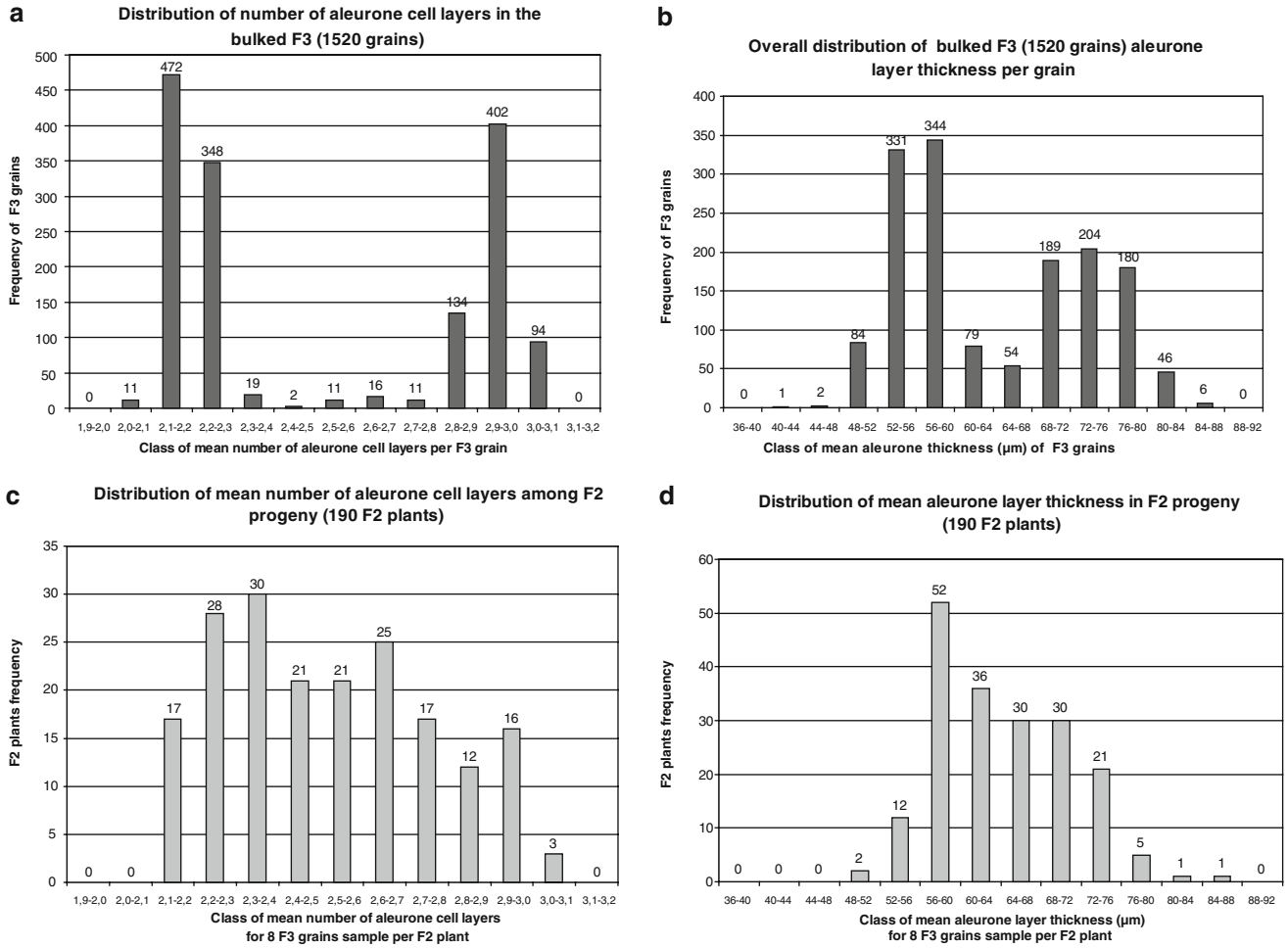

Fig. 3 Progeny of the cross (Erhard Frederichen × Criolla Negra). Distribution of aleurone layer data in bulked F3 generation grain and F2 generation: **a** number of aleurone cell layers in 1,520 F3 grains; **b** thickness of aleurone layer in 1,520 F3 grains; **c** mean number of aleurone layers in F2 progeny in eight-grain samples from each of 190 F2 plants; **d** same as **c** for aleurone thickness

one degree of freedom) and indicates a tendency of underlying genetic factor(s), acting together, to show dominance in favor of the 2-cell genotype in the absence of a gamete selection effect or any other distortion. The thickness of the aleurone layer was measured in the same F3 samples (Fig. 3b). A similar bimodal F3 distribution histogram was obtained. The linear coefficient of correlation on the 1,520 pairs of F3 data on "number of aleurone cell layers" versus "aleurone layer thickness" was $r = 0.908$*** confirming the close association between the two traits. Aleurone layer thickness was not significantly correlated with grain size or plumpness (1,000 kernel weight) even when comparing small grains of the 6-row genotype with those of the 2-row genotype: $r = 0.066$, $P = 0.344$ N.S. (data not shown).

The data from the F2 progeny were obtained by averaging the measurements made on eight F3 grains sampled from each F2 plant; these data present a quite different picture. Except for the F2 plants homozygous for the putative genetic factor(s) controlling aleurone layer structure, the eight-grain samples were still segregating. Hence, the F2 means show a distribution with a much shallower depression in the middle (Fig. 3c, d). For instance, a sample with three grains classified as "D" and five as "T" gives an average of 2.5–2.6 aleurone cell layers (Fig. 3c). The F2 aleurone thickness assessed in the same way gave similar results (Fig. 3d). As observed in the F3, the linear correlation coefficient between the 190 pairs of F2 mean results for "number of aleurone cell layers" versus "aleurone thickness" was highly significant ($r = 0.921$***).

In addition, for F2 plants putatively heterozygous for one or two genes, the final estimate of the mean number of F3 aleurone cell layers depended entirely on the expected binomial proportion of 2-cell versus 3-cell stacks among the eight randomly selected F3 grains. The confidence interval (binomial distribution at $P = 0.95$) of such a small sample mean is very wide, for example, for an observed mean of 5, it ranges from 0.6 to 6.1 2-cell type grains. Such large sampling errors in F2 estimations do have a major

impact on the QTL approach as they markedly lower the observed correlation between F2 aleurone phenotypic values and markers, and hence are expected to generate largely underestimated PVEs (part of the variance explained by the QTLs) on the whole F2 population.

Essential information can however be drawn from the tails of these F2 mean distributions. First, the number of aleurone cell layers and aleurone thickness distributions are dissymmetrical. The aleurone cell layers scored in the 2.1–2.4 range are from 75 F2 individuals which differs highly significantly from the 2.8–3.1 range with only 31 individuals ($\chi^2 = 18.3$; $P = 1.9 \times 10^{-5}$ with 1 *df*). This result confirms that the overall balance of the genetic factor(s) involved is partly dominant for a lower number of aleurone cell layers, as already indicated by the bulked F3 data.

In addition, the F3 distributions (Fig. 3a, b) suggested that the traits considered are probably not governed by a standard polygenic model with additive gene effects as the distributions are far from Gaussian. A monogenic or oligogenic Mendelian model could thus be hypothesized.

In the case of a monogenic Mendelian trait, the F2 plants homozygous for the trait would be expected to always give eight homogeneous F3 grain samples either of the 2-cell or 3-cell type. For a single-factorial trait without dominance, one-fourth of the homozygous 2-cell F2 plants would be expected, that is on average 47.5 F2 plants. In the case of complete dominance of the putative 2-cell allele, we would expect on average 142.5 2-cell F2 plants, that is, three-fourth. In the extreme homogeneous classes, we observed 25 F2 plants with only 2-cell grains and 16 F2 plants with only 3-cell grains. Therefore, a model with simple monofactorial dominance or co-dominance does not fit the results obtained from the 8-grain samples over the F2 population of 190 individuals. In addition, with dominance the expected overall ratio of F3 grains would be 5/8 [AA] and 3/8 [aa] phenotypes. Disregarding the 48 heterogeneous kernels, with the single factor hypothesis, we found 840 putative [AA] and 632 [aa] phenotypes. The statistics testing the adequacy of an overall 5:3 segregation in the F3 gave a χ^2 value of 18.55 with $P = 1.65 \times 10^{-5}$ at 1 *df*, leading us also to reject this 5:3 monogenic hypothesis. These results suggest that inheritance of "the number of cell layers in barley aleurone tissue" is oligogenic.

The quite low frequency (3.16%) of heterogeneous "H" F3 grains was higher in mixed 2-cell/3-cell sample classes than in extreme homogeneous classes, suggesting that these may be associated with more heterozygous genotypes. However the frequencies are too low to test this hypothesis.

Detection of QTLs affecting aleurone tissue traits

QTLs affecting aleurone layer traits with LOD scores above 3.0 detected on chromosomes 5H and 7H, considering the whole 190 F2 population, are shown on Table 3, part a. For the number of aleurone cell layers, the QTL on 5H has a maximum LOD of 5.83 ($P < 10^{-4}$ after 2,000 runs of the permutation test) while the QTL on 7H has a maximum LOD of 3.23 ($P < 10^{-2}$). For aleurone thickness, a QTL on

Table 3 Separate QTLs found using interval mapping of barley aleurone traits on 5H and 7H chromosomes

Aleurone trait	Chr.	Interval	Max. LOD score	Interval length (cM)	QTL position (cM)	95% Conf. Interval (cM)	PVE (%)	Addit. effect	Domi. effect	Resid. error
(a) Whole F2 population of 190 F2										
Nr of cell layers	5H	HvLEU-Bmag357	5.83	11.3	10.0	0–33.9	16.8	0.10	− 0.03	0.18
	7H	Bmac64-Bmag120	3.23	10.3	4.0	62.5–110.8	12.3	0.03	− 0.11	0.18
Thickness	5H	HvLEU-Bmag357	4.21	11.3	6.0	2.0–42.8	13.5	2.95	− 0.75	6.20
	7H	Bmac64-Bmag120	4.45	10.3	4.0	63.7–101.8	14.6	1.64	− 3.52	4.78
Frequency of 2-cell stacks in eight F3 grains	5H	Bg357-EBmac854	4.71	6.4	0.0	16.3–40.9	13.0	−1.15	0.23	2.39
	7H	Bmac64-Bmag120	(2.00)	10.3	4.0	–	(6.5)	–	–	–
(b) Sub-population of 88 F2 (tails)										
Nr of cell layers	5H	HvLEU-Bmag357	5.29	12.2	6.0	0–29.9	28.4	0.18	−0.06	0.22
	7H	Bmac64-Bmag120	3.51	10.3	4.0	72.6–92.8	22.0	0.09	−0.19	0.05
Thickness	5H	HvLEU-Bmag357	4.76	12.2	4.0	0–29.9	25.3	5.39	−2.22	7.45
	7H	Bmac64-Bmag120	4.20	10.3	4.0	72.6–90.6	25.4	2.21	−6.78	5.85
Frequency of 2-cell stacks in eight F3 grains	5H	Bg357-EBmac854	5.27	12.2	6.0	0–22.7	28.2	−2.18	0.71	2.42
	7H	Bmac64-Bmag120	3.21	10.3	4.0	70.6–92.6	20.4	−0.67	2.41	2.42

Phenotypic data of eight-grain F3 samples from: (a) 190 (Erhard Frederichen × Criolla Negra) F2 genotypes, and (b) a sub-population of 88 F2 individuals from the tails of this F2 distribution

In brackets reported for the record, but inferior to threshold LOD = 3 for QTL acceptance

PVE percentage of variance explained by the QTL

7H has a maximum LOD of 4.45 ($P < 10^{-4}$), against a LOD of 4.21 on 5H ($P < 0.5 \times 10^{-3}$). The corresponding LOD values are quite similar for the third, discrete, trait "Frequency of 2-cell stacks in eight F3 grains". The maximum LODs and locations of the three traits studied map nearly to the same position. As these traits are strongly correlated, the QTLs concerned are likely to be the same.

With a multipoint and interval mapping analysis, no above-threshold QTLs were found on chromosomes 1H, 2H, 3H, 4H or 6H, with the maximum LOD being 1.4, 2.0, 0.9, 1.2, and 2.1, respectively. The only near-telomeric unlinked marker on 3HL, EBmac708, was not correlated with the traits studied, unlike Hvm36 on 2HS that was also unlinked. The mean number of aleurone cell layers and aleurone thickness correlated with Hvm36 marker ($r = 0.255$***), but not with *eog*, the nearest mapped marker with reference to our map and consensus maps mentioned above. A *t* test to compare the three genotype classes at locus Hvm36 confirmed the probable existence of a QTL on chromosome arm 2HS: the two homozygote classes were significantly different ($P = 0.012$**). As the classes [AA] (Criolla Negra genotype) and [AB] (heterozygotes) were not significantly different ($P = 0{,}406$ N.S.), dominance of Criolla Negra allele for this QTL is suggested while [AB] (heterozygotes) and [BB] (Ehrard Frederichen) classes were also significantly different ($P = 0.043$**). The minimum part of the variance (PVE) explained by this QTL on 2HS is $r^2 = 6.5\%$***. This QTL is not reported in Table 3: as interval marker is not applicable, Hvm36 being an unlinked marker, the precise location of the QTL on 2H cannot be estimated, and its true PVE cannot be determined as well.

The 3-layer phenotype appeared inherited by the three QTL alleles from parent Ehrard Frederichen. Concerning the PVE explained by the two 5H and 7H QTLs, the large bias introduced by the sampling error on segregating F3 lines led us to consider a "selected genotyping model" on sub-populations of F2 phenotypes from the right and left tails of the total distribution. Such a sub-population of 88 F2 individuals (46.3% of the entire F2 population) gives a fairly high joint PVE of 52.1% by the QTLs for "number of aleurone cell layers", as shown on part b) of Table 3. In contrast, over the whole 190 F2 progeny, the overall PVE of the characters explained together by the two QTLs on 5H and 7H is only 24.8%.

As the LOD score curve of the QTL on 5H was bimodal, a model with two linked QTLs was tested. A two-linked-QTL model tested with 200 permutations using Multi-QTL led to a non-significant LOD score increment of 1.32 at the $\alpha = 0.05$ level.

A QTL search with the constraints of particular genetic sub-models was carried out (Fig. 4). The QTL on chromosome 7H for number of cell layers and aleurone thickness showed the best LOD score under constraints with the dominant sub-model with the 2-cell type as dominant, with an insignificant LOD decrease of 0.54 from the "free" model. In contrast, the additive or "3-cell type as dominant" sub-models led to a LOD decrease of 2.45 or 3.21, respectively, from the "free" model. The situation was less clear-cut for the other QTLs on 5H. An additive sub-model showed a non-significant LOD decrease of 0.31 from the "free" model LOD score of 5.83, while the "2-cell type dominant" and "3-cell type dominant" sub-models showed LOD decreases of 0.84 and 3.45, respectively. Results were similar for aleurone thickness.

Multi QTL software was used for bootstrap estimations of confidence intervals ($P = 0.95$) of QTL locations on 5H and 7H, shown in Table 3 as affecting the "number of aleurone cell layers". For aleurone thickness, the corresponding confidence intervals were 0.0–82.0 and 33.9–109.2,

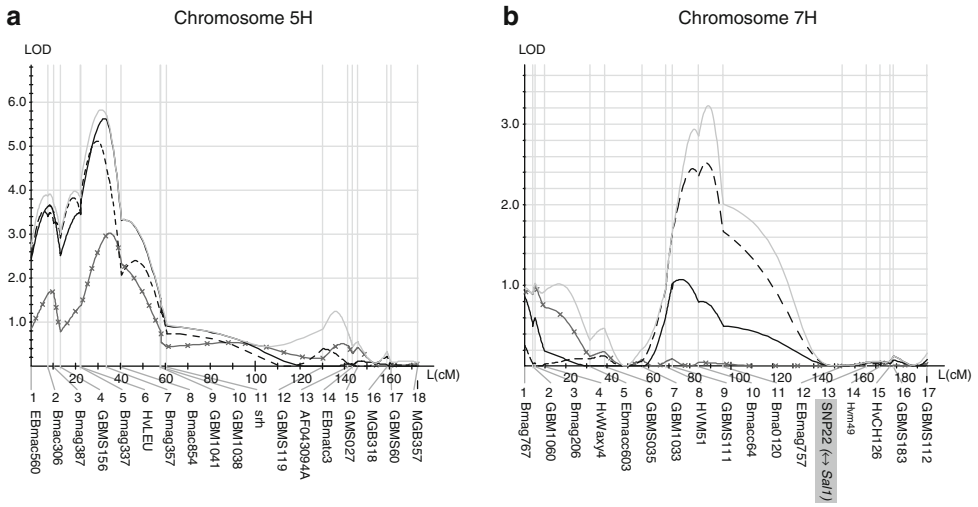

Fig. 4 Sub-models of QTLs for number of aleurone layers: LOD curves of the 190 (Erhard Frederichen × Criolla Negra) F2 population: **a** QTL on chromosome 5H; **b** QTL on chromosome 7H. *Plain grey curves* show default free model without constraints, *black plain curves* show additive sub-model, *black dotted line* the "D" 2-cell dominant sub-model, and *grey line* with *crosses* the "T" 3-cell dominant sub-model

respectively. Some bootstrap draws extended the confidence intervals considerably, but most bootstrap samples were restricted to the relevant interval and its neighbors for QTLs on 7H and 5H.

Concerning interactions between 5H and 7H QTLS, results on a sub-population of 125 F2 individuals, out of the 190, is described (Table 4) for each of the nine assumed QTL5H × QTL7H genotypic classes. As shown above, the key feature shaping the variations is the proportion of 2-cell (D) versus 3-cell (T) layers, a point that has been summarized for each class.

A bi-factorial ANOVA showed highly significant main effects for QTL of 5H ($F = 7.77$, Pr = 0.00068*** with 2 and 116 *df*), significant for QTL of 7H ($F = 3.15$**, Pr = 0.047 with 2 and 116 *df*), but non significant for overall QTL5H × QTL7H interactions ($F = 1.48$, Pr = 0.22 N.S.; 4 and 116 *df*). These results were confirmed using a non-parametric factorial variance analysis on unbalanced weighed ranks based on Kruskal–Wallis statistics: main effects were both highly significant, with Pr (χ^2); 2 *df* = 0.00014*** for QTL on 5H, and 0.013*** for QTL on 7H. The interaction effect was also nonsignificant: Pr (χ^2); 4 *df* = 0.11.

Interestingly, the two parental double homozygote classes AA/AA and BB/BB for QTLs on 5H and 7H (10 individuals) never included all individuals with eight similar parent-like F3 kernels, as in purely parental types, and never widely differed in values either. There were one to three 3-cell kernel among the eight kernels of the two AA/AA F2 individuals, which were however a BB or AB genotype at Hvm36 marker linked with the QTL on 2H. Even four intermediates were present among the eight double recessive BB/BB F2 individuals. These four intermediates were all AA or AB genotypes at Hvm36 marker, contrary to the others. This would match well with the effect of QTL on 2H, which revealed dominance of AA and AB genotypes for 2-cell layer types. However the interactions between QTL on 2H and other QTLs were difficult to estimate due to lower density of markers around QTL on 2H.

Finally, the mapping position of *sal1* on the 7HL chromosome arm between the markers Ebmag757 and Hvm49 does not co-locate with that of the QTL found on the same chromosome. This QTL was mapped to an interval between Bmac64 and Bmag120 more than 60 cM from *sal1*.

Discussion

To maximize the chances of finding QTLs that affect the barley aleurone layer, parent lines that differed as much as possible in their aleurone traits were chosen. In addition, the two parents were polymorphic for 52% of amplifying barley SSR markers. However, other genotypes including European cultivars, such as Isaria, and Asian accessions, such as *Pallidum*10343, also have an average of less than 2.3 aleurone cell layers, whereas cv. Russia I, or particular formae, for example, *erectum, nudum, intermedium*, have the 3-cell type aleurone (Sawicki 1950). The QTLs described above could also be assayed on these backgrounds.

Novel information on the inheritance of the aleurone tissue structure in barley has been gleaned from this study. A simple single-factor Mendelian model of the inheritance of the number of aleurone cell layers and the overall thickness of the aleurone layer can be ruled out. Based on a sub-population found at the tails of the distribution, a model with

Table 4 Joint effects of QTLs on 5H and 7H chromosomes on a sub-population of 125 F2 genotypes displaying alleles of the same parent on both flanking markers of each QTL locus

Item	QMal-7H	QMal-5H			QMal-7H sums weighed means
	F2 Genotypes	AA	AB	BB	
F2 Frequency	AA	2	11	7	20
Average nr of aleurone layers		2.30	2.31	2.57	2.40
% 2-cell kernels in F3 samples: high/med./low		50 / 50/ 0	27 / 72 / 0	14 / 43 / 43	
F2 Frequency	AB	20	30	23	73
Average nr of aleurone layers		2.32	2.25	2.52	2.35
% 2-cell kernels in F3 samples: high/med./low		35 / 65 / 0	50 / 50 / 0	22 / 43 / 35	
F2 Frequency	BB	11	13	8	32
Average nr of aleurone layers		2.35	2.56	2.61	2.50
% 2-cell F3 kernels in F2: high/med./low		27 / 64 / 9	15 / 62 / 23	0 / 50 / 50	
F2 Frequency	QMal-5H	33	54	38	125
Average nr of aleurone layers	Weighed means	2.33	2.34	2.55	2.40

Frequency of the nine genotypic classes, respective average aleurone layers number and % distribution of individual 8-kernel F3 samples according to 2-cell layer (D) kernel frequency (high = 7 or 8 D; medium = 2–6 D; low = 0 or 1 D)

2 major QTLs can explain more than 50% of the trait variance. A third QTL on 2H chromosome was also evidenced using single marker analysis. Linkage with Hvm36 marker allows estimating its PVE as 6.5% as a minimum, but a better coverage by additional markers of 2HS arm would be desirable. A larger overall PVE of the traits would probably have been attained if interval mapping could have been applied also to this third QTL on 2H.

Regarding the interactions between QTLs on 5H and 7H on 125 appropriate F2 genotypes, the overall dominance of QTL on 7H on the whole 190 F2 population still appears on the 125 F2 sub population, in which in turn the A allele of QTL on 5H shows dominance (average: 2.33 layers for genotype AA at QTL 5H, and 2.34 for AB).

A sound analysis of epistasis was not easy, because the phenotypic values were estimated on small samples in F3 generation: for example the double heterozygote F2 class AB5H/AB7H is expected to have fixed one half of its loci in F3. Hence, as both QTL loci on 5H and 7H tend to show dominance of 2-cell type, the occurrence of a somewhat lower average layer number in this AB/AB F2 class should be expected (see Table 4). In addition, the absence of information on which genotypes have actually evolved in F3 from each individual, in F2 classes with QTL on 5H and/or QTL on 7H heterozygotes, hampers a more precise approach, as does the role of QTL on 2H. Therefore further studies would be requested for a better understanding of the interactions between these 3 QTLs. Several factors make it difficult to suggest at the present stage a detailed scheme of genetic control.

1. As there was evidence for a contribution of co-dominance and as the aleurone, an endosperm tissue, is triploid, giving 16 possible different genotypes, a gene dosage effect might play a role in either direction.
2. According to Olsen (2004), the developing aleurone is influenced by its position. This implies potential genetic and epigenetic interactions. Thus, identical F3 grain genotypes produced by different segregating maternal F2 genotypes might lead to different F3 phenotypes. Such an influence could be caused by effects at the periphery of the aleurone, contact with maternal tissue, sporophytic effects via the chalaza, or by transported nutrients or signaling molecules.
3. The sampling error in the 8-grain F3 progenies is very high.
4. The F2 frequencies for numerical traits, such as the average number of cells or the average thickness, belong to arbitrarily designated classes and do not show true discontinuity. Therefore these classes cannot be assigned to defined genotypes with certainty (Fig. 3). Further studies using doubled haploids or recombinant inbred lines might provide a better inference from phenotype to genotype and if possible a denser map in the segments already likely to carry candidate genes (Stein et al. 2007) could be used.

Instead of phenotyping the F3 generation, it might have been preferable to phenotype F2 half-grains while growing the other half-grain containing the embryo for genotyping. This may have introduced bias due to selective survival rates in these conditions. In addition, phenotyping the F2 would then rely on single grains and could fluctuate, especially in heterozygous individuals. In addition, as experienced with F3 grains from segregating F2 plants, it would not have been possible to infer the true genotype of an F2 grain from its observed 2-cell or 3-cell type phenotype.

To limit the work involved in phenotyping aleurone traits, careful scoring of aleurone cell numbers in individual microscope fields was preferred to a more painstaking stack-by-stack count as preliminary observations on microscope sections of parental tissue had proved this scoring approach to be reliable. The high correlation of the number of cell layers with aleurone thickness confirmed this approach to be valid as the latter measurement is less prone to human error. Despite the use of a scoring approach, phenotyping F3 grains required a lot of microscopy work. This was why the sample sizes of F3 observed per F2 plant were small, resulting in a large sampling error. However, it was not judged appropriate to reduce the number of F2 plants genotyped in favor of a wider F3 grain sampling per F2 plant.

The three QTLs might explain a large part of the variation in aleurone traits in the progeny studied. However, if well-mapped chromosomes 1H and 4H do not show any possible QTL, mapping gaps on chromosomes 3H, 5H, 6H and 7H might have hampered the detection of other QTLs. This seems unlikely for chromosomes 5H, 6H and 7H, as a QTL located in the middle of a segment with an appreciable LOD score would probably not have escaped detection if compared to observations on 2H. However on the 3HL chromosome arm, out of 11 SSR markers tested, only one, EBC708, was amplified and was polymorphic. Although EBC708 is not correlated with any aleurone trait, the missing 3HL segment represents about 100 cM on consensus maps and might then contain an undetected QTL.

The barley factors found on 2H, 5H and 7H were named Qmal-2H, QMal-5H and QMal-7H respectively, in accordance with Graingenes database nomenclature. The *sal1* locus appeared to be neutral in affecting the aleurone layer traits. However, other genes might be involved (Shen et al. 2003; Tian et al. 2007).

The multiple-layer aleurone in maize is controlled by a dominant- or partially dominant-single gene (Wolf et al. 1972; Nelson and Chang 1974) and by two or more partially dominant genes (Duangploy et al. 1976). de Miranda (1980)

found in contrast that two linked dominant genes plus a recessive one influenced this trait, although these results depend on the cross studied (de Miranda 1980). Bertoia and Magoja (1985) crossed maize with teosinte, a wild relative (*Zea perennis* Reeves & Manglesdorf). In the F2, transgressions over the aleurone-layer values of both parents were observed. Multilayer single kernels showed varying single layer/multilayer zones. We observed this effect in barley too, although the frequency was much lower (only ~3% of kernels were heterogeneous in the barley F2). A single recessive mutation explains the multilayer maize-teosinte variation.

Our inheritance study does not provide new information on the morphogenesis of barley aleurone. Stacks of two, three or even four cells aligned periclinally in aleurone layers suggest that mitoses in a periclinal direction could produce such morphology. Certainly a number of factors are part of the genetic "black box" programming the phenomenon (Wisniewski and Rogowsky 2004; Consonni et al.2005). A series of genes affecting maize aleurone layers has been documented and some genes been cloned and their function assessed or suggested. Shen et al. (2003) describe a Mutator-suppressed mutant of *sal1* having one to seven aleurone layers. The loss of function of *crinkly 4* blocks aleurone formation (Becraft et al. 1996), while *dek1* (Becraft et al. 2002) impairs the formation of aleurone cells. Other gene mutations affecting aleurone development are: *dil1* and *dil2* (Lid et al. 2004) causing a disorganized aleurone sometimes with multiple layers; *Xcl1*, giving multicellular layers in maize protoderm (Kessler et al. 2002); *Dap3* and *Dap7*, which generate aberrant aleurone layers (Wisniewsky et al. 2004); and *Wpk1* which alters aleurone development close to the embryo (Suzuki et al. 2006). Recent studies (Geisler-Lee and Gallie 2005; Gruis et al. 2006) suggest that aleurone-type differentiation is conditioned by the external surface position rather than by signalling from surrounding maternal tissue. Such studies have seldom involved barley, although barley shrunken endosperm mutants (Bosnes et al. 1987) might be useful in studying the interactions between these *sex* factors and the factors reported in this paper.

Even though they are confined to the interval with the highest LOD scores, it would be difficult to align the barley aleurone QTLs found here with a reasonable number of candidate genes. A further step of denser mapping is certainly required before finding a convincing sequence. This is a worthwhile objective due to the importance of aleurone in human and animal nutrition and other strategies involving TILLING or Mutator gene techniques can be followed concurrently.

Acknowledgments We are grateful to W. Podyma and I. Loskutov for providing seeds of barley germplasm, A. Graner and M. Röder for SSR primers from IPK, L. Zhang and P. Sourdille for CFE markers, C. Dupré (†) and P. A. Meunier for cryogenic microtome sectioning and microscopy, G. Gay and N. Guilhot for microscopy and QTL pictures, and FX. Oury and F. Vear for critical reading of the manuscript.

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