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Development of SSR markers for the short arm of rye chromosome 1

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Introduction

Simple sequence repeats (SSR) are DNA sequence stretches in which a short motif (1-6 bp) is tandemly repeated. SSR gain and lose repeat units at high rates through a mutational mechanism called "DNA replication slippage" (Ellegren 2004). Primer pairs used for PCR reaction are designed for the highly conserved regions flanking the SSR, which may amplify fragments of varying length in different individuals, even within populations. Due to their ease of application, codominance and hypervariability, microsatellites have become the marker of choice in such diverse fields like genotype-identification, construction of genetic maps, QTL analysis, population-genetic studies, marker assisted selection (MAS).
The main disadvantage of SSR-markers is the required amount of labor and the high cost for their development. A recent review from Squirell et al. (2003) estimated the necessary effort to develop ten polymorphic SSR-markers: one hundred sequenced clones and thirty tested primer pairs. These values might even be higher for polyploid species with a large amount of repetitive DNA like wheat. We attempt to develop 100 SSR markers specific for the short arm of rye chromosome 1R, which is present in hundreds of wheat varieties world wide as a 1BL.1RS translocation. They will be mapped physically (bin) as well as genetically. Plant genomes are notorious for their low SSR frequency, therefore enrichment procedures are widely used for plant SSR-marker development (Zane et al. 2002). We present a nebulizer based SSR enrichment procedure.

The SSR development procedure

Prior to chromosome arm specific SSR development, chromosome arm specific DNA is required. At present this can only be accomplished with flow sorting of mitotic chromosomes. Mitotic chromosomes in suspension are stained with fluorescence dyes and passed through a flow cytometer. A detector determines the fluorescence intensity which correlates with DNA content and size of the chromosomes. Subsequently the chromosomes pass a sorter where they get separated according to their estimated size (Dolezel et al. 2004). For sorting 1RS chromosomes, Chinese Spring/Imperial ditelosomic 1RS addition lines have been used (CS+1RS''). Because 1RS chromosomes are clearly smaller than the remaining 42 CS chromosomes, they can readily be separated from the CS background with flow sorting.
The actual SSR development procedure is primarily based on the protocol of Kumpatla et al. (2004). The 1RS specific DNA has been physically sheared with a nebulizer into small fragments ranging from 200-500 bp. Nebulization, compared to enzymatic digestion, has the advantage that the resulting DNA fragments are randomly cleaved and not defined by restriction sites. Since nebulization also generates random fragment ends (sticky or blunt ends) standardization of the fragment ends is required. "Mung bean nuclease (MBNase)" creates blunt ended DNA fragment which can easily be ligated to blunt ended adaptors. PCR reactions are performed with primers complementary to the adaptor sequence. The necessity

for PCR amplification of the fragments arises from the significant DNA losses which occur during the washing steps after nebulization and MBNase treatment. The most crucial step in the whole SSR development procedure is the "Dynabeadfishing" (Figure 1). The baits are small 5'-biotinylated SSR oligo motif repeats (SSR-oligos) which hybridize to DNA fragments containing a sequence complementary to the oligo motif (eg.: oligo motif: GA; complementary SSR motif: CT; Figure 1A). After adding streptavidin coated paramagnetic "Dynabeads" to the reaction tube, the biotin of the SSR-oligos forms a very strong bond with the streptavidin of the "Dynabeads" (Figure 1B).

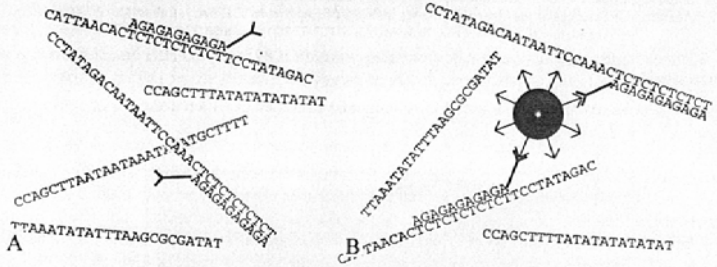

Figure 1. Principle of "Dynabeadfishing": A) 5'-biotinylated SSR-oligos are hybridized to DNA fragments containing a complementary SSR; B) Fragments hybridized to an SSR-oligo are bound to a streptavidin coated "Dynabead", whereas fragments without complementary SSRs remain unbound in the reaction tube.

Subsequent washing steps are performed utilizing a magnet. Fragments bound to a paramagnetic "Dynabead" are caught by the magnet and retained in the reaction tube whereas unbound fragments are washed away (Figure 1B). Thereafter "fished" fragments are ligated into the pSTBlue-1 vector and transformed into competent cells. After size screening of the clone library, fragments larger than 300 bp are sequenced, if necessary in both directions. Sequence quality after a long SSR deteriorates drastically. Primer pairs, for SSRs with a total length of at least 12 -15 nucleotides, are designed using the free available software Primer3 (Rozen and Skaletsky 2000).

1RS specificity of the primer pairs is tested with: flow-sorted 1RS specific DNA, genomic DNA of rye, CS+1RS'' and CS. 1RS specific markers have to amplify a discrete band on 1RS-, rye-, CS+1RS''-DNA but not on CS-DNA (Figure 2A). Testing for polymorphism is carried out with different rye varieties from diverse geographic origin (Figure 2B).

Preliminary results and further work

So far, we sequenced 500 clones from a partial library enriched for the motif GA, further 400 clones were sequenced from a library enriched for the motif CA.

From the 500 sequenced GA clones, until now 270 have been analyzed. As a first attrition only 180 clones contained an SSR. Because, as a second attrition, not every microsatellite has enough space in its flanking sequences for successful primer pair development, only 130 SSR primer pairs could be designed.

So far, 30 primer pairs have been tested: 17 showed a discrete band, 12 were 1RS specific and 7 showed in average 2 to 3 alleles. From the 13 primer pairs which did not show a discrete band (in most cases a smear), 10 were associated with the retrotransposon "Sabrina_231A16-1" (AY188331) or "Sabrina_115G1-1" (AF459639). More than 50% (23 from 44) of the perfect GA-micorsatellites were associated with the retrotransposon "Sabrina".

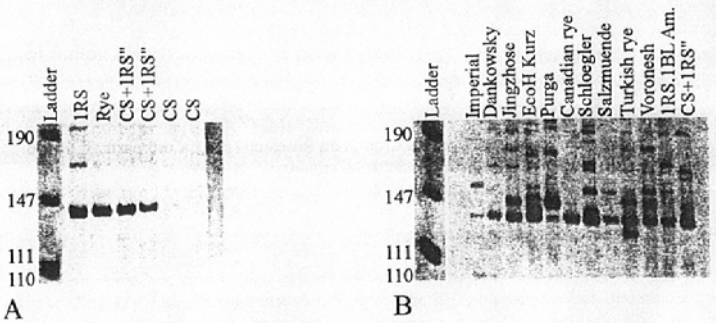

Figure 2. Testing of an SSR primer pair: A) Testing for 1RS specificity with DNA from flow sorted 1RS chromosomes, rye, CS+1RS'' and CS; B) Polymorphism in rye varieties of diverse geographic origin

Additionaly, 27 primer pairs have been developed from 1RS specific BAC end sequences. The BAC library was developed from 1RS specific DNA (Safar et al. 2006) and the BAC end sequences were kindly provided by Dr. Jan Bartos, Olomouc. Further 17 primer pairs were designed from rye ESTs homolog to wheat ESTs mapped to 1AS, 1BS or 1DS wheat chromosomes. Homology has been identified through a BLAST search by Dr. Maria Berenyi, Seibersdorf. Rye ESTs are available at the NCBI homepage through the GenBank sequence database (Benson et al. 2006). Primer evaluations are in progress.

Further work includes the analysis of the remaining sequences; construction of SSR enriched libraries for the motifs AAG, AAC and AT; and the testing of the SSR primer pairs for 1RS specificity and polymorphism.

A physical (bin) and genetic map will be constructed with all newly developed 1RS specific SSRs.

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