

Barley genomics and its impact on breeding.

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Abstract

Significant international collaborative efforts and public sector funding have been devoted to the development of barley genomics over the last ten years. This investment has delivered a broad range of tools and resources into the public domain where they are re-energising research on a broad range of fundamental and applied research questions. One of the most relevant technologies that we have recently developed is a highly multiplexed genotyping platform which allows the robust detection of >1500 single nucleotide polymorphisms (SNPs) in a single assay at an affordable cost. This platform is a prime example of the translation of ‘genomics information’ into a precision tool that can be accessed and applied by the global barley genetics research and breeding communities to germplasm, trait or project specific questions. I discuss how we have developed, used and are using this technology for fundamental scientific research, and in collaboration with the international genetic resources and breeding communities for germplasm characterisation and crop improvement. Because the SNP markers are all derived from expressed genes whose sequences represent a ‘common currency’ linking all of biology, I illustrate how we can leverage considerable additional information from the rice genome sequence by exploiting the conserved synteny between their genomes.

Keywords:

Genomics technologies, molecular markers, germplasm characterisation, breeding.

Introduction

The majority of the world's dietary carbohydrate is derived from crop plants belonging to the Poaceae. In the Pooideae subfamily, the closely related Triticeae species (wheat and barley) synthesize and store large quantities of calorie rich carbohydrate, together amassing more than 720MT annual production. Barley is a true diploid inbreeder with 7 pairs of metacentric chromosomes and is widely considered a model for classical genetic studies. Historical investigations and more recent and significant public investment have resulted in the development of tools and resources that can facilitate genetic discovery and, at least potentially, impact upon the development of new cultivars that are better suited to the demands of sustainable agricultural production. As a genetic model Barley boasts one of the most extensive collections of classically described mutant stocks (Davies et al., 1997), and an expanding collection of key genomics tools and resources including BAC libraries, reverse genetics populations and functional genomic tools such as VIGS (Yu et al, 1999, Caldwell et al, 2004, Hein et al., 2005). EST-based gene discovery programs have generated 437,713 publicly available entries (July 6, 2007, http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) which probably represents in the order of 50% of all barley genes and based on these, the AFFYMETRIX barley1 GeneChip (Close et al, 2004) has been developed to provide a technologically robust and integrating platform for highly parallel investigations of the transcriptome (Druka et al, 2006). Furthermore, the global barley community has recently produced a coordinated strategy for sequence analysis of the barley genome (http://www.public.iastate.edu/~imagefpc/IBSC%20Webpage/publications/whitepaper_IBSC_061110.pdf) which it is envisaged will accelerate the discovery of genes controlling important academic and commercial barley traits. While these developments are impressive in their own right and are

delivering good quality science, it is pertinent to ask if and how they are being used to address key concerns of the end user communities.

Current barley genetics

Since the early 1990's we have firmly believed that many of the advances in molecular biology and genetics will ultimately deliver outputs through plant breeding. Consequently we have focussed considerable effort on developing and applying technologies, specifically in the form of molecular markers, to facilitate barley genetics-based research (Ramsay et al., 2000, Macaulay et al., 2001). By using these marker technologies on bi-parental segregating populations we and others have located genes controlling many traits, and in several cases have extended observations on linkage to develop molecular diagnostics that have been used for marker assisted selection (MAS) in public and commercial crop improvement programs (Thomas et al., 1998, Swanston et al 1999)(Box 1). Although bi-parental mapping populations have successfully identified many trait - marker associations, an alternative population-based gene-mapping approach known as linkage disequilibrium (LD) or association mapping, is becoming attractive for use in barley (LD is defined as the non-random association of alleles at distinct loci in a sample population)(Rostoks et al 2007). The major attraction of LD-mapping is that it has the potential to locate genes responsible for a wide range of traits in a single sample population (e.g. a collection of well known cultivars from a region of interest) using pre-existing or newly collected but highly replicated phenotypic data that has been assembled during crop improvement and cultivar registration programs. In the process of developing effective LD-mapping based approaches for gene discovery, we have been establishing i) the extent of LD in the genepool under study and ii) what is an appropriate number and type of markers that will allow us to conduct LD-based studies effectively and at reasonable cost. Addressing the first question Kraakman et al. (2004) presented preliminary evidence that LD extended up to 10 cM (1% of the genetic map) in European spring barley using AFLP markers.

Box 1: Marker Assisted Selection (MAS):

Molecular markers are currently deployed for either positive (i.e. for a gene) or negative (i.e. for a background) selection. While random markers can be utilised for the latter, for the former linkage between a marker and a trait must be detected, the frequency with which the marker genotype successfully predicts the trait phenotype assessed, and if high enough (e.g. 95%) then the marker frequently needs to be converted into an assay that can be run on thousands of samples cheaply and easily. 'Perfect markers' are those that assay polymorphism in the gene that is responsible for the trait phenotype and are 100% associated. Examples of marker used for MAS in barley include:

- *Rym4/5*: An SSR marker approximately 1cM away from the *Rym4/5* locus has been used for the last 8 years in Europe to select for resistance to BaYMV. The gene has been cloned and diagnostic, functional SNPs identified that could be converted to perfect markers (Kanyuka et al., 2005).
- *mlo11*: A length polymorphism caused by a MITE insertion in the 5' promoter region of *mlo11* is diagnostic. There are no diagnostic SNPs in the coding sequence of *mlo11* to differentiate it from the wild type (Piffannelli et al 2004).
- *VrnH1*: A series of deletions in the first intron of *VrnH1* differentiate the dominant and recessive alleles, captured by a PCR length polymorphism assay (Fu et al., 2005).
- *VrnH2*: Presence / absence of a gene family called *ZCCT-H* which is a dominant repressor of flowering. A presence absence PCR assay has been developed (von Zitzewitz et al., 2005) but several SNPs provide alternatives.
- *PpdH1*: The causal single nucleotide polymorphism in *PpdH1* was putatively identified during validation of functional gene. A SNP assay differentiates the two alleles (Turner et al., 2005)
- *Epi*: The *Epi* locus in barley synthesises epi-heterodendrin, a precursor of cyanogenic glycosides produced through an interaction with copper stills during Scotch whisky distillation. The gene has been cloned and a diagnostic PCR-based marker developed (our unpublished results).

Molecular diversity in cultivated barleys

About 4 years ago, we initiated a program of research to develop a gene-based genotyping platform to approach association-based genetic analyses in barley and to simplify all of the other types of genetic analysis that we routinely conduct. Rapid advances in genomics had taught us that the DNA sequence of a gene is a currency that is relatively easily exchanged across species, and developing gene based markers would allow us to exploit comparative genetics with the rice genomic model. Initially our approach was based on EST-SSRs. However, we soon switched to develop bi-allelic, co-dominant markers based on single nucleotide polymorphisms (SNPs) in barley genes because of their potential for multiplexing. Furthermore, in human studies, biallelic SNPs have been shown to be equivalent to multiallelic SSR markers at moderate, and superior to them at high, densities.

To estimate the number, and decide finally upon the type of markers required for association mapping, we first investigated the level of polymorphism within the genepool under study. In particular, we wanted to know whether genotyping one SNP per locus would allow us to estimate the overall diversity present in cultivated barley, or if we would need to assay several. We re-sequenced alleles from a random set of 88 genes from 19 cultivated European barley lines. In ca. 35 kb of aligned sequence the germplasm contained 193 polymorphic sites organised into an average of 2.6 haplotypes per locus (with a maximum of six haplotypes). Combined with our preliminary understanding of the extent of LD in barley (<10cM), we concluded that assaying a single SNP per gene (locus), at approximately one gene per cM across the 1100 cM barley genome would initially provide suitable coverage for assessing LD mapping. We also know that for certain genome regions SNP haplotype information rather than a single SNP will be required and that for others additional SNPs in certain genomic regions may need to be developed to cover gaps in the existing set.

Robust, high-plex, low cost genotypic analysis.

A major advantage of SNPs is that they are amenable to automation using any of the gel-free high throughput genotyping technologies developed for the human HapMap project. For SNP discovery, we initially re-sequenced EST-derived unigenes after PCR amplification from a number of genotypes, and mapped the discovered SNPs one by one onto several barley reference genetic linkage mapping populations (Rostoks et al. 2005, Stein et al, 2006). The results from these studies combined with their potential for multiplexing encouraged us to explore innovative ways of exploiting the vast amount of EST data available in international sequence databases to identify electronic SNPs (eSNPs). SNPs are present in the EST datasets because they are derived from several different varieties (Close et al, in preparation). Using information from both sources we have currently assembled >15000 putative barley SNP markers and used 3060 of them to produce two pilot oligonucleotide pool arrays (Pilot-OPAs or POPAs) for use with the Illumina Golden Gate BeadArray platform (Fan et al. 2003; Oliphant et al. 2002). We have used the Pilot OPAs in a number of studies.

Reference Genetic Mapping

To date we have genotyped the parents and progeny of three reference doubled haploid mapping populations, Steptoe x Morex, OWBD x OWBR and Morex x Barke. Of the 3060 SNPs on the Pilot OPAs over 2430 assays generated high quality genotypic data, confirming the utility of the technology for HTP genotypic analysis. Of these, we were able to incorporate over 1950 onto an integrated barley genetic linkage map. As marker order is of paramount importance for all subsequent genetic studies we routinely construct graphical genotypes of all of the progeny. Based on these we made the following observations about the OPA barley gene map:

- The data quality is high and frequency of missing data very low
- There is good overall coverage of the genome, with a couple of gaps over 10cM remaining.

- There are few, if any, single marker double recombinants remaining after manually checking the data
- There is considerable clustering of genes around the genetic centromeres

We conclude that the OPA platform is robust and effective for barley genetic marker analysis and encourage its use by the barley genetics community.

Diversity analysis and LD

We have also looked at over 100 cultivated European barley lines, including 13 exotic types, using the POPA platform and based on the resulting information assessed population structure, patterns of genetic diversity and the extent of LD. We observed only 0.3% heterozygous genotypes consistent with the inbred nature of barley. We also observed a surprisingly large amount of diversity in what we considered was a relatively narrow germplasm selection. There were a large number of SNPs with a minor allele frequency (MAF) of <0.1 and these were excluded from our subsequent LD analyses. For that we used both principal coordinate analysis (Figure 1) and the program ‘Structure’ to investigate population substructure within the data. These similarly identified three major subgroups within the germplasm, European spring and winter material ($n=91$) and more exotic lines. Based on the data from Pilot OPA 1, we derived measures of LD (R^2) in the European germplasm subset. The extent of LD was strongly affected by population structure. Highly significant intra-chromosomal LD ($p>0.001$, $R^2>0.5$) extended over more than 60cM in the combined set of European spring and winter barley with approximately 20% of all significant ($p>0.001$) associations ($R^2>0.05$) being inter-chromosomal. However, in the spring 2-row subset ($n=53$), similar to that observed by Kraakman et al. (2004), LD extended only up to 10-15cM and the proportion of inter-chromosomal associations was reduced to 2% (Rostoks et al, 2007). The extent of LD varied across the chromosomes with an obvious relationship between genetic distance and LD. In contrast, there was no obvious relationship with physical distance: regions with reduced recombination, such as centromeres, showed strong LD even though the physical distances are hundreds of megabases.

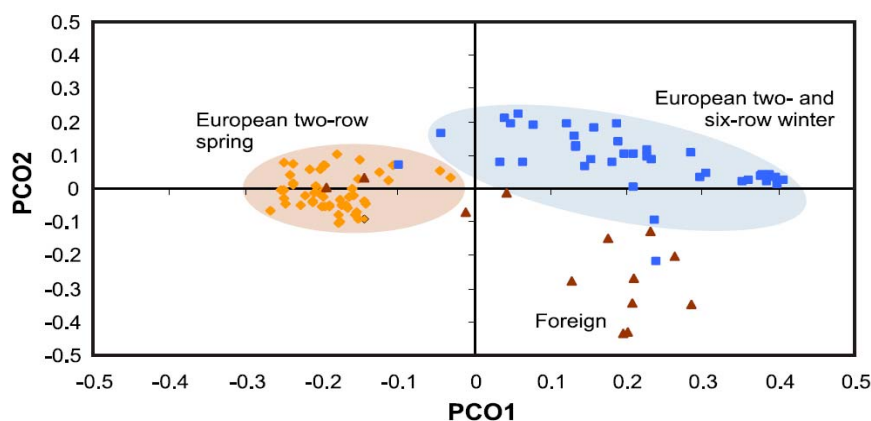


Figure 1. PCO analysis of 103 barley accessions using genotypic data from Pilot OPA1. The spring and winter genotypes show relatively tight and discrete clustering. The foreign or exotic genotypes are clearly separated from the European cultivated lines.

To test whether we could use LD to locate genes we attempted to re-map 100 random ‘test’ loci with known linkage map locations using LD at different R^2 value cut-offs. We then compared the locations predicted by LD-mapping with their positions determined by bi-parental meiotic mapping. Based on approximately 1501 markers that had been meiotically mapped in the three bi-parental mapping populations (mentioned above), and an arbitrary R^2 cutoff of 0.505, we found that 61% of the markers were located by LD analysis to within 5cM of their previously mapped location, $<5\%$ mapped further away than 5cM and 34% fell below the cutoff. However, of the 100 test loci, including those that fell below the R^2 cut-off of 0.505, the highest R^2 value peak mapped to within 5cM of its established

position 73 times (see figures 2a and 2b). This result suggests that mapping the majority of bi-allelic single gene loci is likely to be possible using LD-based approaches with this number of markers, but also stresses that for a comprehensive whole genome scan additional markers will be required in certain regions of the genome.

Impact on breeding

The development of the OPA mapping platforms has been generated as a collaboration between the authors and partially achieved under the banner of two large applied research projects focussed on the exploitation of genetic markers in plant breeding, one in the UK (<http://www.agoueb.org/>) and one in the US (<http://www.barleycap.org/>). These are now joined by several other projects around the world that will utilise this technology (e.g. *ExBarDiv* in Europe and *GenoBar* in Germany). In each of the former projects the objective is to use contemporary genotyping technologies to understand diversity in elite breeding material and to identify markers linked to traits segregating in such elite material for use in crop improvement (based on MAS). Combined, over 5000 cultivated barley lines will be both genotyped at 3000 SNP loci and phenotyped for a range of characteristics over the next three years.

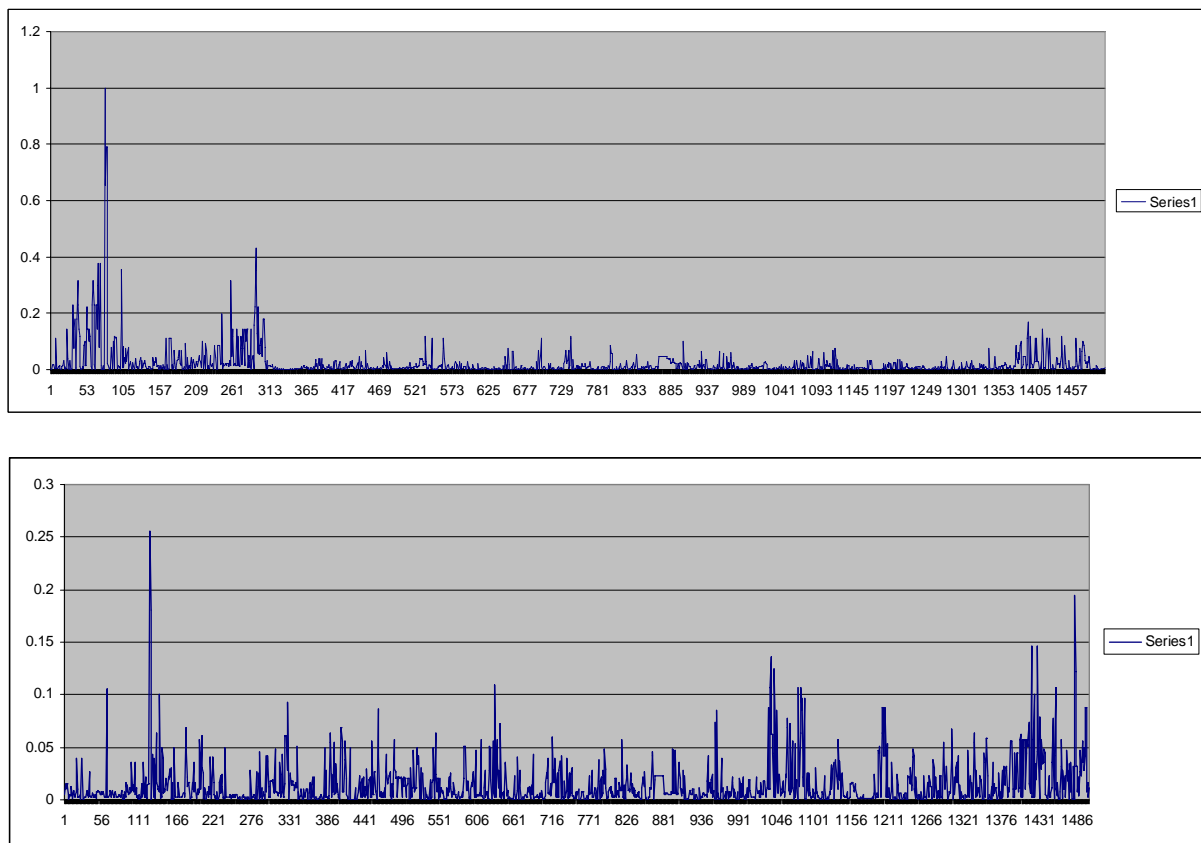


Figure 2. a) LD-scan of Pilot OPA 1 marker 1_0166. X-axis represents marker order of 1501 mapped markers from the tip of the short arm of chromosome 1H through the tip of the long arm of 7H. Y-axis represents the R² value. In this case SNP 1_0166 mapped in a bi-parental mapping population to 76cM on chr 1H and exactly the same place by LD mapping. b) LD-scan of Pilot OPA 2 SNP marker 1_0693. X and Y-axes are as above. By LD-mapping, SNP 1_0693 mapped 6.3 cM away from its location determined by bi-parental population mapping. Its R² value is 0.255.

While it is early days, the data emerging from these studies has been welcomed by breeders and geneticists who can already see links between different sets of germplasm. Thus, it is likely that the initial value of this type and volume of data will reside in the characterisation of breeders germplasm

and will help guide and structure the crossing schedules of individual programmes. To contribute towards that end the development of comprehensive breeders databases (e.g. GERMINATE, Lee et al., 2005) with graphical displays and analytical tools (such as Genotype Visualisation Tool (GVT - D. Marshall, unpublished), Figure 3) that can help choose parents and identify the likely outcomes of individual crosses will be particularly valuable at the practical end. The second output will likely be the identification of markers linked to traits or trait components and their conversion to single or low-plex markers that can be easily and cheaply followed in a plant breeding program. One important feature of the gene based SNP markers is that with relatively little work it is possible to back-convert them from one component of the 1536-plex OPA assay to single marker assays based either on CAPs or SSCP assays. c. 50% of the original SNPs of Rostoks et al (1995) were mapped as CAPs markers. Given that all of the DNA sequences from which the SNPs were designed are publicly available, individual ‘users’ also have the possibility of using simple computer software such as SNP2CAPS (Theil et al, 2003) to automate and simplify the process of conversion of DNA sequences to low-plex low-tech SNP or deletion detection assays. Indels and additional SNPs closely neighboring those represented on the OPAs generally also exist due to multiple polymorphisms between haplotypes, providing further opportunities for marker conversion. Finally, the ability to generate robust, high throughput data at a relatively low cost is already beginning to encourage thinking about novel breeding strategies where one or more intermediate generations in a breeding program may be selected mainly by genotype leaving phenotypic selection to the final population development phase.

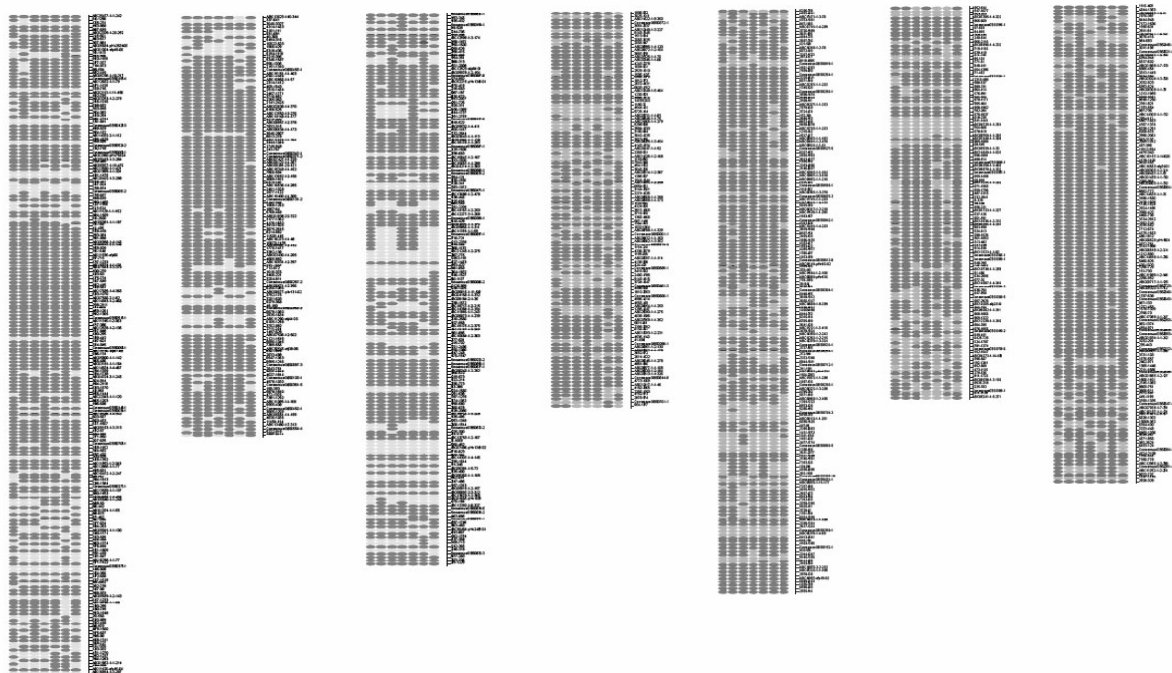


Figure 3. Graphical Genotypes of 7 elite European barley cultivars showing chromosomes (1H to 7H left to right). Within each chromosome block the genotype of each of the cultivars is shown as a vertical panel of grey/white bars representing alternate alleles at a given SNP. SNPs are presented in map order.

Exploiting Genomic Models

Because of its small genome, Rice has been erected for some time as a genomic model for the large genome crops such as wheat and barley, and its genome is fully sequenced (IRGSP, 2005) . In the coming year or so it will be joined by *Brachypodium* which phylogenetically is more closely related to the Triticeae crops. Exploiting genomic models is possible due to the conservation of synteny which facilitates inferences of gene content and order across large segments of the genome of related species. (Bossolini et al, 2007). As sequence conservation lies primarily in the gene sequences, having a

detailed barley gene map coupled with gene-centric marker technologies that are freely available to the research and breeding communities will open the door to broad exploitation of genomic models for a range of scientific endeavours such as new molecular marker development, positional cloning, gene family characterisation, regional DNA sequencing, physical mapping and evolutionary studies. As the quest for additional ‘perfect markers’ continues, simply because they leave little doubt about the impact of a specific allele on a phenotype, it is clear that gene isolation and validation of function will continue to add power to the application of genetic markers in applied breeding programmes. ‘Genomic models’ will continue to play a role in this until the barley genome itself is characterised at the sequence level. When this will happen is just a matter of time. (http://www.public.iastate.edu/~imagefpc/IBSC%20Webpage/publications/mission_statement.pdf).

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