FINAL REPORT

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UA00108

Barley quality: Characterisation of genetic variation for alpha amylase alleles

PROJECT DETAILS

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Summary

This project has characterised genetic variation for alpha-amylase within germplasm consisting of current Australian barley varieties, mapping population parents and wild barley. The investigation into biochemical characteristics revealed significant differences in enzymatic thermostability between various isoforms. Genetic studies and gene sequence variation identified eight different alpha-amylase alleles in current Australian varieties. Functional significance of the alternative alleles was validated and their suitability for specific markets was recommended to breeding programs. Novel alleles with improved thermostability were identified in wild barley. Molecular markers were developed as a selection tool for the identified alleles.

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Conclusions

Alpha-amylase allelic variation has a significant impact on enzyme thermostability, which has considerable influence on other key malt quality traits such as diastatic power and fermentability. Eight different alpha-amylase alleles were identified within current Australian malting barley varieties. Allele 2A, which is present in varieties such as Flagship(Þ), Buloke(Þ) and Skipper(Þ), has the highest thermostability contributing to relatively high levels of alpha-amylase activity in kilned malts, diastatic power (DP) and fermentability. Alleles 4A (Navigator(Þ)) and 4B (Gairdner) have a medium level of thermostability and allele 1D (Schooner, Sloop) has the lowest thermostability which is associated with medium and low DP and fermentability, respectively. Novel alleles with superior thermostability have been identified and validated for superior performance in backcrossed lines. Molecular markers for the identification of those alleles have been developed based on single nucleotide polymorphisms (SNPs) in the open reading frame (ORF) of alpha-amylase genes. The availability of these markers allows barley breeders to actively select for alpha-amylase alleles with specific quality profiles. In combination with the availability of genetic markers for other quality traits such as beta-amylase, the alpha-amylase markers will enable breeding programs to develop barley varieties better suited to specific market requirements.

Recommendations

The outcomes of this project demonstrate the potential for tailoring variety development to suit specific markets by employing knowledge of alpha-amylase genetic variation. Highly thermostable alleles such as 2A (present in Flagship(Þ), Buloke(Þ), Skipper(Þ)) and 1B (in Admiral(Þ)) are recommended for the starch-adjunct brewing style (export markets). The other alleles such as 4A (Navigator(Þ)) and 4B (Gairdner), which confer medium thermostability, are recommended for all-malt or sugar-adjunct brewing (domestic market). The availability of molecular markers as a diagnostic tool allows for fast and efficient selection of those alleles at early stages in breeding programs.

Outcomes

Economic outcomes:
Malting barley is internationally traded on a variety-specific basis and the quality characteristics of each variety dictate market acceptance and demand. All brewing customers desire varieties that are free from processing difficulties and exhibit high levels of malt extract but different levels of fermentability are required depending on the brewing systems and beer styles. Brewing methods range from the use of sugar adjuncts, as in the domestic industry, which requires low fermentability, through to all-malt brewing systems to the use of starch adjuncts, which requires very high fermentability. The alcohol content of the beer product being produced, ranging from low to full strength alcohol content, also influences the fermentability requirements of brewers. It is of vital importance for the Australian barley industry that Australian varieties meet customer requirements to maintain market preference and competitiveness.

The first outcome of this project is the provision of in-depth knowledge on genetic variation in alpha-amylase alleles in barley.

Extensive allelic variation (27 isoforms) has been identified, with nine alleles present in current Australian varieties. Two novel alleles from wild barley have been extensively characterised in the aspects of enzyme functionality and genetic basis. Statistically significant relationships between alpha-amylase thermostability and alpha-amylase activity in kilned malt, DP and fermentability (measured by apparent attenuation limit - AAL), have been demonstrated (linear regression correlation, $P< 0.05$). This provides barley researchers and breeders with the knowledge needed to make informed decisions on the selection of alpha-amylase alleles with desired quality profiles in the context of malting quality.

Secondly, this project also provides germplasm and selection tools in the form of molecular markers. The availability of these markers allows barley breeders to actively select for alpha-amylase alleles with desired characteristics. In combination with the availability of genetic markers for other quality traits such as beta-amylase, the alpha-amylase markers enable breeding programs to develop barley varieties better suited to specific market requirements. The availability of malting barley varieties that are preferred by end users is of benefit to the whole Australian barley industry.

Achievements/Benefits

This project aimed to characterise the allelic variation for alpha-amylase in barley and understand the impact of alternative alleles on key malt quality traits.

A detailed description of alpha-amylase allelic variation in current Australian varieties, elite breeding lines, mapping population parents and exotic germplasm.

Alpha-amylase allelic variation was investigated within germplasm compiled from University of Adelaide (UA) breeding lines, mapping population parents and wild barley accessions (*Hordeum spontaneum*). Additional lines were obtained from other barley breeding programs and the Winter Cereal Collection. A total of 260 samples were screened using isoelectric focussing (IEF) in conjunction with activity staining. For high-pI alpha-amylase the variation was extensive, with a total of 27 IEF isoforms being identified, each with a distinct pattern of alpha-amylase protein bands. The difference in IEF banding patterns was generally quite subtle in cultivated barley and far more apparent in wild barley. Novel, unique variant forms of alpha-amylase banding pattern were identified in most of the wild barley accessions.
Variation in enzyme activity and thermostability was analysed for all IEF isoforms at different germination times (3, 4 and 5 days). The means of initial alpha-amylase activity for each group ranged from 174 to 773 U/g with the highest activities found in wild barley. Thermostability at 55^{o}C expressed as the percentage of retained enzyme activity ranged from 10% to 76%, with the highest observed in wild barley. With regards to temporal effects, statistical analysis showed strong positive correlation between germination time and initial enzyme activity ($P<0.001$) but not thermostability.

Molecular information detailing the genetic basis of observed alpha-amylase variation.

Genetic mapping of variation in IEF isoforms:
The variation in IEF banding patterns was mapped to amy1 locus on chromosome 6H in three segregating populations (Baudin$^{\emptyset}$ x Dhow, Clipper x Sahara and a population derived from backcrosses of Flagship$^{\emptyset}$ with two wild barley accessions), indicating that the segregation of IEF isoforms was mainly controlled by the structural gene at amy1 locus.

Genetic analysis:
Two F2 populations of the variety Navigator$^{\emptyset}$ crossed with wild barley accessions were examined. 150 single seeds from each population were scored for their IEF banding patterns. The IEF patterns each segregate as discrete units, with heterozygote individuals exhibiting a combination of all bands observed in the parents. The segregation ratios of IEF banding isoforms were 32:84:35 for Navigator:heterozygote:Caesarea CPI 77132 -38 and 39:81:27 for Navigator:heterozygote:Talpiyyot CPI 77144-2. Chi-square tests confirmed that each of the variant wild barley isoforms segregated in a 1:2:1 ratio with respect to the Navigator isoform, indicating that they were attributed to co-dominant alleles.

Characterisation of alpha-amylase gene family:
Alpha-amylase is a multigene family. To date, all published literature states that the genes coding for the high pI group are located on chromosome 6H and the low pI group on 7H. The copy numbers for both groups are unknown. The availability of the barley genome sequence (2012) has facilitated our investigation of this matter. A Basic Local Alignment Search Tool (BLAST) search was performed using a scaffold of the barley genome and the results revealed six genes on five different chromosomes (2H, 3H, 5H, 6H and 7H) coding for alpha-amylase. Three genes located next to each other on 6H code for the relatively well-characterised amy2 (high pI). Those three genes share 96.4% sequence identity and were named amy1-1, amy1-2 and amy1-3. The gene on chromosome 7H (amy2) codes for the previously published amy1(low pI) enzyme. The gene on

chromosome 2H codes for a recently published amy4 enzyme. This gene shares less than 50% sequence identity with the genes on chromosome 6H and 7H. The two genes on chromosome 3H and 5H have not been characterised in barley to date. The gene on chromosome 5H shares up to 78% sequence identity with those on chromosome 6H and is an ortholog of the rice amy3A. The gene on chromosome 3H is quite novel, with less than 40% sequence identity to those on chromosome 6H.

Expression profiles of five alpha-amylase genes were investigated in three commercial varieties (Flagship, Baudin and Commander[Φ]) over a five-day germination with measurements at 12-hour intervals using quantitative real-time polymerase chain reaction (PCR). Differential expressions were observed between the genes as well as between varieties. In general, 6H genes amy1-1 and amy1-3 had the highest expression levels followed by amy1-2 and 7H gene amy2. All other genes on chromosomes 2H and 3H had insignificant expression level during the germination time-course. The gene on 5H could not be amplified with the primers used. The results re-emphasise the major role of the genes at amy1 locus on 6H during germination.
The three genes on chromosome 6H were sequenced for 23 cultivated barley varieties and two wild barley lines. Twenty SNPs were identified, of which seven led to changes in amino acids. Possible effects of these amino acid changes on protein stability were studied using Swiss-PdbViewer 4.1.0 and I-Mutant 2.0 softwares. In summary, protein stability was estimated based on four main factors: the energy of hydrogen bonds, the energy of electrostatic interactions (ion pairs, salt bridges), the hydrophobic effect and the conformational entropy due to the restricted motion of the main and side changes. The most prominent effect was observed in the mutation of amino acid 368:glutamine to histidine, leading to an increase of 1.21 Kcal/mol in the unfolding Gibbs free energy.

Based on the SNPs leading to amino acid changes, eight alleles have been identified in commercial malting varieties and breeding lines. These are alleles 1A, 1B, 1C, 1D, 2A, 2B and 4A and 4B, where the numbers 1, 2 and 4 indicate the IEF groups to which the alleles belong. Two wild alleles, Tel-Shoqet 77146-32 and Bar Giyyora 71284-48, have also been identified.

Recommendations to breeders on preferred alleles for specific brewing styles and markets.

In preliminary studies the eight alleles were categorised into three groups: high thermostability (2A, 1A, 1B), medium thermostability (1C,2B, 4A, 4B) and low thermostability (1D). The functional significance of these alleles in different genetic backgrounds was validated by analysing four doubled haploid (DH) populations. In summary, allele 2A (present in Flagship, Buloke[Φ], Skipper[Φ] and 1B (present in Admiral[Φ]) were associated with relatively high thermostability which contributed to high diastatic power (DP) and fermentability and should be selected for starch-adjunct brewing style (export markets). The other alleles such as 4A (Navigator), 4B (Gairdner) had medium thermostability which would be suitable for all-malt or sugar-adjunct brewing (domestic market).
This project also identified a novel highly thermostable allele from wild accession CPI77146-32. In backcrossed lines with Flagship as the recurrent parent, the introgression of wild barley at amy1 locus led to a significant increase in alpha-amylase activity in kilned malt, DP and fermentability compared to isogenic lines carrying the Flagship allele (2A). Molecular markers for the selection of this allele have also been developed.

A detailed description of alpha-amylase allelic variation in important malting barley varieties from Australia and overseas.

Allelic variation was investigated for a barley germplasm selection comprised of 23 important barley varieties including current Australian commercial and historic varieties, newly accredited malt varieties and significant malt varieties from Canada and Europe. IEF analysis classified the 23 varieties into three groups (1, 2 and 4) and gene sequencing identified eight alleles within them.

Investigation of the relationship between alpha-amylase alleles and malt processing performance.

One hundred and fifty malt samples from nine varieties representing six alleles (1A: Baudin; 1C: Commander, 1D: Schooner, Sloop; 2A: Flagship, Buloke, Vlamingh[Φ]; 4A: Navigator, 4B: Gairdner) were obtained from three malting companies (BBM, Malteurop and JWM) and assayed for alpha-amylase activity and fermentability. Protein and Kolbach index (KI) values were provided by the maltsters. There are significant differences for alpha-amylase activity and fermentability ((measured by the apparent attenuation limit - AAL) between the alleles (analysis of variance - ANOVA, $P<0.05$). Alleles 1A and 2A had the highest levels for both parameters, followed by 1C, 4A and 4B. The lowest levels were observed in allele 1D.

There are many confounding factors that affect apparent attenuation (AA) and the AAL, and the commercial malt samples were grown in different environments and malted differently to suit customers' requirements. Two important confounding

factors are KI and grain protein. While grain protein level is similar between AA alleles, KI is significantly different (ANOVA, P< 0.05). Notably, 1A had significantly higher KI than any other allele.
In the relationship between KI and AA activity, five alleles (1A, 1C, 1D, 4A and 4B) followed a similar trend while 2A allele stood out as having greater response to KI. This indicated that within a similar KI range, 2A allele should have higher AA activity than any of the other five alleles. Though 1A allele had similar AA activity and AAL to 2A, these measures could be confounded by its high KI value.
There are six different alpha-amylase alleles present in current Australian malting varieties. Analysis of commercial malt samples confirms that most of these alleles show a similar relationship between the level of alpha-amylase activity and the level of modification. The allele 2A, present in Buloke, Flagship and Vlamingh, shows a significantly different relationship, with higher levels of enzyme activity as KI values exceed 45. The observations support the view that selection for specific alpha-amylase alleles can be used to tailor variety development to suit particular markets.

Genetic and functional characterisation of the Navigator and VT Admiral alpha-amylase alleles.

An F1 derived DH population of Navigator and Admiral comprised of 300 lines was micromalted and analysed for quality parameters including fermentability, DP, alpha and beta-amylase, protein, KI, hot water extract, beta-glucan and viscosity. This population has been genotyped and the genetic map has been constructed by the barley program at The University of Adelaide (UA). Genetic mapping shows four significant quantitative trait loci (QTL) for alpha-amylase, one of which is located in the position of amy1 locus (6H). Fermentability and DP each has five significant QTL, with the one at bmy1 locus having the strongest impact on both parameters; accounting for 71% explainable variation for fermentability and 50% for DP. The results indicate that fermentability and DP were both strongly driven by beta-amylase alleles in this particular population.

Other research

There are three main starch de-hydrolysing enzymes that influence key malt quality traits such as DP and fermentability. With alpha-amylase and beta-amylase now well characterised, research in this area should focus on the last enzyme, limit dextrinase. There is evidence suggesting that this starch de-branching enzyme not only has significant impact on DP and fermentability but also influences some characteristics of final products such as taste or mouth-feel. An investigation into genetic variation of limit dextrinase and its impact on malt quality traits and flavour and taste of the final product would be of interest to the barley industry. This research will potentially provide opportunity for improvement in the area of malting quality for Australian barley varieties.

Limit dextrinase is a large protein encoded by a large complex gene. The enzyme activity is subject to the action of an endogenous inhibitor. These characteristics have made limit dextrinase a difficult target for barley improvement but recent technological advances make it possible for natural variation to be characterised and exploited to tailor barley varieties for different markets.

Intellectual property summary

Project outputs are to be treated as Tier 1 traits as defined under the Australian Winter Cereals Pre-Breeding Alliance and will be made available on a royalty free non-exclusive basis.

Additional information

Cu, T.S., Roumeliotis, S. & Eglinton, J. K. 2012. In: G. Zhang et al.(eds) Advances in Barley Sciences: Proceedings of the 11th International Barley Genetics Symposium. Springer.

Three manuscripts are in preparation for publication in refereed journals:

1. Cu, T.S., Roumeliotis, S. & Eglinton, J. K. Identification of exotic alleles for the improvement of alpha-amylase and related malt quality traits. Journal of Cereal Sciences

2. Cu, T.S., Roumeliotis, S. & Eglinton, J. K.The impact of alpha-amylase alleles on related malt quality parameters: a study on current Australian barley varieties. Journal of Brewing and Distilling.

3. Alpha-amylase gene family: differential expression and sequence polymorphism. PLoS ONE.