Genetic and Physical Mapping of Genic Microsatellites in Barley (*Hordeum vulgare* L.)

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Abstract: Due to the availability of sequence data from large-scale EST (expressed sequence tag) projects, it has become feasible to develop microsatellite or simple sequence repeat (SSR) markers from genes. A set of 111 090 barley ESTs (corresponding to 55.9 Mb of sequence) was employed for the identification of microsatellites with the help of a PERL5 script called *MISA*. As a result, a total of 9 564 microsatellites were identified in 8 766 ESTs (SSR-ESTs). Cluster analysis revealed the presence of 2 823 non-redundant SSR-ESTs in this set. From these 754 primer pairs were designed and analysed in a set of seven genotypes including the parents of three mapping populations. Finally, 185 microsatellite (EST-SSRs) loci were placed onto the barley genetic map. These markers show a uniform distribution on all the linkage groups ranging from 21 markers (on 7H) to 35 markers (3H). The polymorphism information content (PIC) for the developed markers ranged from 0.24 to 0.78 with an average of 0.48. For the assignment of these markers to BAC clones, a PCR-based strategy was established to screen the "Morex"-BAC library. By using this strategy BAC addresses were obtained for a total of 127 mapped EST-SSRs, which may provide at least two markers located on a single BAC. This observation is indicative of an uneven distribution of genes and may lead to the identification of gene-rich regions in the barley genome.

Keywords: EST-SSRs; genic microsatellites; genetic mapping; physical mapping

The analysis of DNA sequence variation is of major importance in genetic studies. In this context, molecular markers represent a major tool for genome mapping and have revolutionised the genetic analysis of crop plants. In the past, a variety of molecular markers including RFLPs (*R*estriction *F*ragment *L*ength *P*olymorphism*s*), RAPDs (*R*apid *A*mplification of *P*olymorphic *D*NA*s*), AFLP*s* (*A*mplified *F*ragment *L*ength *P*olymorphism*s*) and microsatellites or SSRs (*S*imple *S*equence *R*epeats) were developed in barley (Varshney *et al*. 2004). Among different classes of molecular markers, SSR markers have proved as markers of choice for a variety of applications in plant genetics and breeding because of their multiallelic nature, co-dominant inheritance, relative abundance and extensive genome coverage (reviewed by Gupta & Varshney 2000). In barley, about ~400 SSR loci have been mapped (Ramsay *et al*. 2000; Pillen *et al*. 2000; Li *et al*. 2003).

With the establishment of EST sequencing projects, a wealth of sequence information is generated allowing the identification and development of SSR markers from ESTs (Kantety *et al*. 2002; Varshney *et*

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al. 2002). EST-SSR markers are superior to genomic SSRs in terms of transferability and comparative mapping in related species as they are derived from conserved portions of the genome (Varshney *et al*. 2005). To further increase the resource of barley microsatellite markers, we utilized a set of barley ESTs for the computer-assisted identification of SSRs and the development of corresponding markers (Thiel *et al*. 2003).

In this paper, we report on the identification of SSRs in 111 090 barley ESTs generated at IPK and on the development and genetic mapping of a non-redundant set comprising 185 genic microsatellite markers. In addition, a PCR-based strategy was employed to screen the "Morex"-BAC library with SSR (PCR-based) markers and BAC addresses were obtained for 127 SSR-ESTs.

MATERIAL AND METHODS

Plant materials. For detection of SSR polymorphism, a set of 7 barley (*Hordeum vulgare* L.) cultivars was used. This set included Barke, Igri, Franka, Steptoe, Morex, Oregon Wolfe Barley OWB_{Dom} and OWB_{Rec}. Barke was used as a standard because this cultivar was used for the construction of most EST libraries, while the other six genotypes represent the parents of three doubled haploid (DH) mapping populations, i.e. Igri × Franka (I/F, Graner *et al*. 1991), Steptoe × Morex (S/M, Kleinhofs *et al*. 1993), OWB_{Dom} and OWB_{Rec} (D/R, Costa *et al*. 2000).

Genomic DNA isolation was carried out as described in Thiel *et al*. (2003).

Database mining. A total of 111 090 barley EST sequences, generated from 22 cDNA libraries (Michalek *et al*. 2002; Zhang *et al*. 2004; http://pgrc.ipk-gatersleben.de/b-est/), were screened for microsatellites by using the *MISA* software module described by Thiel *et al*. (2003) and available under http://pgrc.ipk-gatersleben.de/misa/. To improve the efficiency of the identification of polymorphic SSRs, we included another set of 207 449 non-IPK barley ESTs present in GenBank (NCBI, USA) which were developed from other cultivars than Barke (Table 1).

Redundancy analysis, primer designing and genetic mapping. Cluster analysis and primer designing for the development of a non-redundant set of markers were performed by help of stackPACK2.1 and PRIMER3 programmes, respectively, as described earlier (Varshney *et al*. 2002; Thiel *et al*. 2003). PCR amplification of microsatellite loci, their separation, visualisation and linkage mapping were performed according to Thiel *et al*. (2003).

PCR-based screening of barley BAC library. To identify gene-containing clones in an ordered BAC library of barley (Morex cultivar) with more than 300 000 clones (Yu *et al*. 2000) a four-step, PCR-based screening protocol was established. The same primer pairs that were used for the mapping of SSR markers were applied for the screening of the BAC library using a touchdown PCR protocol (95°C, 3 min/10 cycles: 95°C, 1 min; 65°C–0.5°C per cycle, 1 min; 72°C, 1 min/25 cycles: 95°C, 1 min; 60°C, 1 min; 72°C, 1 min/72°C, 3 min/15°C) in a total volume of 20 µl (buffer: 10mM Tris-HCl, 50mM KCl, 0.1% Triton X-100, 2mM $MgCl_2$, 0.2mM each of dATP, dCTP, dGTP and dTTP, 100 pmol of each primer and approximately 1 U of Taq polymerase). The first round of screening was performed on 90 super pools comprising BAC-DNA of 3456 clones from 9 consecutive 384-well microtitre plates (Table 3). PCR products with a typical size of 100–500 bp were analysed on 3% agarose gels. For those super pools that yielded a fragment of the same length as genomic DNA from *H. vulgare* cv. Morex all 9 individual plate pools of BAC DNA were examined during the second round of screening. The third round of screening was performed on 16 row and 24 column pools running through a positive plate. These row and column pools were derived from rows and columns running through a rectangular arrangement of 24 by 34 microtitre plates of the BAC library to minimize the number of DNA preparations.

RESULTS AND DISCUSSION

Occurrence of SSRs in ESTs

In the context of barley genomics project, > 110 000 barley ESTs were generated from 22 cDNA libraries constructed from different tissues (Zhang *et al*. 2004). The main objective to generate the large number of ESTs was to catalogue as many as possible non-redundant gene sets (unigene) of barley. Genome sequencing of the plant species such as barley and wheat, which have large genomes with 80% repetitive DNA, is not feasible in the near future and therefore EST sequencing in such plant species has been proved an important option for genome analysis (Ogihara *et al*. 2003; Rudd 2003). Additionally, ESTs can be exploited to develop molecular markers such as

SSRs (see Varshney *et al*. 2005) and SNPs (single nucleotide polymorphisms, Rafalski 2002) after database mining approach and a putative function can generally be deduced for such markers, they are popularly termed as functional markers (Anderson & Lubberstedt 2003).

In the present study a set of 111 090 barley ESTs, corresponding to 55.9 Mb, was employed for the searching of microsatellites as a source for marker development. With the help of the *MISA* software tool, 9564 microsatellites (EST-SSRs) were identified in a total of 8766 ESTs (SSR-ESTs). Cluster analysis revealed 2823 non-redundant SSR-ESTs that contained 3122 (2.8%) non-redundant SSRs. The overall frequency of microsatellites obtained in barley ESTs in the present study is in agreement with earlier studies conducted in grape (Scott *et al*. 2000), sugarcane (Cordeiro *et al*. 2001), wheat (Nicot *et al*. 2004; Yu *et al*. 2004), etc. As expected, trimeric SSRs constituted the major portion as 52.6% and 63.4% of the total SSRs identified in non-redundant and redundant SSR-EST sets, respectively. Pentameric and hexameric microsatellites were present at less than 1% of total SSRs searched. Although the relative frequencies of different types of SSRs largely depend on the search criteria used in database mining (search tool) as well as on the dataset, the obtained results are in accordance with earlier studies on database mining of SSRs in ESTs in cereal genomes (Kantety *et al*. 2002; La Rota *et al*. 2005; Varshney *et al*. 2005).

Genetic mapping of EST-SSRs

A set of seven genotypes was used to screen for polymorphism with the primer pairs for EST-SSRs. For designing and analysing the primer pairs, SSR-ESTs were selected by using two approaches. In the first approach, a set 665 SSR-ESTs was selected from the non-redundant SSR-ESTs from IPK-dataset. Of the 464 primer pairs (69.8%), which yielded an amplicon in the analysed genotypes, 156 primer pairs (33.6%) displayed polymorphism between the parents of at least one mapping population (Table 1).

To enhance the level of polymorphism in the genotypes of our interest, we adopted a second strategy based on additional non-IPK barley ESTs from the public domain. While the IPK-ESTs were generally derived from the 'Barke' genotype, non-IPK ESTs were developed from a series of different cultivars (Kota *et al*. 2003). Hence, a comparison of Barke ESTs with non-Barke ESTs allows a preselection of polymorphic SSRs. After clustering the 18 041 SSR-ESTs containing 24 623 SSRs (out of 207 449 non-IPK barley ESTs, available in the public domain at that time) with 8766 SSR-ESTs of the IPK set, we identified a total of 197 mixed clusters containing SSR-ESTs that showed variation in SSR length between IPK and non-IPK ESTs. Of this set, we selected 89 IPK SSR-ESTs for the amplification of the microsatellite locus in the set of 7 genotypes (Table 2). Amplicons with 61 (68.5%) primer pairs were obtained. Of these, 29 (47.5%) detected polymorphisms that could be mapped in the populations used in this study. Thus the level of polymorphism detected in parental genotypes of at least one mapping population was increased by 13.9% and this higher level of polymorphism was statistically significant (χ^2 test, $P < 0.01$) compared to the first strategy. Therefore, the presence of a polymorphic SSR in the EST databases was a good predictor for the presence of a polymorphism in any of the three mapping populations used in this study. This observation is in accordance with the results obtained from database mining for SNPs,

Table 1. An overview of polymorphism and development of EST-SSR markers in barley

	First approach (database mining of IPK- ESTs)	Second approach (clustering of IPK SSR-ESTs with public SSR-ESTs*)	Total
Identified potential SSR-ESTs	3122	197	
Primer pairs designed	665	89	754
Functional primer pairs	464 (69.8%)	61 (68.5%)	525 (69.6%)
Polymorphic primer pairs in parents of at least one mapping population	156 (33.6%)	29 (47.5%)	185 (35.2%)

*In this approach, 8766 SSR-ESTs from IPK (developed from cv. Barke) and 18 041 SSR-ESTs from the public domain (non-IPK ESTs, developed from other cultivars than Barke) were clustered and mixed clusters were analysed to select the IPK-ESTs that showed variation in SSR length compared to that of non-IPK ESTs

where the presence of a SNP in the EST-database greatly enhanced the probability to detect polymorphism also in our mapping populations (Kota *et al*. 2003).

Using both approaches a total of 754 primer pairs were analysed with the set of 7 genotypes. 525 (69.6%) primer pairs yielded amplicons, out of which 185 (35.2%) primer pairs detected polymorphism in parents of at least one mapping population. The failure of ~30% primer pairs to generate an amplicon can be explained in this way: one or both primers of the primer pairs may have been designed across the exon/intron splice site preventing genomic DNA to be amplified (Cordeiro *et al*. 2001). Further the lower level of polymorphism (~35%) is an inherent feature of EST-SSR markers as these SSRs are located in the conserved proportion of the genome (Varshney *et al*. 2005). Similar results were obtained in other plant species (Scott *et al*. 2000; Cordeiro *et al*. 2001; Thiel *et al*. 2003; Yu *et al*. 2004).

Of the 185 polymorphic markers 129 were mapped in the OWB_{Rec} × OWB_{Dom} (R/D), 47 in the Steptoe × Morex (S/M) and 23 in the Igri × Franka (I/F) population. These results showed that the R/D population was the most polymorphic (Costa *et al*. 2001) followed by the S/M (Kleinhofs *et al*. 1993) and the I/F (Graner *et al*. 1991) is the least polymorphic mapping population. Twelve SSRs were mapped in both I/F and D/R while two SSRs were mapped in S/M and D/R. In addition to these common SSR markers a set of anchor markers was used to construct a consensus map of all three mapping populations. On this map, the EST-SSR markers were fairly evenly distributed with numbers ranging from 21 (7H) to 35 (3H) with an average of 27 per chromosome (Table 2). This is probably an important feature of EST-SSR markers unlike the genomic SSR markers that were mapped earlier and clustered around the centromere (Ramsay *et al*. 2000). Genomic SSRs may be associated with repetitive DNA such as retrotransposons (Ramsay *et al*. 1999) while EST-SSRs mirror the genes (Thiel *et al*. 2003).

For all mapped markers, the polymorphism information content (PIC) was calculated on the basis of observed alleles in 6 (with 76 markers) to 7 genotypes (with the remaining 109 markers). In the studied set of genotypes, the mapped markers detected 2 to 5 alleles with an average of 2.7 alleles per locus. The PIC value for the developed markers ranged from 0.24 to 0.78 (average 0.48) and about 50% markers had a PIC value > 0.50. The observed PIC value was lower than that of genomic DNA-derived microsatellites (Ramsay *et al*. 2000; Li *et al*. 2003; Russell *et al*. 2004) and was comparable to that of EST-derived SSRs (Pillen *et al*. 2000; Thiel *et al*. 2003).

Physical anchoring of EST-SSRs

For 129 EST-based SSR markers an attempt was made to identify BAC clones in a genomic library of barley (Yu *et al*. 2000). A four-step, PCR-based screening strategy was developed, employing DNA of BAC pools and a confirmation step at the level of individual clones (Table 3).

As an initial step this strategy requires the screening of 90 super pools, each containing the DNA of 3456 BAC clones. During the following steps, positive pools are deconvoluted to plate pools and to BAC addresses using special row and column pools. These row and column pools run through the rows of 24 plates and columns of 34 plates of the library, respectively. Finally, BAC addresses for genes are confirmed by PCR at the level of individual clones. In theory, this four-step screening procedure should require on average 390 reactions plus controls and their analysis on agarose gels for

Table 2. Summary of genetic mapping of barley EST-SSRs in different mapping populations

Population	Chromosome							
	1H	2H	3H	4H	5H	6H	7H	Total
I/F	5 (3)*	1	3 (2)	1 (1)	2 (1)	7 (4)	4 (1)	23 (12)
S/M	5	8	12 (1)	7 (1)	4	5	6	47 (2)
R/D	18 (3)	22	23 (3)	20 (2)	17 (1)	17 (4)	12 (1)	129 (14)
Total	25	31	35	26	22	25	21	185

*numbers in parentheses represent the number of common markers mapped in two mapping populations

Table 3. PCR-based strategy to screen the BAC library

Pool type	Numbers	Pool size	Assembly of	PCR reactions
Super	90	3 456	9 MTPs	90
Plate	810	384	1 MTP	6 × 9 = 54
Row	544	576	row through 24 MTPs	6 × 16 = 96
Column	576	544	column through 34 MTPs	6 × 24 = 144
Clone	311 040	1	clone	6

the identification of all BAC clones in the library containing a single-copy sequence. Using that strategy one or more BAC clones were obtained for 127 (98.4%) of the SSR markers analysed.

BAC library screening and genetic mapping revealed closely linked groups of EST-SSR markers (maximum distance of 2 cM on a genetic map) of which more than one hit the same BAC clone. A closer inspection of the markers in the genetic window of < 2 cM provided a total of 8 groups of markers that were present on 3 different chromosomes (3H, 6H and 7H), contained in one or more BAC clones, on which at least two markers of the group were located. Furthermore, PCR screening of BAC clones with the markers from the same group and the cluster analysis of the corresponding SSR-ESTs for these markers showed that independent and physically separated sequences were amplified by the respective markers (at least two from one group), demonstrating again that they belong to different genes. The regions on the genetic map where the 'gene-rich' BAC clones or contigs were observed in the present study could be assigned to recombination hot spots on the translocation-breakpoints based physical map of Künzel *et al*. (2000). Therefore this dataset, although being small, is in agreement with earlier studies where gene-rich regions in *Triticeae* genomes were identified in regions high in recombination (for references see Gill 2004).

In summary, the present study contributes a set of 185 gene-derived microsatellite markers. These can be amplified using a standardised single PCR profile and are uniformly distributed on all seven barley linkage groups without any obvious centromeric clustering. BAC addresses identified for 127 SSR-ESTs will provide useful anchoring points to correlate the genetic map with a "future" physical map of the barley genome. Furthermore, at least eight regions identified on three chromosomes offer a possibility of the preparation of contigs leading to the construction of sub-genome physical maps of gene-dense regions of the barley genome.

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Abstrakt

Varshney R.K., Hähnel U., Thiel T., Stein N., Altschmied L., Langridge P., Graner A. (2005): **Genetické a fyzikální mapování mikrosatelitů u ječmene** (*Hordeum vulgare* L.). Czech J. Genet. Plant Breed., **41**: 153–159.

Vývoj mikrosatelitních a SSR (simple sequence repeat) markerů umožnila dostupnost sekvenčních dat z projektů EST (expressed sequence tag). Soubor čítající 111 090 EST (odpovídajících 55,9 Mb sekvenci) byl využit pro identifikaci mikrosatelitů pomocí programu MISA na bázi „PERL5 script". Celkem 9564 mikrosatelitů bylo identifikováno v 8766 EST (SSR-EST). Shluková analýza odhalila přítomnost 2823 SSR-EST, které v tomto souboru nebyly redundantní. Bylo určeno 754 párů primerů a analyzováno u souboru čítajícího 7 genotypů při zahrnutí rodičů ze tří mapovacích populací. Na závěr bylo 185 mikrosatelitních (EST-SSR) lokusů umístěno do genetické mapy ječmene. Tyto markery vykazují jednotnou distribuci ve vazebních skupinách v rozsahu od 21 markerů (na chromozomu 7H) po 35 markerů (na 3H). Informační hodnota (PIC) pro markery se pohybuje od 0,24 do 0,78, s průměrem 0,48. Pro přiřazení těchto markerů BAC klonům byla vyvinuta strategie na bázi PCR pro skríning v rámci BAC knihovny odrůdy „Morex". S využitím této strategie bylo umístění BAC získáno celkem pro 127 zmapovaných EST-SSR, což umožňuje přiřadit ke každému BAC minimálně dva markery. Tato zjištění indikují nerovnou distribuci genů a mohou vést k identifikaci genově bohatých úseků v rámci genomu ječmene.

Klíčová slova: EST-SSR; mikrosatelity; genetické mapování; fyzikální mapování

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