Molecular Mapping in Barley: Shifting from the Structural to the Functional Level

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Abstract: As a resource for structural and functional barley genome analysis, more than 140 000 ESTs (expressed sequence tags) were generated from 22 cDNA libraries that yielded 25 224 tentative unigenes. About 50% of them belong to gene families. The size of the complete transcriptome is estimated to comprise between 35 000 and 75 000 genes. The barley EST collection is a rich source for the development of novel markers including SSRs (simple sequence repeats) and SNPs (single nucleotide polymorphisms). Several bioinformatic tools have been developed facilitating the computer-assisted analysis of EST databases for the presence of either SNPs or SSRs and the development of SNP-derived CAPS (cleaved amplified polymorphic sequences) markers. In an attempt to systematically map barley genes a high-density transcript map is under construction and presently comprises more than 1000 markers. This map is a gateway to comparative genomics with particular emphasis on the rice genome. 65% of the mapped ESTs showing a significant homology to rice ESTs were found to display a syntenic relationship between barley and rice. Thus, the barley EST resource facilitates the rapid and systematic transfer of genetic information from rice to barley and other *Triticeae*, which can readily be exploited for marker saturation of defined chromosome regions and their detailed comparison to rice. In the context of a functional genomics study, the complex trait "malting quality" is investigated using a barley cDNA array. By correlating the phenotypic malting trait data of selected barley lines with the corresponding expression profiles, a set of candidate genes was identified and further verified by genetic analysis.

Keywords: ESTs; functional genomics; malting quality; DNA-marker; rice; synteny

In the last 25 years the availability of molecular marker maps has provided an unprecedented insight into structural features of the barley genome. Several generations of selectable markers have been included in these maps and a large number of qualitative and quantitative traits were located in the genome some of which are routinely selected in marker assisted breeding programs. The first generation of molecular marker maps mainly comprised genomic DNA fragments since the cloning of low molecular weight genomic DNA for marker development is a straightforward process. In this regard, genomic DNA fragments revealed higher DNA polymorphism than gene-derived fragments, although it could be shown that many genomic RFLP-probes were derived from genes (Michalek *et al*. 1999). With the establishment of large scale EST-programs in several laboratories around the world, a comprehensive resource has been created that provides direct access to genes. In the following, the application of this EST-resource will be described for the construction of a genome-wide barley transcript map, the marker saturation of sub-chromosomal target regions by exploiting synteny between the genomes of rice-barley and the identification of candidate genes by an EST-based functional association approach.

Facets of the barley transcriptome

The salient challenge of applied genetics and genomics is the correlation between genetic and phenotypic information and the subsequent identification of the genes underlying a trait of interest. Since most traits are only defined by a phenotype rather than by proteins or metabolites, map-based cloning has been the strategy of choice to isolate genes of interest. However, positional cloning is a time and resource intense approach that has to be restarted from scratch for any novel trait/gene. Consequently, there is a quest for more systematic approaches to gene identification. In the best case, this results in deciphering the complete sequence of a genome as it was done for *Arabidopsis* and rice (TAGI 2000; Yu *et al*. 2001; Goff *et al*. 2002). Along with the progress in the field of bioinformatics, the availability of the whole genome sequence will greatly accelerate the identification of trait-related genes. Until now, the relative size of the barley genome has hindered any serious effort of systematic sequencing. The available sequence data on selected subgenomic regions show that, despite of very variable gene densities, any genome-wide sequencing effort in barley will likely result in sequencing endless stretches of repetitive DNA. Therefore, the pre-selection of expressed sequences will avoid the issue of genome complexity in barley. As a result of a barley EST project that was initiated at the IPK in 1998 about 200 000 sequences originating from 37 libraries have been released into the public domain. Together with the ESTs released by other groups more than 419 000 ESTs have been deposited until now in the public EST database of the NCBI (http://www.ncbi.nlm.nih.gov/dbEST/). These are derived from more than 80 cDNA libraries covering virtually any tissue and growth stage as well as a series of physiological conditions. Since ESTs reflect the transcriptional status of the tissue they were derived from, the sequences are inherently redundant. EST clustering is applied to remove the redundancy and to sort the sequences into singletons and sequence clusters. The sum of the numbers of singletons and clusters yields the number of tentative unigenes (Tentative Unigene Consensi, TUCs). In this regard, cluster analysis of 330 000 ESTs that were available in 2003 resulted in the definition of ca. 33 000 tentative unigenes. Evidently the outcome of this kind of analysis depends on a number of parameters including the average sequence length of an EST, the quality of the sequences and the contamination of EST data with sequences from other organisms, such as microbes or fungi. Moreover, the result of the analysis is influenced by the stringency of the cluster algorithm. The higher the stringency, the more singletons (which may be due to sequencing errors only) and thus the more unigenes will be defined. Using appropriate software and proper settings meaningful results can be obtained as was shown for the differentiation of the individual members of the transcription elongation factor 1-alpha (eEF1A) gene-family (Michalek *et al*. 2002).

A comparison of the available sequence data to 254 well characterised barley genes from the SWISSPROT database and to 1.2 Mb of annotated BAC-sequence originating from several regions of the barley genome revealed an EST coverage of 87% for the SWISSPROT dataset and 45% for the genomic sequences. Thus, a preliminary estimate of the gene repertoire of barley will lie between 38 000 and 72 000 genes. However, the complexity of the genome is defined not only by the number of its genes but also by the number of its proteins. The latter may be influenced by alternative splicing, which is a common feature of the human transcriptome. In higher plants alternative splicing may be much less frequent since only a few cases have been described so far. Our EST data revealed that about 4% of the barley genes showed alternatively spliced isoforms, a similar figure as was recently reported for *Arabidopsis* (Brett *et al*. 2002; Zhang *et al*. 2004).

From ESTs to markers

RFLPs. The barley unigene set represents a comprehensive source for the development of gene-derived markers. A BLASTN analysis of the barley unigene set itself revealed that about 50% of the genes were putative single copy genes while the remaining genes were members of gene families. Of these, the majority comprises only 2–5 copies (Figure 1). This is in accordance with the fact that cDNAs represent a good source for the development of RFLP markers or conversely that genes can readily be placed as RFLPs on the genetic map. Despite the experimental efforts required for RFLP mapping more than 600 EST-derived RFLP markers have been placed to date on a consensus map consisting of three mapping populations (Igri/Franka; Steptoe/Morex; Oregon Wolfe$_{rec}$/Oregon Wolfe$_{dom}$).

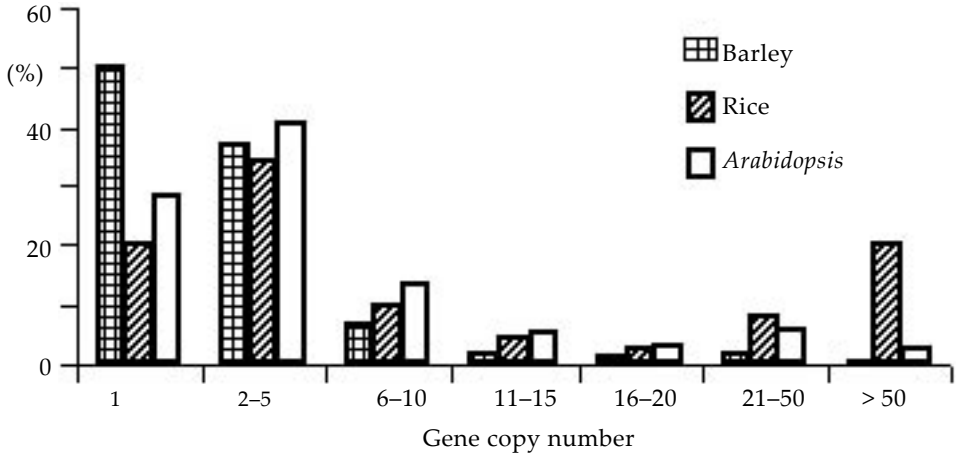

Figure 1. Distribution of the size of gene families. The copy number of each barley unigene was estimated by counting the number of BLASTN matches against the unigene data set itself with an E-value threshold of less than 10^{-20}

SSRs. Data from several plant species showed that, compared to non-coding regions, genes are enriched with microsatellites (SSRs) (Morgante *et al*. 2002). Analysis of the barley EST resource revealed similarities to other grass genomes: the barley transcriptome contains on average 1 SSR every 7.5 kb. This frequency of EST-SSRs is similar to that found in maize, wheat and sorghum, while the frequency in rice is 1/3.9 kb (Varshney *et al*. 2002). Trimeric SSRs represent the largest class of repeat motifs found in barley ESTs accounting for 56%. This may be explained by the suppression of non-trimeric SSRs in coding regions due to the ensuing frameshift mutations. Monomeric and dimeric repeats were observed at frequencies of 19% and 18%, respectively (Thiel *et al*. 2003). For SSR identification and primer development a software tool has been developed (MISA, http://pgrc.ipk-gatersleben.de/misa/) which allows a widely automated search of EST databases for SSR-containing sequences.

SNPs. Similar tools have been developed for the computer-assisted identification of single nucleotide polymorphisms (SNPs). SNPs represent the most common class of genetic variations and have rapidly become the markers of choice because of their high frequency in the genome and low mutation rates. A survey for barley based on the comparison of EST-derived sequence tags in 7 divergent barley cultivars and one *H. spontaneum* accession revealed a mean nucleotide diversity of 3.2×10^{-3} with values ranging from 0 (no SNP) to 3×10^{-2}. A marked increase in SNP frequency could be obtained by the computer-assisted preselection of polymorphic EST-sequences from public databases. This approach is based on the fact that the ESTs deposited in dbEST originated from different genotypes. Thus, sequence alignments can be searched for the presence of SNPs. By this approach it is possible to identify genes or regions within genes that show a higher frequency of polymorphisms. Using a software tool that has been designed to automatically perform this task, 4329 high scoring and putatively cultivar-specific SNPs were identified. Further experimental verification of the results obtained from the data mining approach resulted in the confirmation of 86% of the SNPs detected. The average nucleotide diversity of the SNPs identified in this way amounted to 9×10^{-3}. This increase was mainly due to the reduced frequency of monomorphic EST-alleles since their frequency dropped from 34% in random samples to only 9% after employing the data mining approach (Kota *et al*. 2003).

With the influx of a plethora of SNP genotyping assays in recent years, there has been an imminent need for an assay that is robust, yet cost effective, and could be performed using standard gel-based procedures. In this context, CAPS (cleaved amplified polymorphic sequences) markers have been shown to meet these criteria. However, converting SNPs to CAPS markers can be a laborious process if done manually. Therefore, a computer programme (SNP2CAPS, http://pgrc.ipk-gatersleben.de/snp2caps/) was developed that facilitates the computational conversion of SNPs into CAPS markers (Thiel *et al*. 2004). To investigate the number of potential CAPS markers present in our EST-allele

database, 413 multiple aligned sequences derived from barley ESTs were analysed for the presence of polymorphisms in 235 distinct restriction sites. 282 (90%) out of 314 alignments that contain sequence variation due to SNPs and InDels revealed at least one polymorphic restriction site. After reducing the number of restriction enzymes from 235 to a set of 10 common restriction enzymes, still 31% of the polymorphic sites could be detected. Thus, a significant portion of the available barley SNPs can be assayed as CAPS markers, which might be an option for laboratories that cannot afford or do not need the establishment of high throughput SNP-detection platforms.

The barley transcript map

The major challenge of genomics is the identification of candidate genes for a given trait. On the structural level it requires the identification of all possible genes residing on a chromosomal segment to which a trait has been assigned. In the best of all cases, each point on a genetic trait map can be connected to a defined region in a fully sequenced

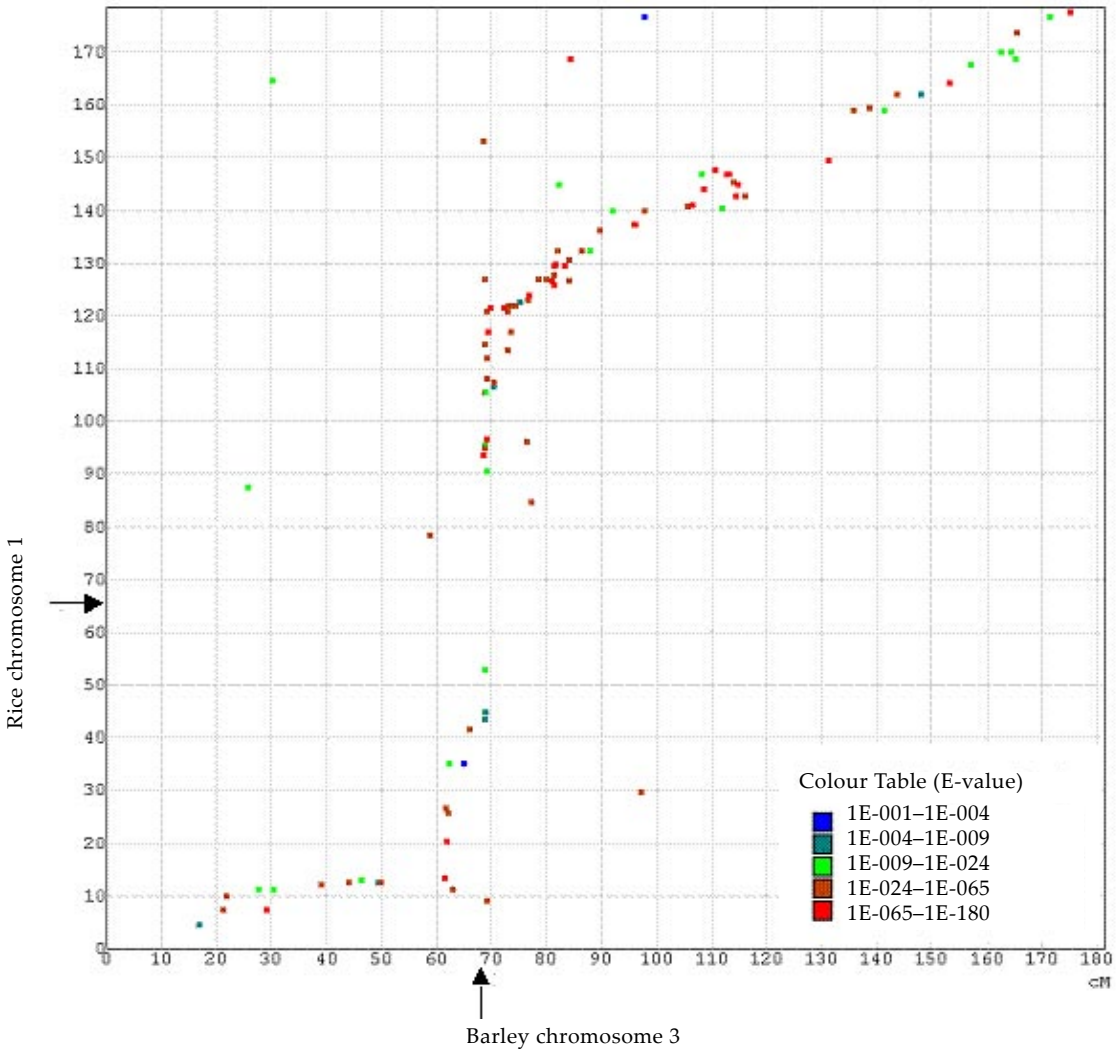

Figure 2. Comparison of the genetic maps of barley chromosome 3 (*x*-axis) and rice chromosome 1 (*y*-axis) (Harushima *et al*. 1998). The data for barley chromosome 3 are based on a consensus map of three populations. ESTs in common to both chromosomes are represented as dots with a colour code indicating the BLASTN similarity between homologous ESTs of the two species. The positions of the centromeres are indicated by arrows. Given similar map distances in both species, colinear markers would be ordered along the bisecting line. However, due to the reduced recombination observed in the proximal regions of the 7 barley chromosomes, colinear markers form a sigmoid curve. Moreover, because of the inaccuracies still present in the barley consensus map underlying this scatter plot, many dots deviate from an intended line that connects the colinear markers

genome – so far a privilege of *Arabidopsis* and rice only. Although the barley genome is far from being fully sequenced, strategies are emerging for sequencing the gene rich regions of the genome. To relate these partial physical maps to the genetic map(s) of barley a high density transcript map is being developed that presently comprises 1023 ESTs and extends over 1131 cM. The corresponding loci represent potential anchor points for sub-genomic physical maps of barley and the mapped markers, in particular the 255 SNPs and 185 SSRs further complete existing resources for trait mapping and marker assisted selection. Most importantly, the EST-loci are the connecting points to relate the genomes of barley and rice. The presence of extensive marker colinearity between these two genomes, which diverged about 60 million years ago, provides the opportunity to tap into the wealth of information and resources available for rice. To explore the sequence similarity between barley and rice, the barley ESTs were compared with both rice ESTs and rice genomic sequences. Out of the 1023 barley ESTs, 769 share a significant match with rice sequences (< 1E-5, > 80% sequence identity) and out of these 505 displayed a syntenic relationship at the chromosomal level as is shown in Figure 2 for rice chromosome 1 and a consensus map of barley chromosome 3. The apparent discrepancy between the numbers of syntenic and colinear markers is mainly attributable to inaccuracies of a few cM in the present consensus map, which disturb the linear marker order. An increase in the number of anchor markers is expected to alleviate this problem.

Application of marker colinearity between rice and barley

Evidently, it is most tempting to use information from the rice genome for the immediate identification of orthologous genes in barley. But colinearity observed at the level of genetic resolution may provide too optimistic a picture. In several cases, the orthologue from barley is no longer present in rice, due to small-scale genomic rearrangements or due to the rapid or divergent evolution of genes that may prevent the detection of their orthologues (Kilian *et al*. 1995; Leister *et al*. 1998). But even in the absence of the orthologous candidate gene, the information from rice can be applied to increase the marker saturation of a defined chromosome region in barley, as it may be required for the identification of a gene by positional cloning. A systematic approach to the EST based marker saturation of a target region around the *Rph16* rust resistance locus based on sequence information from rice was described recently (Perovic *et al*. 2004). In this study, 309 non-redundant candidate ESTs were identified for this region out of a collection of over 320 000 public barley ESTs in a two-step *in silico* selection procedure. For mapping, fifty-four barley cDNA-clones were selected due to the even distribution of their homologues on a putatively colinear 3 Mb rice BAC-contig. Out of them, 97% (30) of the polymorphic markers could be genetically assigned in colinearity to the target region in barley and a set of eleven markers was integrated into a *rph16* high-resolution map. Although the colinear target region of rice does not contain an obvious candidate gene for *rph16*, the results demonstrated the potential of the procedure to efficiently utilise EST resources for synteny-based marker saturation. A similar approach was successfully employed to saturate the chromosomal regions harbouring the GA-insensitive dwarfing gene *sdw3*, which is located on the same chromosome (Gottwald *et al*. 2004). The systematic genome-wide exploitation of the increasing sequence data resources will strongly improve our current view of genome conservation and likely facilitate a synteny-based isolation of genes conserved across cereal species.

Candidate gene identification by functional association

The availability of a comprehensive EST resource for barley also set the stage for the development of functional genomics-based strategies for the identification of trait-related genes. It could be shown that germination of a barley grain is based on the orchestrated, spatio-temporal expression of a large number of genes in embryonal, scutellar and endosperm tissue (Potokina *et al*. 2002). In addition to tissue dependent gene expression, cDNA-array experiments also revealed that gene expression within a given tissue varied between different genotypes. Under the hypothesis that differences in the expression of a quantitative trait are based on differences in the expression of the genes underlying this trait, a DNA-array based "functional association" approach was devised to identify genes whose expression was related to the trait malting quality (Potokina *et al*. 2004). As a result, 19 genes were identified in a pilot study that

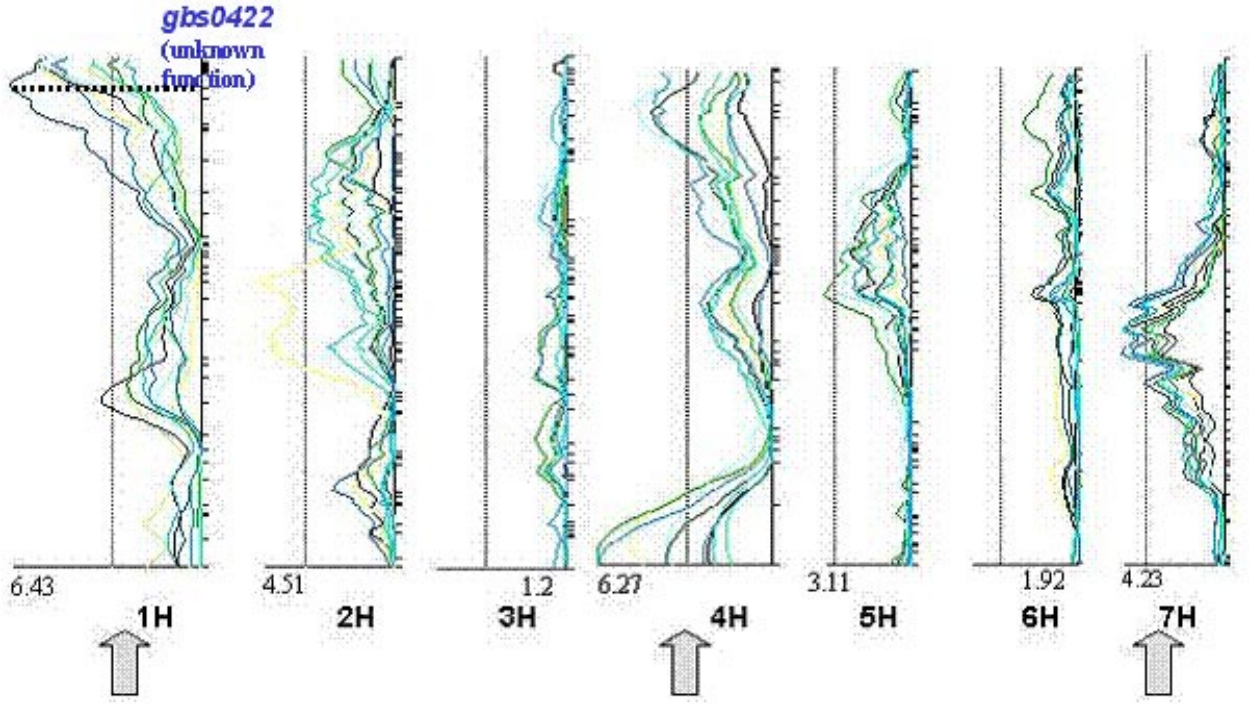

Figure 3. QTL-analysis of the trait "diastatic power" in the Steptoe/Morex progeny. Each LOD curve represents one of 7 environments analysed. Chromosomes are shown as vertical lines with the skeleton markers used for QTL mapping represented as horizontal bars. The dashed line indicates a LOD of 3.0. The maximum LOD that has been reached on each chromosome is indicated. SNP-marker GBS0422, corresponding to the barley EST HY01D13, has been identified by the functional association approach as a candidate gene for malting quality (for details see Potokina *et al*. 2004)

was based on a cDNA array comprising 1400 genes whose expression levels in a set of 10 European barley cultivars significantly correlated with the expression of the 7 malt parameters measured. This set of candidate genes contained genes that were already previously supposed to be related to malting quality (e.g. cysteine proteinase 1), genes hitherto unknown to be related to this trait (e.g. 70 kd heat shock protein) and genes of unknown function. In several cases genetic mapping of these candidate genes in the Steptoe/Morex cross revealed a congruency between the map location of the candidate gene and the presence of a QTL for malting quality (Figure 3).

Although these findings lend further strength to the validity of our functional association approach, it suffers from the limited resolution of QTL (quantitative trait locus) mapping. Hence, additional data from a larger set of genotypes are required for further verification. In addition to the genetic mapping of candidate genes and subsequent QTL analysis in a bi-parental population, which inherently is confined to the segregation of two alleles only, the genetic association of candidate genes may be analysed in a larger panel of genotypes using a case-control design.

CONCLUSIONS

While the development of molecular markers has shifted from anonymous DNA fragments to genes, the development of a comprehensive transcript map of the barley genome is still at its beginning. ESTs are presently used on a large scale for the systematic development of SSR and SNP markers and it will be important to integrate the maps generated in different laboratories into a robust consensus map which needs to be curated in a coordinated and sustainable way. The same applies to the integration of trait data.

As long as there is no comprehensive sequence information available for barley, many attempts of positional cloning will benefit from the availability of sequence information from rice. In this regard, mapped barley ESTs provide an excellent resource to quickly identify the orthologous target region

in the rice genome and to exploit the rice sequence for an EST-based marker saturation in barley.

In addition to gene isolation by positional cloning, the availability of comprehensive cDNA and oligonucleotide arrays now provides an option for the systematic investigation of quantitatively inherited traits, using a functional association strategy. Undoubtedly the verification of the candidate genes obtained by this approach presents further challenges for the future. Complementary to the genetic mapping of the candidate genes, genomics studies are required to investigate whether candidate genes identified from the array analyses are regulated in cis or in trans, since only the cis-regulated genes will be amenable to marker assisted selection in plant breeding.

Despite the many issues that still await a solution, the availability of a large collection of barley ESTs has set the stage for a systematic dissection of the genetic basis of agronomic traits in barley. The identification of the corresponding genes is expected to lead to the development of improved strategies to identify novel and useful alleles from the vast number of genetic resources that rest on the shelves of the gene banks or that are thriving *in situ*. In this way genome research can deliver a significant contribution to the use of biodiversity for the adaptation of barley to the future needs of mankind.

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Abstrakt

Graner A., Thiel T., Zhang H., Potokina E., Prasad M., Perovic D., Kota R., Varshney R.K., Scholz U., Grosse I., Stein N. (2005): **Molekulární mapování ječmene: přechod od strukturální úrovně k funkční**. Czech J. Genet. Plant Breed., **41**: 81–88.

Jako zdroje pro strukturální a funkční analýzu genomu ječmene bylo získáno více než 140 000 ESTs (expressed sequence tags – exprimovaných sekvencí) z celkem 22 cDNA knihoven, což reprezentuje 25 224 potenciálních genů. Asi 50 % z nich patří genovým rodinám. Velikost úplného transkriptomu je odhadována na 35 000 až 75 000 genů. ESTs kolekce ječmene představuje bohatý zdroj pro tvorbu nových markerů, zahrnující SSRs (simple sequence repeats – jednoduché sekvenční repetice) a SNPs (single nucleotide polymorphisms – jednotlivý nukleotidový polymorfismus). Bylo vytvořeno několik bioinformačních nástrojů umožňujících počítačové zpracování ESTs databází s cílem vyhledávání SNPs nebo SSRs a tvorby SNPs odvozených z CAPS (rozštěpené množené polymorfické sekvence) markerů. Ve snaze o systematické mapování genů ječmene je konstruována transkripční mapa s vysokou hustotou, která v současné době obsahuje více než 1000 markerů. Tato mapa je vstupem do komparativní genomiky se zvláštním důrazem na genom rýže. Bylo zjištěno, že 65 % mapovaných ESTs vykazuje průkaznou homologii k rýžovým ESTs, což znamená syntenický vztah mezi ječmenem a rýží. Proto ESTs ječmene umožňují rychlý a systematický přenos genetických (genomických) poznatků z rýže do ječmene a jiných druhů čeledi *Triticeae*. Toho může být pohotově využito pro nasycení definovaných chromozomálních oblastí markery a jejich detailní srovnávání s rýží. Funkční genomika v tomto kontextu může být využita pro studium komplexního znaku jako "sladovnická kvalita" pomocí sady cDNA. Korelací fenotypických údajů sladovnické kvality u vybraných linií ječmene s odpovídajícími profily byl identifikován soubor genů – kandidátů a ten byl dále verifikován genetickou analýzou.

Klíčová slova: ESTs; funkční genomika; sladovnická kvalita; DNA-markery; rýže; synteny

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