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Zhang, X-Q, Li, C., Panozzo, J., Westcott, S., Zhang, G., Tay, A., Appels, R., Jones, M. and Lance, R. (2011) Dissecting the telomere region of barley chromosome 5HL using rice genomic sequences as references: new markers for tracking a complex region in breeding. Molecular Breeding, 27 (1). pp. 1-9.

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Dissecting the telomere region of barley chromosome 5HL using rice genomic sequences as references: new markers for tracking a complex region in breeding

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Abstract

The terminal region of barley chromosome 5HL controls malt extract, diastatic power, free amino acid nitrogen, alpha-amylase activity, seed dormancy and pre-harvest sprouting. Comparative analysis of the barley and rice maps has established that the terminal region of barley chromosome 5HL is syntenic to rice chromosome 3L near the telomere end. The rice BAC (Bacterial Artificial Chromosome) sequences covering the region of chromosome 3L were used to search barley expressed sequenced tags database. Thirty-three genes were amplified by PCR (polymerase chain reaction) with the primers designed from barley ESTs (expressed sequence tag). Comparison of the sequences of the PCR generated DNA fragments revealed polymorphisms including single nucleotide polymorphism (SNP), insertions or deletions between the barley varieties. Seven new PCR based molecular markers

were developed and mapped within 10 cM in three doubled haploid barley populations (Stirling × Harrington, Baudin × AC Metcalfe and Chebec × Harrington). The mapped genes maintain the micro-syntenic relationship between barley and rice. These gene specific markers provide simple and efficient tools for germplasm characterization and marker-assisted selection for barley malting quality, and ultimately lead to isolation and identification of the major gene(s) controlling multiple quality traits on barley chromosome 5HL.

Keywords: Malting quality; Comparative mapping; Micro-synteny; Rice; *Hordeum vulgare;* Marker-assisted selection

Introduction

A high value use of barley is to produce malt as a raw material for the brewing of beer and fermentation and distillation for the production of whisky. Thus, malting quality is the primary objective of various barley breeding programs around the world because the premium is often paid for barley with malt quality. Phenotypic selection for malting quality using laboratory scale malting of grain samples is expensive and the data on malt quality from one harvest is generally not available prior to selection for sowing the next season's yield trials. Thus, malting quality is a major target for marker-assisted selection. The first systematic quantitative trait loci (QTL) mapping in barley was reported by Hayes et al. (1993), in which 62 QTLs for 8 traits were mapped. Since then, efforts have been made to map QTL for malting quality by three major groups around the world (North American Barley Genome Mapping Project, Australia National Barley Molecular Marker Project and several European projects). Numerous genes and QTL have been identified that are related to malting quality and these have been reviewed by various authors (Kleinhofs and Han 2002; Fox et al. 2003; Li et al. 2009). Although there was strong QTL × E interaction for the malting quality traits (Panozzo et al. 2007), the terminal region of chromosome 5HL has been consistently detected to control multiple malting quality traits (Mather et al. 1997; Marquez-Cedillo et al. 2000; Collins et al. 2003; Li et al. 2003; Hayes et al. 2003; Li et al. 2005; Panozzo et al. 2007; Von Korff et al. 2008). These traits

included malt extract, diastatic power, free amino acid nitrogen, soluble nitrogen, alpha-amylase activity, wort viscosity, beta-glucan and beta-glucanase. The same chromosome region was also associated with early vigor, seed dormancy and pre-harvest sprouting (Han et al. 1996; Ullrich et al. 1996; Gao et al. 2003; Li et al. 2003). Thus, the chromosome 5HL region has been a key target for improvement of malting quality in the breeding programs by marker assisted selection (Collins et al. 2003; Coventry et al. 2003). However, the association in repulsion of malting quality factors and seed dormancy/pre-harvest sprouting presented a big challenge for the breeding program. Rapid germination and conversion of starches to sugars is a favorable attribute of barley used in brewing with starch adjuncts. Malt with this attribute is desirable in markets where other starch sources are less expensive than barley or malt. On the other hand, rainfall and high humidity at harvest can cause sprouting and losses of viability during storage. Identification of suitable recombinants in the 5HL region might resolve the problem.

In a previous study, we have established that the terminal region of the barley chromosome 5HL is syntenic to rice chromosome 3L near the telomere end (Li et al. 2004). The rice BAC sequences covering this region of chromosome 3L were thus utilized in this study to dissect the telomere region of barley chromosome 5HL by using the rice syntenic gene sequences as a reference and developing gene-specific, robust and polymorphic molecular markers. The closely linked simple PCR markers could reduce the cost and enhance throughput by marker assisted selection for malting quality. In addition, increased marker density in this chromosome region facilitates the understanding to determine if the multiple QTL cluster for malting quality is due to multi-locus clusters, pleiotropic effects or reduced recombination in the region. The PCR markers were mapped in multiple mapping populations to understand micro-syntenic relationship between barley chromosome 5HL and rice chromosome 3, and ultimately lead to isolation and identification of the major gene(s) controlling multiple quality traits on barley chromosome 5HL.

Materials and methods

Plant materials

Three mapping populations and their parents were used in this study. The first population was derived from the cross of Chebec × Harrington with 117 DH (doubled haploid) lines. The population was generated with anther culture and provided by University of Adelaide. A molecular linkage map has been constructed with 258 Restricted Fragment Length Polymorphism (RFLP), 47 Amplified Fragment Length Polymorphism (AFLP) and 41 Simple Sequence Repeat (SSR) markers covering the seven chromosomes with approximately 1,330 cM and average marker distance 3.9 cM (Barr et al. 2003). QTL for malting quality and pre-harvest sprouting tolerance have been mapped in this population in previous studies (Barr et al. 2003; Li et al. 2003).

The second DH population was derived from the cross of Baudin × AC Metcalfe with 178 DH lines. A molecular linkage map was constructed with 163 AFLP and 70 SSR markers (Cakir and Li, unpublished). Total length of the map was 1306.6 cM with an average marker density of 5.6 cM per marker. This population has also been used to map QTL controlling seed dormancy and pre-harvest sprouting tolerance.

The third population was derived from the cross of Stirling × Harrington with 183 DH lines. The population was mapped with 128 SSR markers covering 1145.3 cM with an average marker spacing of 8.9 cM (Bonnardeaux et al. 2008). This population has been used to map QTL controlling seed dormancy.

Micromalting analysis of malting quality

Two sets of the Baudin × AC Metcalfe mapping population were selected for micromalting after initial analysis of grain protein, grain brightness and screenings for suitability of micromalting. The detailed analysis method and results are reported in a separate paper.

Candidate gene search

Comparative analysis has established the synteny between rice, barley and wheat genomes. Based on the synteny between rice chromosome 3L and barley chromosome 5HL (Li et al. 2004), the BAC clone sequences from rice chromosome 3L end region were used to identify barley or wheat ESTs from database (www.ncbi.nlm.nih.gov). The EST and genetic map information for barley and wheat were obtained from GrainGenes (www.graingenes.com). The rice genome BAC clone sequences were retrieved from the Institute for Genomic Research (www.tigr.org). Six rice BAC clones from rice chromosome 3 (between 160.1 and 167 cM) were used in the primary candidate gene search.

There are about 100 genes or gene models predicted in this region (by the rice genome browser of the rice genome database, www.tigr.org). ESTs from barley or wheat were used to design primers for PCR from barley genomic DNA. The ESTs syntenic to rice chromosome 3 BAC clone OSJNBa0015N08 (accession AC096688) were chosen first for designing the PCR primers as this BAC clone defines the upper border of the chromosome 3L end region (Table 1). Then the other syntenic genes distal to OSJNBa0015N08 were further used to develop additional markers.

PCR from barley genomic DNA and sequencing of the amplicons

Gene-specific primers (from barley EST sequences) were used to amplify the genomic DNA of five barley varieties Stirling, Harrington, Chebec, AC Metcalf and Baudin (Table 1). The PCR products were checked on agarose gel for amplification quality. The major DNA band was cut from agarose gel and purified with a QIAEX II gel extraction kit (QIAGEN GmBH, Hilden, Germany). If no significant background was detected in the PCR products, the purified amplicons were sequenced directly from both strands with the same primer for the PCR using ABI sequencing system at SABC sequencing Lab, Murdoch University.

DNA sequences analysis and identification of the polymorphisms

A suite of programs in BioManager provided by Australia National Genomic Information Service (www.angis.org.au) were used for DNA sequence editing, comparison and alignment (Cattley and

Arthur 2007). The sequence comparison identified polymorphism among varieties. The subsequent PCR markers were developed based on the polymorphisms.

PCR amplification for the polymorphisms

The PCR was conducted with BIOTAQ DNA polymerase system from BIOLINE (Australia) following the supplier's recommended protocol. The PCR products were separated on 1.5% agarose gel or 8% polyacrylamide and visualized by ethidium bromide staining. Detailed PCR protocols and primers are listed in Table 2.

Data analysis

Software package Map Manager QTX (Manly et al. 2001) was used to determine linkage groups using the Kosambi mapping function (Kosambi 1944) with a search linkage criterion of $P < 0.001$. The default settings of MapManager were used to order the markers. The location of markers in each population was further validated by the program RECORD (van Os et al. 2005). Association of the markers with malting quality traits was calculated using stepwise regression analysis by the 'Fit-model' function of JMP software (SAS Institute). A probability of $P < 0.01$ was used to claim association between the markers and traits.

Results

Development of gene-specific markers

Using 100 genes or gene models predicted in this region, a BLAST search identified a total 33 barley genes/ESTs in the database. Comparison of the sequences of the PCR generated DNA fragments revealed polymorphisms including single nucleotide polymorphism (SNP), insertions or deletions between the parents. Seven genes were further selected as candidates for marker development based on their location, potential function and polymorphism, and covering the entire region for the malting quality QTL (Table 1). Preliminary PCR primers were designed to amplify the genomic DNA

fragments of the seven genes from five barley varieties AC Metcalfe, Baudin, Chebec, Harrington and Stirling (Table 1).

PCR primers, polymorphism and PCR reaction conditions for the new markers are listed in Table 2. The PCR products for E6041 contained a large intron and showed size difference between Harrington and Stirling (Fig. 1), or Harrington and Chebec or AC Metcalfe and Baudin. There is an insertion/deletion of 114 bp within the intron. The PCR products for E6099 included two introns. The 1st intron was 145 or 139 bp and the 2nd was 1,132 bp. There were two small sized insertion/deletion polymorphisms in the 1st intron and several SNPs in both introns between Stirling and Harrington. The PCR products for E6100 included a large intron and showed a small InDel polymorphism between Stirling, Chebec or Baudin and Harrington or AC Metcalfe. The PCR products were 220 bp from Stirling, Chebec and Baudin, 245 bp from Harrington and AC Metcalfe.

There was only one SNP in one intron of the 811 bp PCR amplicon between barley varieties Stirling and Harrington for E6104. Marker E6104 was developed on the SNP site, with allele-specific primers for both alleles on the opposite strand of the ds DNA, working with two gene specific primers (non-allele-specific). This bi-directional allele-specific marker identified either allele by the PCR product size difference in a 4-primers-multiplex PCR reaction under a stringent condition. There was a 31 bp InDel polymorphism between the Stirling and Harrington PCR products from genomic DNA for E6118. This InDel was also identified between Chebec and Harrington, and between Baudin and AC Metcalfe. The polymorphic primers of E6118 were designed to flank the InDel and the polymorphism could be scored from the PCR amplicons separated on 2% agarose gel (578 bp from Stirling, Chebec and Baudin, 547 bp from Harrington and AC Metcalfe).

The PCR products with the preliminary primers showed 3 SNPs at the intron region between barley varieties (Table 1). Marker E6126 was developed at one of the SNPs with allele-specific primers for both alleles on the opposite strand of the ds DNA, working with two gene specific primers (non-allele-specific). This bi-directional allele-specific marker identified either allele by the PCR product size difference in a 4-primers-multiplex PCR reaction under a stringent condition (Table 2). The amplicons from Harrington or AC Metcalfe allele showed two DNA bands of 663 and 220 bp, while

the amplicons from Stirling, Chebec or Baudin allele were two DNA bands of 663 and 496 bp. The 660 bp band is an indicator of the successful PCR for both alleles. The 220 bp band is the allele-specific amplicon from Harrington or AC Metcalfe. The 483 bp band is the allele-specific amplicon from Stirling, Chebec or Baudin.

Sequence comparison of the preliminary PCR products from Stirling and Harrington showed five small InDel polymorphisms in one intron and six SNPs in the second intron (Table 1). Polymorphic primers were based on a part of the first intron sequence, flanking a 14 bp InDel (Table 2). This primer pair amplifies a 169 bp DNA fragment from Stirling, Chebec and Baudin, a 155 bp DNA fragment from Harrington and AC Metcalfe.

Mapping the gene-specific markers in three DH populations

The seven PCR markers were mapped in three DH populations of Chebec × Harrington, Stirling × Harrington and Baudin × AC Metcalfe. All markers were designed as co-dominant (Fig. 1).

Six of the seven gene-specific markers were mapped in the Chebec × Harrington population. All the markers were mapped to the QTL region between the RFLP marker CDO506 and SSR marker GMS001 covering approximate 10 cM based on the distances determined in the original map (Barr et al. 2003). Markers E6099, E6100 and E6104 co-segregated in the population while markers E6118, E6126 and E6136 co-segregated with GMS001. The genetic distance between the two marker groups was 3.4 cM (Fig. 2b).

All the seven markers were mapped in the Stirling × Harrington population. No segregations were detected between E6100 and E6104, E6118 and E6126, E6136 and GMS001. The six common markers mapped in the other two populations covered 10.2 cM. E6041 was 7.3 cM away from the major marker group toward the centromere (Fig. 2c).

Six markers were mapped in the Baudin × AC Metcalfe population. Markers E6099, E6100 and E6104 co-segregated in the population while marker E6118 co-segregated with E6126. The total genetic distance covered by the six markers was 8.6 cM which is 5.2 cM larger than that in the

Chebec × Harrington DH population. The previous SSR markers GMS001 and GMS002 were mapped between E6126 and E6136 (Fig. 2d).

Micro-synteny between barley and rice

Although the genetic distance varied between the markers, the order was the same among the three mapping populations (Fig. 2). In addition, the map positions of the barley markers and the corresponding rice gene sequences indicated that synteny was maintained (Fig. 3). Thus, these genes on the chromosome 5HL were colinear with the rice genes on chromosome 3L (Figs. 2, 3). In most cases, the syntenic genes display the similar structures of exon/intron distributions (data not shown). This region in rice was about 6.9 cM.

Association of the new markers with malting quality traits

Associations of the new markers with 10 quality traits were analyzed in 178 DH lines from the Baudin × AC Metcalfe population. All the markers showed significant correlation with the quality traits (Table 3). Genetic variations explained by the markers varied from 4% for malt beta-glucan (BG) to 89% for pre-harvest sprouting tolerance. Each marker explained similar genetic variation for a specific trait. This is not surprising as all the markers were mapped within 10 cM. Thus, the additional markers provide an efficient tool for germplasm characterization in this chromosome region and for marker assisted selection in various breeding populations. Although the most significant linked marker varied with different traits, it seems that the key gene(s) controlling malting quality traits except malt yield and KI should be between the markers E6126 and GMS001. The gene specific markers have well defined the boundary for the target gene, which will provide the basis for map-based cloning of the target gene(s).

Discussion

The chromosome region 5HL, controlling multiple malting quality traits, has been a key target for improvement of malting quality in breeding programs by marker assisted selection (Collins et al.

2003; Coventry et al. 2003; Emebiri et al. 2009). The SSR marker GMS001 has been the most popular marker for marker assisted selection in various breeding programs. In most cases, the polymorphism for this marker was detected using polyacrylamide gels. The present study provides six new PCR-based molecular markers in less than 10 cM. These markers show similar accuracy for selection of various malting quality traits compared to GMS001 except for soluble protein (Table 3). Furthermore, polymorphism for most of the markers could be detected using simple agarose gel electrophoresis in a basic molecular laboratory. These markers can be easily adapted to multiplex high throughput molecular marker system for marker-assisted selection (Hayden et al. 2008). Furthermore, combinations of these new markers provide a solution to overcome the problem of lack polymorphism in some breeding populations. Significantly, the markers provide a basis for characterizing barley germplasm and identifying different haplotypes using association mapping approach. Previous studies showed that there were multiple alleles for this chromosome region with various quality profiles (Marquez-Cedillo et al. 2000; Li et al. 2003). Thus, the marker combinations could also be used to select multiple alleles in complex crosses in breeding programs to understand the relationship between the polymorphism and malting quality.

The terminal region of chromosome 5HL affects two critical aspects of malting barley, high enzymatic activity and dormancy/pre-harvest sprouting, for which major genes controlling them in repulsion (Li et al. 2003, 2004). Rapid germination and conversion of starches to sugars is a favorable attribute of barley used in brewing with starch adjuncts. Malt with this attribute is desirable in markets where other starch sources are less expensive than barley or malt. On the other hand, rainfall and high humidity at harvest can cause sprouting and losses of viability during storage. The new molecular markers developed in the present study could provide a tool to dissect suitable recombinants if the repulsion is not based on pleiotropic effects.

Due to the syntenic relationship between wheat and barley, these new markers might also be used for marker-assisted selection in wheat, as demonstrated recently in a study to select the wheat major seed dormancy QTL on chromosome 4AL using the same marker sequence of E6118 (Zhang et al. 2008).

It is unclear if the multiple QTL cluster is due to multi-locus clusters, pleiotropic effects or reduced recombination in this region. The present study demonstrated that there was small difference of the closest linked markers for different traits in the traditional QTL region for malting quality (Collins et al. 2003; Li et al. 2003, 2005; Mather et al. 1997; Marquez-cedillo et al. 2000; Panozzo et al. 2007; Von Korff et al. 2008) when more molecular markers were mapped in the chromosome region. The results in Table 3 indicate that there exist multi-locus clusters for different quality traits in this chromosome region. As these markers were so closely linked, the recombinant lines from the mapping populations should be re-evaluated for various malting quality traits under an experiment with multiple replications and sites to understand if there exist multi-locus clusters for malting quality in this region.

Conclusion

The barley chromosome 5HL telomere region was dissected using candidate gene markers syntenic to the rice chromosome 3L telomere region. Seven new molecular markers were developed and mapped in three DH populations. These markers showed polymorphisms in three different mapping populations. The markers were also closely linked with multiple malting quality and seed dormancy/PHS resistance. These robust, polymorphic markers could be used in marker assisted selection in barley breeding to develop elite malting barley varieties with tolerance to PHS and proper dormancy for the malting industry. These gene-specific markers provide additional useful genetic information for this chromosome region and could further lead to the isolation and identification of the major gene(s) controlling multiple quality traits on barley chromosome 5HL.

Acknowledgments

This research was supported by Molecular Plant Breeding CRC, Australian Grain Research and Development Corporation and Natural Science Foundation of China (30828023).

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Fig. 1 Polymorphism of a new PCR marker E6041 segregating in a barley DH population of Stirling × Harrington. Sizes of the PCR products were 322 bp for Stirling and 437 bp for Harrington. The other lanes were the DH lines from the Stirling × Harrington population

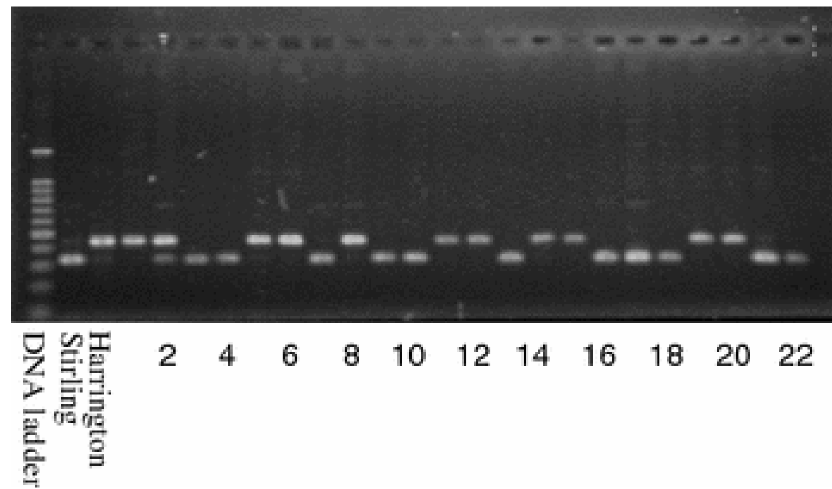

Fig. 2 Alignment of molecular linkage maps for the new markers in three mapping populations.

Figure 2a was cited from Li et al. 2003 and the LOD score is for the pre-harvest sprouting

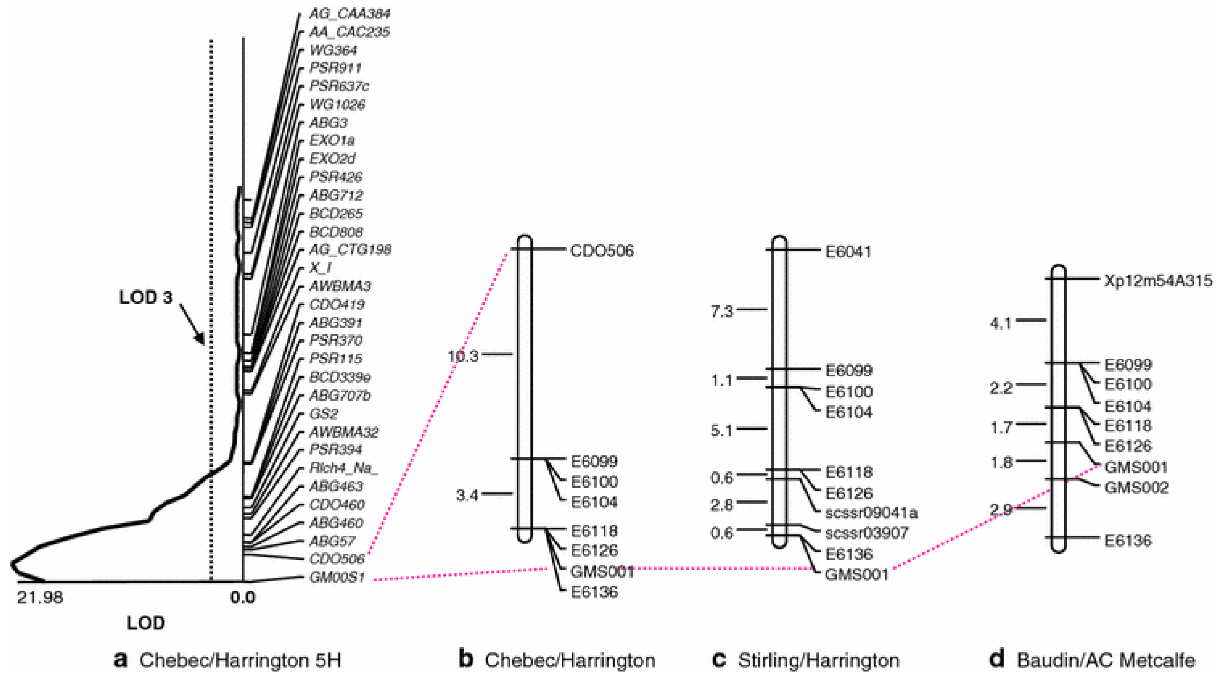

Fig. 3 Colinearity of the genes between the rice chromosome 3L and the barley chromosome 5HL telomere region

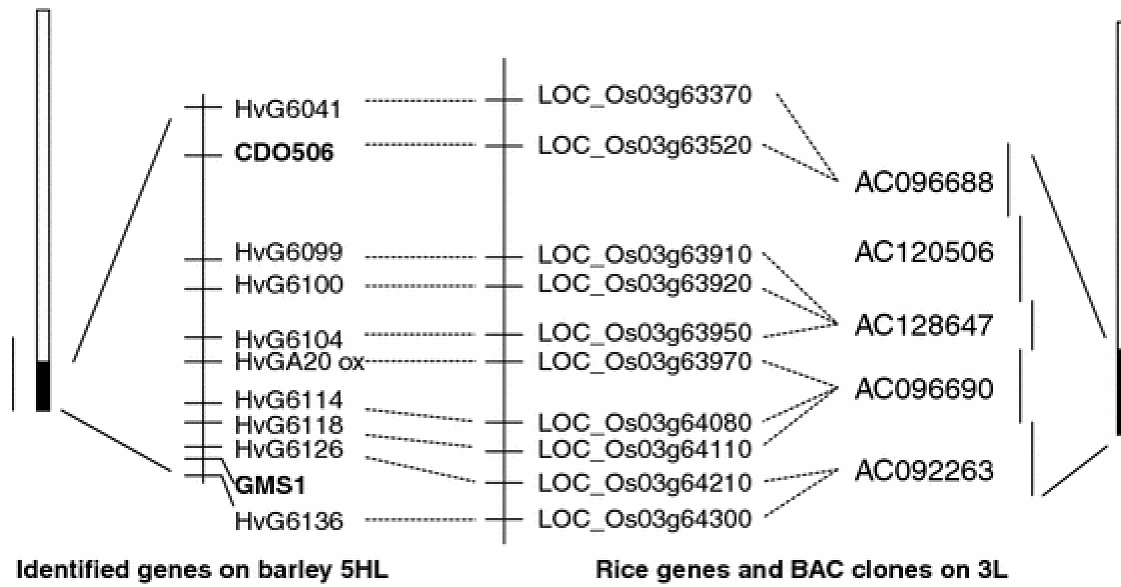

Table 1 Barley ESTs for designing preliminary primers and corresponding gene loci on rice chromosome 3L BAC clones

Locus	ESTs for primers	Primer sequences	Rice locus identifier	Putative gene function	Rice BAC clone/accession
E6041	AV913224 AV908850	5′-TGCTCAAGGGTGTTGCTATG 5′-CACTATAGGAATAGCAGGGTC	Os03g63370	Polyphosphoinositide-binding protein	OSJNBa0015N08/AC096688
E6099	DN186848 BU983710	5′-ATGATTCTGGCCGTGCTCTTC 5′-AACAATGTACAGGCAGACGTC	Os03g63910	Pentatricopeptide	OSJNBb0062G19/AC128647
E6100	BE195390 BM097815	5′-TCGCATGAAGTCAAAGTGGAT 5′-ACACATCGCAGGATTGCAACT	Os03g63920	KAP-2	OSJNBb0062G19/AC128647
E6104	BM441720 AV924224 BU982242	5′-TCAAGGGCACCAAGGAGTG 5′-GCTACAGCTAGTGATTGACG	Os03g63950	Plastid 30S ribosomal protein 1	OSJNBb0062G19/AC128647
E6118	BU975917 BE195567 CB859847	5′-CTAGACCTCAACACCCTCAG 5′-TCCGCATACATGTAGAGCGA	Os03g64110	WD domains,G-beta repeats containing protein	OSJNBa0059G06/AC096690
E6126	CA009681 BQ470791 BU988954	5′-ACTGAGACGGAGCTTGAGGA 5′-ACCACACGCCCTTAGAATTG	Os03g64210	RuBisCO subunit binding-protein alpha subunit	OSJNBa0033P04/AC092263
E6136	AJ475579 CA032628 AV940297	5′-GACGTATACATCTACGACTACC 5′-GCAATAACTGGAGTCTCATCTG	Os03g64300	Transcriptional corepressor LEUNIG	OSJNBa0033P04/AC092263

Table 2 PCR primers, polymorphism (Poly) and PCR reaction conditions for the new markers on barley chromosome 5HL

Marker	Primer sequence (5′-end to 3′-end)	Poly	PCR conditions
E6041	Forward: TGCTCAAGGGTGTTGCTATG Reverse: GGAAAGACTCACTAGACTGTC	InDel	94°C 3 min, followed by 30–35 cycles of 94°C 30 s, 52°C 30 s, 72°C 30 s, and final 5 min incubation at 72°C
E6099	Forward: GCCTCACGAATCGAATCTCATC Reverse: TGTGGGAAGCGACGAGGAGCT	InDel	94°C 3 min, followed by 30–35 cycles of 94°C 30 s, 59°C 30 s, 72°C 30 s, and final 5 min incubation at 72°C
E6100	Forward: TGCAAACATTTTACCCCACTAG Reverse: TTGTCCAACCTACTGTCGT	InDel	94°C 3 min, followed by 30–35 cycles of 94°C 30 s, 56°C 30 s, 72°C 30 s, and final 5 min incubation at 72°C
E6104	Forward: TCAAGGGCACCAAGGAGTG PASF: GAATAGAATTCATATACTCCGT P3ASR: TCTTAACACAGCAACATAGTG Reverse: GCTACAGCTAGTGATTGACG	SNP	Using IMMALASE system. 96°C 7 min, followed by 35 cycles of 95°C 30 s, 61°C 30 s, 72°C 30 s, and final 5 min incubation at 72°C
E6118	Forward: AGCGACGCCTGCAACCTAG Reverse: CATCGATCCTGAATACTCTCAC	InDel	94°C 3 min, followed by 30–35 cycles of 94°C 30 s, 56°C 30 s, 72°C 30 s, and final 5 min incubation at 72°C
E6126	Forward: ACCACACGCCCTTAGAATTG PASF: GCTGATATCATTCAGAAGGTATTGCT PASR: CAGTGTTGTTGTCTTCGTTTGGT Reverse: ACTGAGACGGAGCTTGAGGA	SNP	94°C 3 min, followed by 30–35 cycles of 94°C 30 s, 62°C 30 s, 72°C 30 s, and final 5 min incubation at 72°C
E6136	Forward: ACACCCAGCCTTACACCTAG Reverse: CGTCGAAGATGTCCCAGAAG	InDel	94°C 3 min, followed by 30–35 cycles of 94°C 30 s, 56°C 30 s, 72°C 30 s, and final 5 min incubation at 72°C

Table 3 Association (R^2) of the new markers with malting quality traits in the DH population of Baudin× AC Metcalfe ($P < 0.01$)

Marker	**Dorm**	**PHS**	**MYD**	**AA**	**BG**	**HWE**	**VIS**	**FAAN**	**SP**	**KI**
E6100	0.19	0.83	**0.23**	0.21	0.04	0.09	0.08	0.24	0.25	**0.42**
E6102	0.19	0.83	**0.23**	0.22	0.04	0.09	0.08	0.24	0.25	**0.42**
E6104	0.19	0.83	**0.23**	0.22	0.04	0.09	0.08	0.24	0.25	**0.42**
E6118	0.16	0.87	0.21	**0.23**	0.04	0.09	0.08	**0.27**	0.25	0.39
E6126	0.19	**0.89**	0.21	**0.23**	0.05	**0.10**	0.08	**0.27**	0.26	0.40
GMS001	**0.20**	0.87	0.20	**0.23**	**0.06**	0.08	0.07	**0.27**	**0.31**	0.39
E6136	0.18	0.84	0.16	**0.23**	0.04	0.10	0.08	0.25	0.25	0.36

The bold text showed the most closely linked markers with specific malting quality traits

Dorm Dormancy, *PHS* pre-harvest sprouting, *MYD* Malt yield, *AA* alpha-amylase activity, *BG* malt beta-glucan, *HWE* hot water extract, *VIS* wort viscosity, *FAAN* Free amino acid nitrogen, *SP* soluble protein, *KI* Kolbach index