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Genomic and RNA divergences of *Revolver* transposon-like gene offer chromosome tags in Triticeae

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ABSTRACT

Revolver is a new class of transposon-like genes in the Triticeae genome[1,2]. By RT-PCR and TA cloning, *Revolver* cDNAs were obtained from self-fertile rye and several Triticeae species. The total lengths of the *Revolver* cDNAs are 665 to 723 bp, and they are classified into three sub-families wherein the regions of the second and third exons are almost identical, while the region of the first exon exhibits low homology of 60% among the families due to duplication or deletion. Such a length divergence in *Revolver* allows the development of rye chromosome markers. PCR was performed using the 3'-flanking region of *Revolver*-2 as a single primer, and 4 DNA fragments (2.3 kb, 2.8 kb, 3.3 kb and 4.3 kb) were amplified from the rye genome, but nothing was amplified from the wheat genome. These variants, assumed to be non-autonomous elements of *Revolver,* have the downstream region of the second intron, but they have structural modifications at the 5' side and first exon region as in the cDNAs. Moreover, when PCR was performed with the same primer using rye chromosome addition wheat lines, 4 DNA types were recovered from 1R, 5R, 6R and 7R addition lines, respectively. By using PCR primers comprising the sequences flanking each element of *Revolver* scattering in the genome, the chromosome on which each *Revolver* is located can be determined or tagged. Furthermore, an anchored AFLP approach, called Sequence-Specific Amplification Polymorphism (SSAP) or transposon display, was applied to exploit the variation in the sequences flanking the insertion site of *Revolver*. The regions between *Revolver* elements and adjacent *Eco*RI-cleaved host sites were amplified by a *Revolver* primer labeled with fluorescent dye and an *Eco*RI adaptor primer. The SSAP autoradiograph from an inbred rye and callus derived from it showed numerous DNA fragments including a few callus-specific SSAP, which means mobilization of *Revolver*.

INTRODUCTION

Revolver is a new class of transposon-like gene discovered in the Triticeae genome[1,2]. *Revolver* encompassing 3041 bp has 20 bp of terminal inverted repeated sequence at both ends and contains a transcriptionally active gene encoding a DNA binding-like protein. The entire structure of *Revolver* does not share identity with both the class I and class II autonomous transposable elements found[3,4,5]. Southern blot analysis in the Triticeae shows that *Revolver* is abundant in the genomes of a wheat relative, *Secale* (RR), and is present at intermediate levels in wheat ancestor species, *Triticum monococcum* (AA), *T. turgidum* (AABB) and *T. tauschii* (DD). However, *Revolver* is rare in the genome of bread wheat *T. aestivum* (AABBDD). These facts indicate that *Revolver* has existed since the diploid and tetraploid progenitor of wheat, and then has been lost from bread wheat[1]. The considerable quantitative change of *Revolver* among the wheat related species strongly indicates its propagation, activity and diversity in recent evolutionary times.

In this paper, genomic and RNA divergences of *Revolver* were analysed and then length variants of *Revolver*-non autonomous elements were utilized for chromosome tags. Furthermore, an anchored AFLP approach, called Sequence-Specific Amplification Polymorphism (SSAP) or transposon display, was applied to exploit the variation in the sequences flanking the insertion sites of *Revolver*. *Revolver*-based molecular markers have been detected by SSAP, which is the most popular transposon-based marker method.

MATERIALS AND METHODS

RT-PCR: The primers for amplification of *Revolver* cDNA were designed from both ends of a positive cDNA clone. Total RNA for RT-PCR extracted from seedlings was treated with DNase I. First strand cDNAs were synthesized by AMV reverse transcriptase (Life Science) using an oligo(dT) primer. Reaction mixtures contained 10 ng of template cDNA, 50 pmole of each primer (5’-GGCACGAGGGTACGAGTCCGAG-3’, 5’-GGCACAACTCATGTAAAAGAGGG-3’), 0.4 mM dNTPs, 1× LA PCR buffer Ⅱ, 2.5 mM $MgCl_2$, and 0.5 U of *LA Taq* polymerase (Takara) in a volume of 25 µl. The PCR reaction program consisted of 30 cycles of 30 sec at 95˚C, 30 sec at 63˚C, 1 min at 72˚C. The RT-PCR products were purified, ligated to the pGEM-T vector (Promega) and sequenced.

Genomic PCR: The single primer for amplification of *Revolver* genomic DNA was designed from the 3'-flanking region of a typical clone of *Revolver* (*Revolver*-2). Reaction mixtures contained 10 ng of template genomic DNA, 50 pmole of each primer (5’-GTAGTCGTCAGG-AGTCCTCACCA-3’), 0.4 mM dNTPs, 1× LA PCR buffer Ⅱ, 2.5 mM $MgCl_2$ and 0.5 U of *LA Taq* polymerase (Takara) in a volume of 50 µl. The PCR reaction program consisted of 30 cycles of 30 sec at 95˚C, 1 min at 70˚C, 3 min at 72˚C. The 4 types PCR products (2.3 kb, 2.8 kb, 3.3 kb and 4.3 kb) amplified from the rye genome and rye chromosome addition wheat genomes were purified, ligated to the pGEM-T vector (Promega) and sequenced.

SSAP: Genomic DNA was restricted with *Eco*RI and ligated with the *Eco*RI adaptor. selective amplification was carried out using the 17-bp *Eco*RI adaptor primers plus one selective nucleotide. The total 25 μl volume of reaction mixture contained 30 ng of template DNA, 0.8 μM of adaptor primer, 200 μM dNTPs, 1× LA PCR buffer II and 1 U LA *Taq* polymerase. Touch-down PCR was applied using the following conditions: 2 min at 95 C; 24 cycles of 30 sec at 94 C, 30 sec at 56 C; 1 min at 72 C. The final elongation step was done at 72 C for 5 min. Selective amplification was carried out using the preselected PCR product as a template. The preselective amplification product was diluted 10 times with distilled water. Selective *Revolver* primers were labelled at the 5' end with fluorescent dye. The labelled primers were combined with *Eco*RI adaptor primers extended with two selective nucleotides. The 10 μl mixture included 2 μl template DNA, 1 μM of labelled and 1 μM of unlabelled primer, 200 μM dNTPs, 1× LA PCR buffer II and 1 U LA *Taq* polymerase. The PCR conditions were 37 cycles of 30 sec at 94 C, 30 sec at 70 C (–1 C per cycle until 60 C); 1 min at 72 C. After PCR, the samples were loaded on 6% Long Ranger sequencing gel. The electrophoresis was carried out in 1× TBE at a constant power of 10 W.

RESULTS & DISCCUSSION

Structural diversities of Revolver mRNA

In order to characterize the diversity within *Revolver*, structural analysis of the cDNA clones was performed. As a result, the total lengths of typical *Revolver* cDNAs were 665 to 723 bp, and they were classified into three sub-families wherein the regions of the second and third exons are almost identical, while the region of the first exon exhibits the low homology of 60% among the families due to duplication or deletion.

Moreover, cDNAs exhibiting completely different structures at the first exon were found. Five cDNAs cloned from *Secale silvestre*, a wild species of rye, have a total length of 1,597 bp and contained the second intron (1,210 bp) and the third exon (301 bp) of *Revolver*, but at the 5' terminal they have an 86 bp sequence not observed in *Revolver*. The homology among the 5 clones was 96%. On the other hand, four cDNA clones cloned from rye cultivated species *S. cereale* have a total length of 395 bp, and lack the second intron compared to the above cDNA clones of 1,597 bp.

Meanwhile, another cDNA clone screened from a cDNA library of leaf (total length of 2,182 bp) has the second exon (90 bp) and the third exon (260 bp) of *Revolver*, but the region corresponding to the first exon is extremely long and it has no homology with the other cDNA clones. As above, the members of the *Revolver* family, having a common structure downstream of the second intron, are actively transcribed and various structural diversities are observed at the 5' end.

Chromosomal markers using Revolver

PCR was performed using the 3'-flanking region derived from a typical genomic clone of *Revolver* (*Revolver*-2) as a single primer, and 4 DNA fragments (2.3 kb, 2.8 kb, 3.3 kb and 4.3 kb) were amplified from the rye genome, but nothing was amplified from the wheat genome. The genome of rye and that of wheat can be distinguished by this primer easily. Furthermore, when PCR was performed with the same primer using genomic DNA from rye chromosome addition lines, DNA fragments of 2.8 kb, 3.6 kb and 4.3 kb were amplified from 1R, 5R and 6R chromosomal addition lines, respectively. By PCR with this primer, rye chromosomes 1R, 5R and 6R can be distinguished and identified. Each DNA fragment derived from chromosome addition lines of 1R, 5R and 6R and four types of DNAs amplified from the rye genome, were cloned and sequenced. As a result, all of them were non-autonomous elements of *Revolver* which have the second intron of *Revolver* and the downstream region thereof, but they have considerable structural changes at the 5'-end. Such clones were converted to STS on rye chromosomes.

Revolver-3 comprises a total length of 4269 bp, and at the 3'-end, it has a region of 2112 bp from the middle of the first intron of *Revolver* through the third exon and reaching to the 3' terminal region. At the 5' end, it has the homologous region of 150 bp including the inverted repeat sequence. However, in the 2 kb between these sequences, it lacks the region from the first exon to the middle of the first intron. However, it includes the region of 370 bp showing 65% homology to the sequence of LARD LTR-1 (4960 bp), which is regarded as solo LTRs of the non-autonomous retrotransposon element LARD (large retrotransposon derivative) in barley[6]. In the region from 631 to 2,176 bp at the 5' end of *Revolver*-3 and the region from 598 to 2,353 bp of LARD LTR-1, there exist short repetitive sequences occurring repeatedly and both clones exhibit 53% homology, but no homology was observed with typical *Revolver*. *Revolver*-3 and LARD LTR-1 have a unique consensus region at the 5’ end, and they exhibited a high homology in total. *Revolver*-3 was converted to STS on the 6R chromosome because it was amplified with the 3'-flanking region primer 5’-GTAGT-CGTCAGGAGTCCTCACCA-3’ of *Revolver*-2 only from the rye 6R chromosome addition line. *Revolver*-2 was located on the rye 7R chromosome because 492 bp of DNA was amplified only from the rye 7R chromosome addition line by PCR using the 5'-flanking region of *Revolver*-2, 5’-GCCTTTCGGCCTTCCTCTC-AGGCGG-3’, and its internal sequence of 5’-GTACTT-GGCATCGGTAGATGTTCGG-3’, as the primers.

The LARD LTRs include the region homologous to both 5' and 3' ends of *Revolver*. At the 5' terminus, LARD LTRs have the region of 123–149 bp homologous to upstream of the transcription initiation site of *Revolver*. At the 3' terminus, LARD LTR-1 has a region of 2112 bp showing 60% homology to *Revolver*, from the middle of the first intron to the 3' terminus of *Revolver*. However, no homology to *Revolver* was observed in 631–2176 bp of LARD LTR-1. As to the region of about 2 kb between these end regions, LARD

LTRs lack the region from the first exon to the middle of the first intron. LARD LTRs have sequences not present in *Revolver* inplace of the first exon of *Revolver* and result in non-coding sequences. *Revolver* and LARD LTR may be evolutionally related, but LARD LTR is a structural part of a LTR retrotransposon while *Revolver* is a single gene consisting of an exon-intron structure. Therefore, *Revolver* is distinguished from LARD LTRs, in which exon 1 and exon 2 of *Revolver* are replaced by different sequences and the coding region is not present, and whose autonomous element has never been reported.

Revolver-4 consists of 3,219 bp, and at the 3' end, it has the region of 1806 bp ranging from immediately before the second exon to the 3' terminal of *Revolver*. However, in the 1,413 bp at the 5' side, the region homologous to *Revolver* is limited to only 101 bp at the 5'terminal. Further, *Revolver*-5 has a total length of 2,665 bp, and at the 3' side, it has a region of 1,826 bp ranging from immediately before the second exon to the 3' terminal of *Revolver*. At its 5' side, the region homologous to *Revolver* is limited to only 37 bp at the terminal region, but the region of about 670 bp is homologous to *Revolver*-4. *Revolver*-5 was located on the rye 1R chromosome because it was amplified with the 3'-flanking region primer of *Revolver*-2 only from the rye 1R chromosome addition line.

Finally, *Revolver*-6 has a total length of 3,503 bp, and at the 3' side it has the region of 1,294 bp ranging from the middle of the second intron to the 3' terminal of *Revolver*, however, at the 5' side there is not a region homologous to *Revolver*, and 121 bp at the 5' terminal is homologous to *Revolver*-4 and *Revolver*-5. *Revolver*-6 was located on the rye 5R chromosome because it was amplified with the 3'-flanking region primer of *Revolver*-2 only from the rye 5R chromosomal addition line. Moreover, 2,973 bp of DNA specific for the 5R chromosome was certainly amplified using the internal sequences of *Revolver*-6, 5'-ATAGCTCCACTGTTGG-CTCCTCTTTC-3' and 5'-CATTCATCCAAAGAACA-CAGAGTCCG-3', as the primers. As the above, by the PCR primers comprising the sequences flanking to each element of *Revolver* scattering in the genome, internal sequences of such element or combination thereof, the chromosome on which each element of *Revolver* is located can be determined, and further such PCR primers can be utilized for detection and identification of the chromosomes.

Revolver SSAP

SSAP is an anchored AFLP approach that amplifies the region between a transposon-specific primer and a nearby cleaved restriction site, to which an oligonucleotide adaptor has been added. Three rye-specific *Revolver* sequences were used to design SSAP primers. These primers and restriction site-specific adaptor primers were used to establish SSAP markers for rye chromosome in a wheat background. *Revolver*-associated polymorphisms were assigned to rye chromosomes by scoring rye chromosome addition lines. In order to selectively amplify and label fragments containing *Revolver* transposons and the adjacent host sequence, two rounds of consecutive PCRs were performed.

Examples of SSAP autoradiographs for transposons are shown in Fig.1. The SSAP procedure based on the *Revolver* transposon amplified numerous products from genomic DNA from an inbred rye line and callus derived from it (Fig.1). The SSAP banding patterns were almost identical between plants and the callus lines derived from this line, but a few callus-specific SSAP products were also found (Fig.1). The SSAP products of the callus were cloned, and a colony transformed with plasmids containing 100-200 bp products derived from callus was selected for sequencing. This amplified fragments contained *Revolver* sequences at one end and a host restriction site at the other. The callus-specific SSAPs driven by *Revolver* mean mobilization of *Revolver*.

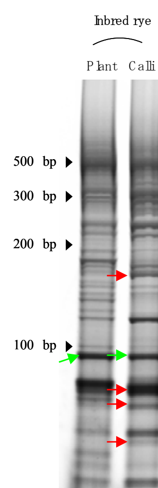

Fig.1. Examples of SSAP autoradiographs for transposon. The SSAP procedure based on the *Revolver* transposon amplified numerous products from genomic DNA from an inbred rye line and callus derived from it. The callus-specific SSAP products were identified.

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