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Study of simple sequence repeat (SSR) markers from wheat expressed sequence tags (ESTs)

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Abstract The increasing availability of expressed sequence tags (ESTs) in wheat (*Triticum aestivum*) and related cereals provides a valuable resource of non-anonymous DNA molecular markers. We examined 170,746 wheat ESTs from the public (International Triticeae EST Cooperative) and Génoplante databases, previously clustered in contigs, for the presence of di- to hexanucleotide simple sequence repeats (SSRs). Analysis of 46,510 contigs identified 3,530 SSRs, which represented 7.5% of the total number of contigs. Only 74% of the sequences allowed primer pairs to be designed, 70% led to an amplification product, mainly of a high quality (68%), and 53% exhibited polymorphism for at least one cultivar among the eight tested. Even though dinucleotide SSRs were less represented than trinucleotide SSRs (15.5% versus 66.5%, respectively), the former showed a much higher polymorphism level (83% versus 46%). The effect of the number and type of repeats is also discussed. The development of new EST-SSRs markers will have important implications for the genetic analysis and exploitation of the genetic resources of wheat and related species and will provide a more direct estimate of functional diversity.

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Introduction

Microsatellites, also often referred to as simple sequence repeats (SSRs), are currently being widely employed for developing genetic linkage maps in several cereal species such as maize (Senior et al. 1996), barley (Liu et al. 1996; Ramsay et al. 2000), rice (McCouch et al. 1997; Temnykh et al. 1999) and wheat (Röder et al. 1998). Despite their numerous advantages—primarily their ability to reveal high levels of polymorphism, user friendliness and successful adaption to automation—they are still very expensive and difficult to produce. Genetic studies on SSR development have generally used anonymous genomic DNA fragments, which has necessitated the creation of enriched libraries or the screening of genomic libraries. In both cases, all of the positive clones must be sequenced. However, there is always a high redundancy frequency among the clones, and primer designing is not always feasible due to the location of the microsatellite—too close to the ends or because of a low complexity of the flanking sequences.

One way to overcome the problem of cost is to explore the huge number of sequences available in public databases. Significant emphasis has recently been placed on generating substantial expressed sequence tag (EST) databases for plant species. In particular, the number of cereal ESTs has considerably increased during the last 3 years, and more than 1.6 million of these sequences have been released into the public domain (http://www.ncbi.nlm.nih.gov/dbEST/). The potential of ESTs as a source of SSRs has already been demonstrated for numerous species, such as grape, wheat, *Arabidopsis*, soybean, rice, maize, barley and sorghum (Scott et al. 2000; Eujayl et al. 2002; Holton et al. 2002; Kantety et al. 2002; Morgante et al. 2002). Furthermore, ESTs constitute a

novel source of markers that are physically associated with coding regions of the genome, and this can enhance the role of genetic markers in germplasm evaluation by enabling the variation in transcribed and known function genes to be assayed. Genomic SSRs are anonymous markers, and the genetic linkage they sometimes exhibit with quantitative trait loci is often only due to a physical linkage and statistical relationships. In addition and contrary to genomic SSRs which are frequently not transferable to closely related species (Röder et al. 1995; Sourdille et al. 2001a), EST-SSRs present a relatively high level of transferability (26–55%, Holton et al. 2002).

The overall purpose of our project was to screen more than 170,000 public and Génoplante wheat ESTs (sequences available at http://www.ncbi.nlm.nih.gov, search Nucleotide for wheat Genoplante) in order to identify a sample of SSR markers and evaluate their usefulness for wheat genotyping. We report here our results on: (1) screening of the libraries and evaluation of the ability of EST-SSRs to detect polymorphism on a set of hexaploid wheats; (2) effect of the motif and length of the microsatellite on polymorphism; (3) distribution of these markers throughout the wheat genome.

Materials and methods

Bioinformatic analysis for SSR detection and primer design

The clustering was done with 170,746 EST sequences originating from public and Génoplante databases (92,387 and 78,359 ESTs, respectively). The sequences had been previously masked for vectors and repeats. The minimal overlapping length was 80 bp with 96% identity for an alignment length between 80 bp and 200 bp and 90% identity for an alignment length greater than 200 bp. The CAP3 program (Huang et al. 1999) was used for EST assembly with default parameters on unmasked sequences. This resulted in 19,191 contigs containing a range of 2–395 sequences and 27,319 singletons. The 46,510 consensus sequences ranging from 93 bp to 7,497 bp in length were screened for the presence of SSRs using either REPEATMASKER (Smit and Green, unpublished results, http://ftp.genome.washington.edu/RM/RepeatMasker.html), TRF (Benson 1999) or SSRSEARCH (ftp://ftp.gramene.org/pub/gramene/software/scripts/ssr.pl) programs. A microsatellite was defined as a sequence containing a minimum of three repeats of a motif comprising from one to six nucleotides, with a total length of at least 12 nucleotides. ESTs containing SSRs were then extracted from the pool of 46,510 contigs. Primers flanking the microsatellites were designed using PRIMER (ver. 0.5, Whitehead Institute for Biomedical Research, Cambridge, Mass.). They were chosen with a length of 20 bases, an optimal annealing temperature of 60°C and for an amplification product length ranging between 150 bp and 400 bp.

Plant material

Eight hexaploid wheat lines, the parents of five mapping populations, were used for polymorphism screening of the microsatellites: W7984 (synthetic wheat, Van Deynze et al. 1995) and cvs. Opata, Courtot, Chinese Spring, Eurêka, Renan, Arche, Récital. A subset of 46 recombinant inbred lines (RILs) of the ITMI (International Triticeae Mapping Initiative) population (W7984 × Opata) was used for segregation analysis. DNA extraction was performed from fresh leaves using a CTAB protocol as described by Tixier et al. (1998).

PCR amplification and product analysis

PCR analyses were performed as described by Tixier et al. (1998) at an annealing temperature of 60°C. Microsatellite polymorphisms were visualized either by the silver staining method (Tixier et al. 1997) or using an ABI PRISM 3100 genetic analyzer (PE Applied Biosystems, Foster City, Calif.). For the latter system, each forward primer was 5′-tailed with the M13 forward consensus sequence. The M13-tailed forward primers were then used in combination with a standard M13 primer dye-labeled at its 5′-end (Boutin-Ganache et al. 2001). PCR analyses were performed at an annealing temperature of 60°C for 30 cycles, followed by eight cycles at 56°C.

Statistical analysis

The polymorphic microsatellites were genotyped on 46 RILs of the ITMI population, and the data were integrated into an existing framework map (Leroy et al. 1997). The markers were assigned to genetic bins using the MAPMAKER software program (Lander et al. 1987) 'assign' and 'map' commands at LOD 3 and a recombination fraction of 0.35.

As described in Guyomarc'h et al. (2002), a generalized linear model (GLM) deviance analysis was used to test the effect on the polymorphism of both factors—motif length and number of repeats.

Results

SSR detection and polymorphism

Among the set of 46,510 consensus sequences used for the search for microsatellites, REPEATMASKER detected 4,128 contigs (9%) containing a SSR. However, 14.5% of these SSRs were not found by the other two programs (TRF and SSRSEARCH), suggesting that REPEATMASKER was over-sensitive. Only 3,530 contigs (7.5%, sequences available at http://www.ncbi.nlm.nih.gov, search Nucleotide for wheat Genoplante) contained a SSR detected by all three programs.

Among these 3,530 contigs, 927 were used for primer pair design. From these 927 contigs, only 688 primer pairs (74%) were developed; for the remaining 239, no primer pair could be designed because of a low complexity (i.e. repeated DNA) or the occurrence of too much missing data in the flanking regions or because the microsatellite was located close to one of the ends. The 688 primer pairs were tested on eight reference cultivars in order to detect polymorphism. Only 70% of the primer pairs led to an amplification product, with 53% exhibiting polymorphism for at least one cultivar. The combination W7984 × Opata was the most polymorphic (Fig. 1), whereas Eurêka × Renan gave the lowest level of polymorphism.

Amplification analysis

The quality of each amplification product was scored (Fig. 2, Table 1) on a scale from A (the best quality obtained, one clear polymorphic band) to E (no amplification product). Only 29% of the primer pairs produced a high-quality pattern (A), while 30% were not able to

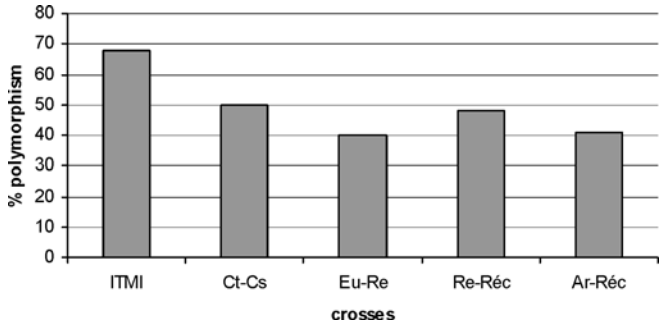

Fig. 1 The level of polymorphism between parents evaluated for each cross. *ITMI* Synthetic × Opata, *Ct-Cs* Courtot × Chinese-Spring, *Eu-Re* Eurêka × Renan, *Re-Réc* Renan × Récital, *Ar-Réc* Arche × Récital. A total of 482 EST-SSRs were amplified in the eight parental cultivars

amplify any product at all. Of the primer pairs exhibiting an amplification product, 68% were scored as A or B.

Some differences were observed between the expected and the observed size of the amplification product. Among 413 primer pairs, 35 (8.5%) gave an amplification product that was more than 10% larger than expected, suggesting the possible simultaneous amplification of an intron during the PCR. Conversely, 22 primer pairs (5%) gave an amplification product that was smaller than expected, suggesting: (1) the occurrence of deletions within the genomic sequences; (2) a lack of specificity of some primer pairs which may have resulted in the amplification of a different copy belonging to the same multigenic family or (3) a slight variation between the amplified copy and the consensus sequence.

Influence of the type of the motif on polymorphism detection

Among the 688 EST contigs from which primers were designed, 555 contained one SSR and 133 contained two or more SSRs. Within the set of 555 microsatellites, most consisted of trinucleotide motifs (66.5%), while only 15.5% consisted of dinucleotide motifs. However, even if these latter were less represented, they showed a much larger polymorphism level (83%) than both the trinucleotide motifs (46%) and the other classes. However, the number of repeats did not increase the level of polymorphism rate (data not shown). Among the dinucleotide motifs, $(GA)_n$ repeats were more frequent (45%) than $(CA)_n$ (38%) or $(AT)_n$ (17%) repeats, with the $(CG)_n$ repeat being totally absent in our set of ESTs. $(CA)_n$ and $(GA)_n$ repeats located either within genomic or EST sequences were compared, but no significant difference in polymorphism was observed (data not shown). $(CCG)_n$ repeats represented 36% of the trinucleotide motifs, whereas the two repeat classes $(AAT)_n$ and $(TAC)_n$ were only rarely represented (0.5 and 1%, respectively)

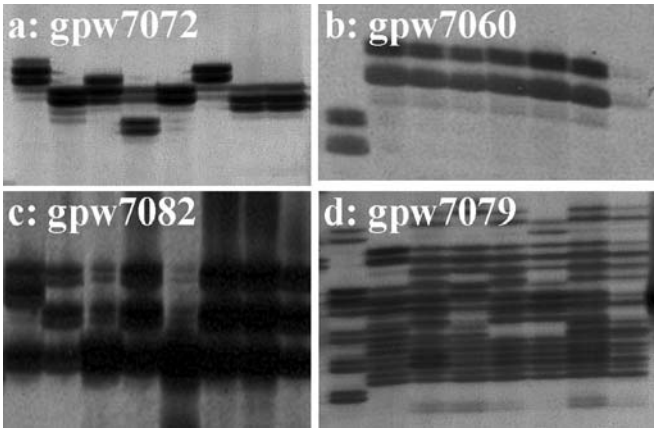

Fig. 2a–d Examples of microsatellites representative of each quality score. **a** Quality A (gpw7072), **b** quality B (gpw7060), **c** quality C (gpw7082) **d** quality D (gpw7079). Quality E indicates a lack of amplification and is thus not shown

Mapping

A set of 112 polymorphic microsatellites amplifying 126 loci was genotyped on the ITMI population (W7984 × Opata). These loci were distributed over all 21 chromosomes with some variations, ranging from 3 to 11 per chromosome: 11 loci were placed on chromosome 3D, which was the highest number of loci on one chromosome, while only three loci were located on chromosomes 1D, 6A and 7D. These differences were not significant and were probably due to the low number of EST markers mapped at the time.

BLAST (see http://wheat.pw.usda.gov/wEST/) was used to compare the sequences of the mapped EST microsatellites against the ESTs assigned to wheat deletion lines during the course of the NSF programs. The e-value used was $1e^{-150}$ in order to conserve only those with a good homology. Only 31 ESTs matched significantly with one physically assigned EST. They all mapped on the same chromosome as reported by the NSF physical mapping project. For example, the SSR GPW7055 gave two bands, only one being polymorphic. The resulting locus *Xgpw7055* (Table 1) mapped in the ITMI population on chromosome 4A. This EST-SSR was 99% identical to EST BE590484 which was assigned to chromosomes 4AL and 7AS. Thus, the polymorphic locus mapped on one of the chromosomes where the EST was physically assigned, while the non-polymorphic band, which probably corresponded to the other locus, was assigned to chromosome 7A.

Among the 112 mapped microsatellites, only 11.8% gave more than one polymorphic locus. In most of the cases (91%), the detected loci were located in homoeologous positions. These results are in accordance with the mapping positions of the ESTs that had been previously physically determined. The remaining 9% were located on the same chromosome, suggesting a duplication of the sequence.

Table 1 EST-SSRs primer pair data and accession number, type of repeat and chromosome location

SSR	Accession no.	Motif	Forward	Reverse	Location
gpw7055	BE414053	$(CAA)_6$	CATGCAACAACAATCCCAAC	TGTCAACTGTCCTGCAACTG	4A
gpw7060	BE430341	$(GTT)_{10}$	CCTTATTGCCTCAAACTGGG	TCTGCATCAACAACACCACC	6D
gpw7072	BE398510	$(GCA)_6$-$(CAA)_6$	AATGCAGCGATGTCTTGCTA	ACTGTTGGGGTTGTTGTTGC	1A
gpw7079	BE398777	$(CAA)_9$	AGCTGGTGCCATTGTGTTGT	CCTGGCCTGATGGATATTGT	6D
gpw7082	BE399376	$(TGT)_6$	AGGTACGGAGTGCAATGGAA	CCCAACAGTCAGGTCAAGGT	1D

Discussion

As described by Holton et al. (2002), EST sequences could be a very good source of SSR markers for wheat and could therefore be related simultaneously to agronomically important characters. In order to improve the number of microsatellite markers on the ITMI map and also to map non-anonymous loci, we investigated SSRs present in wheat EST sequences. SSRs were detected in more than 7.5% of the contigs analyzed. Kantety et al. (2002) found a lower frequency of SSRs in ESTs of different species (from 1.5% for maize to 4.7% for rice), probably because they looked at SSRs which contained a minimum length of 18 bases in contrast to this study where they had a minimum length of 12 bases.

We did not observe significant differences among the coding regions (5′ or 3′) with respect to the frequencies of microsatellites (data not shown). Morgante et al. (2002) showed that 3′ and 5′ untranslated regions (UTRs) had a higher frequency of SSRs than the rest of the genome. However, this result was inferred from predicted open reading frames, while we used short expressed sequences for which we were unable to precisely identify their location (5′ or 3′). We were also unable to associate the occurrence of SSRs to a specific class of genes such as transcription factors or retroelements since about 40% of our ESTs did not significantly match (e value −50) with any known sequences (SwissProt and Trembl).

Thirty percent of the primers designed for the microsatellites did not amplify a product; this is rather high compared to the rate obtained with genomic sequences (15%, Guyomarc'h et al. 2002) but is identical to the one mentioned by Holton et al. (2002). The lack of amplification could be due to several reasons: (1) one of the primers designed from EST sequences could overlap two exons; (2) the primer pair encompasses a long intron producing a PCR product that could not be visualized on the electrophoretic profile; (3) sequence errors or problems during primer synthesis could occur; (4) as consensus sequences obtained from the compilation of several ESTs were used, some of them may result from the addition of different copies of the same gene (homoeologs or paralogs).

With respect to polymorphism, 53% of the primer pairs were polymorphic for at least one of the eight cultivars tested. Guyomarc'h et al. (2002) found a 55% polymorphism rate using the same genotypes and SSRs issued from the D genome, which is relatively similar to our results. However, the genomic sequences they used originated from *T. tauschii* and were mainly mapped on the D genome of bread wheat, which is known to be less polymorphic than the other two genomes (A and B); the polymorphism level among EST-SSRs (53%) compared to genomic SSRs (61%, Sourdille et al. 2001b) was lower. This is consistent with the results mentioned by Eujayl et al. (2002), who found that EST-derived SSRs were less polymorphic than those derived from other sources.

The dinucleotide motifs showed more polymorphism in both the ESTs (83%, this study) and the genomic sequences (79%, Guyomarc'h et al. 2002) than did the other motif types. This was surprising since increasing (or decreasing) the number of dinucleotide motifs would result in the modification of the reading frame of the gene and may even lead to a stop codon, which is not the case for the trinucleotide motifs. However, the dinucleotides may vary following a step of three motifs which do not modify the reading frame (3×2=6 bp=2 codons) or they may be located in 5′ UTRs as stated by Morgante et al. (2002).

The effect of sequence length on the polymorphism between di- and trinucleotide motifs was not significant in our investigation. The average number of repeats for polymorphic or non-polymorphic dinucleotide microsatellites was 15 and 12, respectively. The average repeat number for trinucleotide motifs was eight for both polymorphic or non-polymorphic SSRs.

EST-SSRs generally generated high-quality amplification products since 68% of the amplification products were scored as types A or B (Fig. 2). This is consistent with the results of Eujayl et al. (2002) and suggests that ESTs are more suited for specific primer pair design than genomic sequences. The lower quality obtained for genomic sequences could also be due to a large number of low-complexity repeated regions framing the microsatellites, which is consistent with the results of Ramsay et al. (1999) who showed that microsatellites are associated with cereal retrotransposon-like sequences.

We noticed that in some cases the amplification product was either smaller or larger than the expected product as determined from EST sequences analysis. When there was an increase in the product size (8.5% of the cases), this was probably due to the amplification of a small intron. If the intron was too long, the amplification product would either be out of the detection range or would even not be detected. For the 5% which had an amplified product smaller than expected, this may have resulted from: (1) a small deletion within the sequence framed by the two primers; (2) non-specific annealing of the primers because

of the low complexity of the flanking sequences; (3) duplication of the EST sequence in the genome on the same or on another chromosome; (4) the EST belonging to a multigenic family and the primers being designed in the conserved domain.

As was found for other species (Temnykh et al. 1999; Kantety et al. 2002), the most abundant dinucleotide motif was GA/CT, while the CG motif was not represented. Although $(AT)_n$ microsatellites are thought to be very abundant in the genomic sequences of plants (Lagercrantz et al. 1993; Morgante and Olivieri 1993), this was not the case in wheat EST sequences (16%) compared to $(GA)_n$ (45%). Presumably, this was due to the self-complementation existing for $(AT)_n$ microsatellites, making them less easy to isolate. Kantety et al. (2002) suggested that the high level of the GA/CT motif is due to its translation into Ala and Leu, depending on the reading frame, two very frequently occurring amino acids in proteins (Lewin 1994).

We found the trinucleotide motifs, $(CCG)_n$, to be very abundant in the wheat EST sequences, although Kantety et al. (2002) found that in wheat the most abundant trinucleotide motifs were AAC/TTG. However, our results are consistent with those of Morgante et al. (2002) and Murray et al. (1989) who found a high GC% level in the monocotyledonous genes. Among the ESTs analyzed, 21% were issued from seed tissues. In seeds, the most frequent amino acids are Pro and Gln (Feillet 1965). Pro is coded by CCG and Gln by CAA and CAG. These three motifs represent 60% of all the trinucleotide microsatellites we found in wheat ESTs. The difference between our results and those of Kantety et al. (2002) are probably due to the tissues used for EST production. The two motifs ATT/TAX and CTA/TAG were more poorly represented (0.54% and 1.36% respectively), presumably because they constitute a stop codon.

Among the 112 microsatellite primers genotyped on the ITMI population, ten amplified two loci and two amplified three loci. A low proportion of multilocus microsatellites was also mentioned by Gupta et al. (2002) for genomic sequences. In our case, all the multilocus markers were found to map on homoeologous chromosomes; for the two primer pairs giving three bands, the three loci mapped on the three homoeologues. This confirms that coding sequences, even if they are framing a microsatellite located within a gene, are well conserved between homoeologous copies. Most of the genes exist in three homoeologous copies, and this is also probably the case for those bearing a SSR. The fact that we observed only one mapped locus for 89% of the EST-SSRs may be due either to the lack of polymorphism of the other two loci or to the specificity of the primer pair designed. In this latter case, modifying a few bases or even changing the primer pair may lead to the amplification of the three homoeologous loci.

The in silico mapping results obtained through our BLAST analysis of EST sequences containing a microsatellite against the ESTs assigned on deletion lines are promising. Microsatellites developed from EST sequences that were previously mapped on deletion lines were located in the same deletion bin by segregation analysis. Microsatellites located in assigned ESTs and that have not been developed yet could be used as a potential source of markers. Mapping a large number of EST-SSRs could become very useful because genes of known function could then be related to phenotypic variation (Eujayl et al. 2002) and QTLs. Future investigations include using EST-SSRs for germplasm evaluation and linkage disequilibrium analysis.

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