Identification and Improvement of Components of Barley Malting Quality.

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Introduction

Barley ranks amongst the five major crop species in both Europe and the world. Its most prominent features are high yield potential and excellent adaptability to divergent environmental conditions. Barley destined for the production of beer and whisky demands a premium at the farm gate but, in return, must exceed a range of malting quality (MQ) parameters laid down by the processing (malting, brewing and distilling) industries. MQ has been the subject of several studies. These reveal that it is a complex character, influenced by a number of genes and also by environmental conditions. It is subject to processor and consumer preference and to growers' varietal preference in the regions of production. Genetic factors which influence malting quality have been mapped, primarily in North American and European germplasm (Hayes *et al.* 1993; Han and Ullrich, 1994; Tinker *et al.*1994; Backes *et al.* 1995; Mather *et al.* 1997; Bezant *et al.* 1997; Powell *et al.* 1997; Thomas *et al.* 1995, 1996, 1998). Preliminary studies have been performed to assess the feasibility of marker assisted selection (MAS) for quality traits (Lee and Penner, 1997; Han *et al.* 1997a,b; Ellis *et al.* 1999). As a result, a framework understanding of the genetics of barley quality currently exists.

We report here some of the technologies we have been developing or applying in an attempt to facilitate the longer term improvement of the quality of malting barley. The first involves establishing marker technologies which can be routinely implemented in genetics and breeding. The second involves developing resources to enable us to apply the 'candidate gene approach' for the identification of genes involved in determining quality. It has two main components: i) a gene discovery program aimed at identifying sequences expressed during grain fill or when barley undergoes malting ii) a technology development program for high throughput transcript mapping, to correlate the genetic location of candidate genes with established QTL. Our goals are to develop a thorough understanding of the genetics and biochemistry of malting and to identify gene alleles which confer improved quality and will facilitate its manipulation through contemporary genetic or biotechnological improvement schemes.

Results And Discussion

Simple Sequence Repeat (SSR) - based Marker development

In 1993, Morgante and Olivieri demonstrated that SSRs were useful as genetic markers in plants. These authors identified a difference in the SSR repeat types and a much lower frequency in plants as compared to animals. Given the latter finding, a method for specifically enriching DNA populations for small restriction fragments which contain SSRs was developed and applied to (AC)n, (AG)n, (ACC)n, (AAC)n, (AAG)n and (ATC)n repeat motifs. Over 1800 positive clones were selected and sequenced. A

significant number of the (AC)n and (AG)n SSRs exhibited an intimate association with retrotransposons and other repetitive elements (Ramsay *et al.* 1999) and were discarded. In total over 700 primer pairs have been designed and tested with more than 550 amplifying the correct sized product from barley DNA. Saghai Maroof *et al.* (1994) showed the potential of SSR markers for application in barley genetics. Since then, two other reports by Becker and Heun (1995) and Liu *et al.* (1996) have confirmed these results. These authors described the development of approximately 50 SSRs, the majority of which were mapped on doubled haploid populations. Struss and Plieske (1998) recently identified 15 SSRs from barley which have not yet been mapped. Thus, there are currently over 600 barley SSRs available to the barley community.

SSR Genetic linkage mapping

Mapping the SSRs was performed on a Lina x *H. spontaneum* (Canada Park) doubled haploid (DH) population. Over 300 SSR loci have been placed onto this genetic map. Between 20 and 100 have also been mapped on other populations. The SSRs show clustering around the centromeric regions of each linkage group. While this has been observed for other marker types the effect has not been as severe as seen here. Whether the observed distribution is due to genetic or physical effects is not clear. However, studies with two sets of tightly linked centromeric SSRs in an intraspecific population revealed that the genetic distance between the SSRs increased in one case from 0 cM to 9 cM and in the other from 4 cM to 17 cM. In addition the genetic distance at some centromeric locations can be depressed in DH populations compared to normal F2 populations (A. Graner, pers comm).

QTL Analysis of quality traits

A number of DH populations have been used for linkage studies. For each of the populations, genome wide linkage maps were constructed using a combination of AFLP, RFLP and SSR markers. Common markers were used to align the maps, which allowed the identification of common QTL in different studies. QTL for quality have been detected on all linkage groups. By comparing results from these studies, some QTL are detected frequently in different populations, whereas others are specific to a single population. The obvious possibility is to combine positive factors using genetic markers flanking the QTL. SSRs flanking some important QTL have been identified and are currently being used for this purpose.

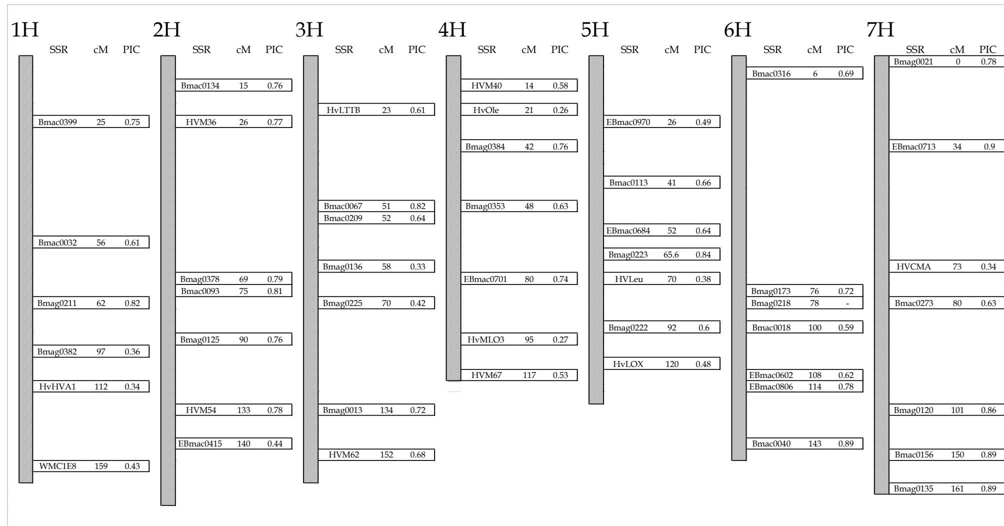

Figure 1: Name, Location and PIC values (in the cultivated genepool) of SSRs in the first genotyping set of 48 barley SSRs

A genotyping set of SSRs.

Unlike other molecular markers, SSRs and SSR allele information is essentially comparable across all labs. We have developed a preliminary 'genotyping set' of SSRs and distributed them to the academic research community. The chosen SSRs are high quality, robust, highly informative in the cultivated genepool (i.e. high PIC values) and are evenly distributed across the barley genome. They have been fluorescently labelled with three different fluorochromes to allow simultaneous examination and detection of up to nine different SSR loci on an ABI automated DNA sequencer. The location of the SSRs [i.e. cM from the end of the short arm of the Lina x H. spontaneum (Canada Park) population] and their PIC values in the cultivated barley genepool is given in Figure 1.

Germplasm analysis

Mapping genes or QTL onto genetic linkage maps provides primary information on a gene or locus that may be involved in a given trait. The application of this information is, however, frequently restricted to crosses or populations involving the original parent that was used in the linkage analysis. To add an extra dimension, mapped SSRs have been used to carry out a germplasm analysis of cultivars, landraces and *H. spontaneum* lines. Graphical genotypes for chromosome 3H for 16 lines are shown in Figure 2. In addition to obvious statutory value (i.e. for cv. identification, purity etc), the results are helping us address the following types of question:

- Are there key areas of the genome common to leading varieties?
- Are specific regions of the genome associated with performance in specific environments selected?
- Can we identify the 'source' genotypes of these segments?
- Can we identify chromosomal segments harbouring genes responsible for disease, quality and agronomic traits?
- Can we define alternative alleles available in these regions?

- How do the genepools change with geographical location or local ecology?

W1	W2	W3	W4	W5	W6	S1	S2	S3	S4	S5	S6	S7	S8	Hs1	Hs2
214	214	214	214	213	216	215	214	216	214	214	215	214	214	216	216
142	142	142	142	142	142	154	142	142	154	142	154	142	142	154	154
197	197	197	197	197	197	197	197	199	197	197	197	197	199	197	199
174	174	176	176	174	170	174	174	172	174	174	174	174	174	174	160
179	177	179	171	171	177	179	177	181	179	173	179	177	181	181	195
156	156	156	156	164	162	156	156	164	156	156	163	156	164	161	136
147	151	147	135	135	139	147	151	151	151	151	151	151	151	127	127
114	114	114	114	114	114	114	114	114	114	114	114	114	114	114	116
195	195	195	195	199	199	195	195	197	195	191	197	195	197	193	193
179	179	179	193	193	191	179	179	193	179	179	191	179	193	185	181
171	171	171	171	171	169	171	171	163	171	163	163	171	163	165	169
160	157	157	160	160	160	157	157	157	157	157	157	157	157	161	161
141	163	163	145	145	141	149	163	163	163	163	151	163	163	143	149
160	160	160	160	160	160	164	162	164	160	160	162	162	164	132	156
128	130	130	120	130	130	128	140	140	128	128	128	140	112	122	112
251	247	247	247	247	247	251	251	251	251	251	251	251	247	256	256
100	100	100	100	100	100	100	100	100	100	100	100	100	102	100	112
169	152	169	169	169	169	169	169	169	169	169	169	169	169	152	147

Figure 2: Graphical genotypes of the 16 parental lines used for initial germplasm screening. Eighteen SSRs mapping to chromosome 3H were assayed. Each column represents the genotype of a single individual. Each row represents the alleles detected at each SSR and these are colour coded for ease of interpretation. The size of each allele in base pairs is indicated in each box

Marker Assisted Selection:

One of our primary objectives was to develop and test markers that could be evaluated for use in barley improvement schemes for marker assisted selection. To that end, we have selected two different chromosomal regions to assess the efficacy of SSRs for MAS. One contains the Mlo mildew resistance gene, the other, the *rym4* and *rym5* resistance locus to the BaMMV/BaYMV complex

Mlo

Searching a 60kb BAC sequence encompassing the *mlo* locus on chromosome 4H of barley (Panstruga *et al.* 1998) revealed five SSRs. From these, three informative primer pairs were used to screen 148 *Hordeum vulgare* genotypes, including 77 spring and 21 winter cultivars and 50 ssp. *spontaneum* accessions representing a wide range of ecotypes (Pakniyat *et al.* 1997). The spring barleys included a sample of accessions featured in pedigrees of *mlo* cultivars (Thomas *et al.* 1998, J. Russell pers. comm.). All three SSRs were polymorphic although variation was limited in the cultivated lines to two alleles at each marker locus. In the *H. spontaneum* lines considerably more variation was found (3, 6 and 17 alleles at the three loci). Because the three SSRs are linked at a very short physical distance (<10kb), recombination is unlikely to have played a major part in reassembling the different alleles into new combinations. It is, therefore, possible to combine the information from all three informative markers to derive a 'haplotype' of the region encompassing *mlo.* Twenty-four haplotypes were revealed, only three of which were present in *H. vulgare* germplasm. The most common haplotype, found in 83% of the cultivated lines was also one of the two most frequent (13%) found in the *H. spontaneum* germplasm. The overall haplotype *PIC* value was very high at 0.93.

However, no linkage disequilibrium was observed between SSR haplotypes and possession of the *mlo11* (nor *Mlo9)* resistance gene alleles. As many *mlo* alleles are spontaneous or induced mutations, it appears that these have occurred in the most common haplotype background. Even though we have isolated three markers within a region spanning *mlo* (two actually in the gene), neither their individual marker genotypes nor combined haplotypes are diagnostic of resistance alleles. Even for such a simple trait, the best marker will probably need to detect the actual mutation that confers resistance.

BaYMV-2

Molecular mapping has located *rym4* and *rym5* conferring strain specific resistance to the BaMMV/BaYMV complex to the end of 3HL (Graner and Bauer 1993, Graner *et al.* 1999). *Rym4* and *rym5* form the genetic basis of resistance to this virus complex in European barley cultivars and breeding lines. Resistance to BaYMV-2 is of particular interest, because the acreage infested by this virus strain is gradually increasing and *rym4* (the most commonly deployed resistance gene) does not confer resistance to this strain (whereas *rym5* does). Since resistance conferred by the *rym5* locus is recessive, the availability of a codominant marker is prerequisite for the identification of heterozygous carriers in the course of a breeding program. One SSR marker linked at a distance of <1.5 cM to the resistance gene has been developed. To determine its utility as a selectable marker, the SSR has been used to screen a collection of barley genotypes containing either *rym4*, *rym5* or the susceptible locus. The rym4/5 linked SSR appears to be diagnostic not only for resistance vs susceptibility but can also differentiate between *rym4* and *rym5* types (Table 1). The use of this marker will make the introgression of corresponding resistance genes independent of environmental factors, which range from low infection frequencies, as observed after a cold and dry autumn, to increased winter killing under optimal conditions for virus infection.

Accession	Phenotype	SSR-Fragment Size	Accession	Phenotype	SSR-Fragment Size
Alraune	susceptible	176	Franka	resistant (rym4)	158
Corona	susceptible	176	Labea	resistant (rym4)	158
Gerbel	susceptible	176	Mokusekko 3	resistant (rym5)	160
Igri	susceptible	176	Resistant Ym 1	resistant (rym5)	160
Trixi	susceptible	176	Japan S-1001	resistant (rym5)	160
Ragusa	resistant (rym4)	158	Misato Golden	resistant (rym5)	160
Brunhild	resistant (rym4)	158	Kanto Nijo 19	resistant (rym5)	160
Colambo	resistant (rym4)	158			

Table 1. Diagnostic DNA-fragment pattern of microsatellite marker linked to rym4 and rym5. Fragment sizes are given for a set of randomly selected barley accessions being susceptible to BaYMV-2 or having different resistance genes on the long arm of barley chromosome 3H (rym4, rym5) (courtesy of A. Graner)

Gene Discovery and the candidate gene approach.

The maximum potential for manipulating a given trait will only be realised when the genes controlling the components of that trait have been isolated and characterised and the extent of the variation at the locus in the genepool established and related to the phenotype. Given the difficulties of the positional cloning of single genes in large genome species such as barley - let alone components of a quantitative trait - we have adopted the opportunities provided by physical methods of gene isolation for gene discovery. In basic terms, we have constructed cDNA libraries from developing and germinating (malted) grain and are systematically determining the DNA sequence of each of the clones in these libraries. Each of the sequences are used to search international DNA and protein databases to assign a potential function. The principle of the approach is that the presence of certain cDNAs, and their abundance in the libraries, will give a snap-shot of the biochemical processes which are going on in the sample used to make the libraries. As a result, we have recently submitted 1000 of our EST sequences to the International Triticeae EST Consortium (ITEC) database. To implicate any of these genes as candidates for involvement in malting quality, they will have to satisfy a number of criteria. First, their putative biochemical function (if known) must be consistent with the

process. Second, their spatial and temporal expression patterns should also be consistent. Third, their map location would be expected to correlate with QTL identified from genetical studies of the trait and, fourth, allelic variants would be associated with the phenotypic extremes of the trait. Once these criteria have been satisfied, the final proof of a candidate's involvement will come from 'quantitative complementation' through genetic transformation or targeted breeding. A current example of a candidate gene for malting quality is Beta amylase. Swanston *et al.* (1999) used a DH population between the cultivars Derkado and a breeding line B83 to locate QTL for 'fermentability' on chromosomes 2H, 3H and 5H. The QTL on chromosome 5H maps to the same region as Beta amylase, which is involved in starch breakdown. It is known to be highly-expressed upon germination. Kihara *et al.* (1998) have recently shown that genetic variation on the thermostability of Beta amylase is correlated with malting quality. Finally, SSR allelic variation at the Beta amylase locus also appears to show a correlation with quality types.

High resolution, high throughput physical mapping.

High resolution, high throughput mapping, which does not rely on the detection of polymorphism, is an objective of many research groups around the world, particularly for the development of candidate gene approaches for identifying genes involved in complex processes. In humans, radiation hybrid (RH) mapping fills this niche. We are currently evaluating RH mapping in barley. In RH mapping, a population of donor cells, which have had a selectable marker gene added (e.g. antibiotic resistance), is irradiated to physically shear the chromosomal DNA and then fused with an unrelated immortalised cell line. During this process, some of the fragmented donor DNA becomes incorporated into the genome of the recipient. Selection for the marker gene identifies hybrid cell lines, which can be picked and multiplied by cell culture. The hybrids are then evaluated using donor specific PCR-based molecular markers to see how much of the donor genome has been retained. As the recipient cell line is unrelated to the donor, PCR products are generated only when the portion of the donor genome that contains the target sequence is present in the hybrid cell line. When a suitable 'retention frequency' is obtained, individual hybrid cell lines are assembled into a 'radiation hybrid panel' which can be used for high resolution genetic linkage mapping using a presence/absence marker assay. The resolution of the mapping panel depends on the average size of the DNA fragments retained from the donor genome. Larger segments of retained DNA will increase the chance of detecting markers which are physically linked on the same piece of DNA, and on the 'retention frequency' of the hybrids. Resolution is, therefore, experimentally manipulable.

We are also developing 'HAPPY mapping' (Dear, 1997) as a feasible alternative. It has a number of potentially significant advantages which include simplicity and universality (features which are particularly important for plants where a wide range of species may be studied). HAPPY mapping involves shearing high molecular weight DNA to give a pool of random DNA fragments. The average length of the fragments depends upon the radiation dose and the sampling window from DNA fractionated by CHEF gel electrophoresis. A panel of very dilute samples is then taken from this pool and these are assayed by PCR for the presence of target DNA sequences. Dilution is determined empirically such that each sample in the panel contains less than a single genome equivalent of DNA. Marker sequences are, therefore, present in some samples but not all. As with RH mapping, the observation is that closely linked markers, presumably amplified from the same DNA fragment, are present in the same samples (unless another sample contains a DNA fragment which has been sheared between the two markers).

Markers, which are physically too far apart, display random assortment. For both RH and HAPPY mapping the availability of the large set of genetically mapped SSRs is critically important for anchoring the genetic to the physical map.

Concluding remarks

Selecting for quality traits in breeding programmes is frequently time-consuming and resource intensive. With recent advances in molecular genetics, it has been possible to map genes or loci affecting quality traits to a number of chromosomal positions. Screening the effective genepool to identify alternative alleles (or haplotypes) at the target loci can enhance this information. Tightly linked markers are potentially valuable for MAS and, if sensibly deployed, could add both value and a competitive edge to a breeding program. Markers which are useful for selection need to be relatively low tech, 'user-friendly' and suitable for high throughput analysis. At the current time, SSRs best fit the bill. Ultimately, the identification of the genes underlying simple or complex characters will provide the ultimate resource for improvement by either traditional or biotechnological routes. With considerable knowledge of the location of QTL for malting quality developing the 'Candidate gene approach' is a potentially valuable route towards that objective.

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