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RYS1, a foldback transposon, is activated by tissue culture and shows preferential insertion points into the rye genome

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Abstract The study of two variable amplicons of rye indicates that *RYS1*, a mobile element, is activated during tissue culture. We propose that *RYS1* could be a foldback (FB) transposon. The FB transposons have been rarely reported in plants; *RYS1* is the first described in rye and also the first active plant FB transposon reported. Preferential integration points in the rye genome exist, because the new insertions seem to be located, in all studied cases, in the same genome positions. We assume that *RYS1* became active in rye very recently, as different plants from in vivo-growing cultivars showed that these elements were present or absent in the same genomic position in which the in vitro-activated element was found. This high rate of modification in these particular loci, both in the in vivo and in vitro populations, could indicate that probably the mechanisms promoting genetic variability in nature are the same that induce variation in vitro, and the modifications induced by somaclonal variation could be already present in vivo populations

Introduction

During the last few years numerous studies involved in the analysis of the so-called epigenetic variation have been carried out (Wolffe and Matzke 1999). In the meantime, in many cases with a direct relation to the above-mentioned studies, mechanisms of defence against 'invasive DNA' and silencing phenomena were identified (Matzke et al. 2000; Chandler and Vaucheret 2001). On the other hand, the sequencing of the genomes of different organisms has revealed the existence of numerous mobile elements which are now considered central players in the structure, evolution and function of plant genomes (Feschotte et al. 2002; Casacuberta and Santiago 2003). These elements constitute invasive DNA, and thus their activation must be carefully controlled and repressed through mechanisms that seem to be related, if not identical, to the ones implicated in other cases of gene silencing. However, the repetitive nature of some of them, as well as their different position on closely related genomes, indicate that, on some occasions, they were and are activated. The circumstances promoting their activation are not entirely known, but several kinds of stress have proved to be implicated. Plant tissue culture has been proposed as one of these stresses (Grandbastien 1998), and the activation of several elements under those conditions has been reported (Kaeppler et al. 2000). The mutations promoted by their activation accounted for, at least in part, the so-called somaclonal variation, a term introduced by Larkin and Scowcroft (1981) referring to the mutation phenomena observed on some occasions among the cultured cells and plants regenerated in vitro. As plant tissue culture is a method by which to obtain plant clones, the plants regenerated from the cultures derived from the same explant must be genetically identical, and therefore any genetic differences between them must be originated through mutations.

We have already shown that in rye (*Secale cereale* L.) the frequency of somaclonal variation is quite high (Linacero and Vázquez 1993). We have tried to ascertain the origin of the genetic changes utilizing RAPD analyses (Linacero et al. 2000), using the possible modifications of the pattern of amplified bands as a criterion to detect the variation, because for a particular primer these must be the same in all the plant clones as well as in the original explant. This study reveals the existence of hot spots of mutation: the same sequence varied in several plants obtained from different cell lines. These hypervariable bands were sequenced, and their se-

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quences compared with the ones present in the sequence databases. The study of these sequences was carried out in order to deduce which type of variation occurred, and thus to advance the knowledge of the mutational events involved in the promotion of somaclonal variation in rye. The analysis of two sequences, C20a and C20b, revealed the implication of a mobile element, a foldback (FB) transposon, in promoting the variation. An important feature which gives a special relevance to these events is that the new insertions seem to be located in the same genome positions. These preferential integration points could explain the hot spots of mutation previously detected in rye (Linacero et al. 2000).

Materials and methods

Plant material

Rye (*S. cereale* L.) regenerated plants were obtained from cell lines derived from immature embryos (cultivars Ailes and Merced), following Linacero and Vázquez (1993). Cell lines were constituted for all the calli derived from the same embryo.

RAPD analysis

The DNA was isolated from leaf tissues following the Dellaporta et al. (1983) method, with some modifications, such as phenol chlorophorm extraction. The RAPD reaction conditions were similar to those described by Williams et al. (1990) using the primer OpC20 from Operon Technologies. The 25-µl reaction mixtures contained 25–30 ng DNA, 0.20 µ*M* primers, 200 µ*M* of each dNTP and 1.25 U Amplitaq DNA polymerase Stoffel fragment (PE Biosystems, Foster City, Calif., USA) or Dinazyme (Finnzymes, Espoo, Finland) with the corresponding buffer and $MgCl_2$. The PCR products were analysed by electrophoresis in a 2% TAE agarose gel and observed under UV light after staining with ethidium bromide. DNA from the variable bands was recovered from the gel (GENECLEAN Kit; Bio 101, Vista, Calif., USA) and cloned into pCR2.1 vector (Invitrogen, Carlsbad, Calif., USA).

PCR analysis

Two oligonucleotides, $C20_1$: 5′TCTTAAACCCTTCTCTTCATAGAG and $C20_2$: 5′TGTGTAGTGGGAATATGAAGTGTT, were designed to amplify an internal fragment of the hypervariable sequences C20a and C20b. The PCR reactions were carried out using DNA obtained from regenerated and in vivo grown population plants. The 25-µl reaction mixtures contained 100 ng DNA, 0.20 µ*M* primers, 200 µ*M* of each dNTP and 1.25 U of *Taq* DNA polymerase (AmpliTaq Gold; PE Biosystems, Foster City, Calif., USA) with the corresponding buffer and $MgCl_2$. PCR conditions consisted of an initial activation of *Taq* polymerase at 95°C for 8 min, followed by 35 cycles at 95°C for 1 min, 55°C for 2 min and 72°C for 2 min. The PCR products were analysed by electrophoresis in a 1.5% TAE agarose gel and observed under UV light after ethidium bromide staining.

Southern blot hybridisation

The PCR-amplified DNA was run in a TAE agarose gel and then transferred to a nylon membrane (Hybond-N^+; Amersham Pharmacia Biotech, Pisacataway, N.J., USA). Likewise genomic DNA, 10–15 µg, was digested with different restriction enzymes, electrophoresed in a 0.8% TAE agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech). The DNA probes were labelled with [^{32}P]-dCTP (3,000 Ci/mol, ICN) using the random prime labelling systems Rediprime II (Amersham Pharmacia Biotech). The radiolabelled probes were hybridised in the presence of 5X SSPE, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 62°C. Washes were performed at 62°C in 2X SSPE, 0.1% SDS; 1X SSPE, 0.1% SDS and 0.1X SSPE, 0.1% SDS. Autoradiography was carried out at 70°C, and the blots were exposed to Amersham Pharmacia Biotech MP hyperfilms with intensifying screens.

Sequence analysis

In all cases, the bands isolated from two plants regenerated from different cell lines, or from plants growing in vivo, were analysed. The studied DNA fragments were sequenced using the Applied Biosystems Prism DNA sequencing kit, and the Applied Biosystems ABI 373 Automated DNA Sequencer at the UCM Genomic CAI (Centro de Apoyo a la Investigación Genómica).

These sequences were compared with the ones present in the sequence databases using BLASTN at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and FASTA at the European Molecular Biology Laboratory (http://www.ebi.ac.uk) programs. The studied sequences have been registered in the GenBank database under accession numbers AY196348 (C20a) and AY196349 (C20b).

Results

The C20a and C20b sequences were two of the amplification products obtained when the oligonucleotide OPC20 was used as primer for RAPD (Linacero et al. 2000). The C20a band (455 bp) was smaller than the C20b band (1,362 bp). Plants obtained from embryo-derived cell lines were studied (Table 1). In most cases (16 lines), all the plants regenerated from one line

Table 1 Regenerated plants with or without the C20a and/or C20b bands

Cultivar	Cell line	Number of plants			Variable lines
		C20a band	C20b band	No band	
Merced	A + B + C + T	0	16	0	–
	D	**6**	**10**	**0**	+
	O	**3**	**1**	**0**	+
	R	4	0	0	–
	S	0	0	4	–
Ailes	L	0	4	0	–
	N + I + K + G	17	0	0	–
	E	0	5	0	–
	F	**1**	**6**	**0**	+
	H	**5**	**1**	**0**	+
	M	2	0	0	–
	U + V + W	0	0	12	–
Total	20	38	43	16	4

Plants regenerated from embryo-derived cell lines in which the band C20a, C20b or none of them appeared in the RAPD pattern. Each line was derived from a single embryo. The variable lines are indicated in *boldface*, among them, some of the regenerated plants presented the C20a band, whereas in others, the C20b band appeared; hence, two different patterns were present in plants originated from one embryo

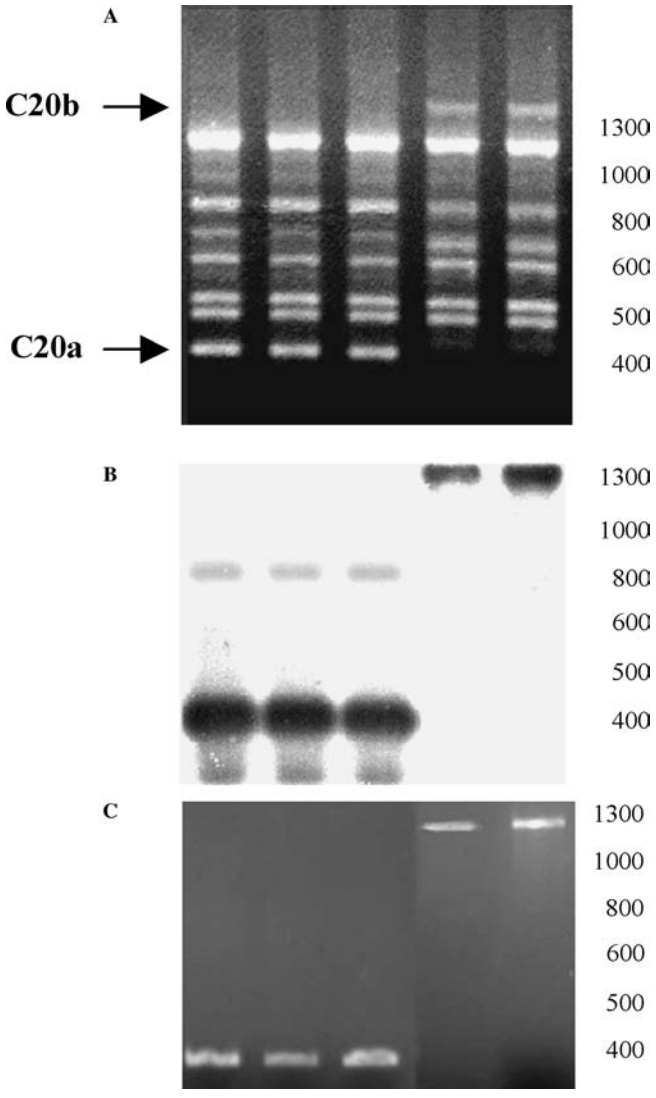

Fig. 1 **a** The RAPD pattern obtained from plants regenerated from one embryo cell line with the OpC20 primer. The *arrows* indicate the polymorphic bands: C20a and C20b. The band C20a was excised from the agarose gel and cloned. **b** Southern blot from the same gel, using the C20a band as a probe. **c** Specific amplification from the same individuals performed with the primers $C20_1$ and $C20_2$

showed the same RAPD pattern, either the C20a band or the C20b band or neither of them were present. However, in four lines, plants originated from a single embryo showed a different pattern: either the C20a band appears and the C20b band was absent, or vice versa (Fig. 1). The variable bands were present in different clonal plants derived from different cell lines, so they must have originated from independent mutation events.

Three C20a and three C20b bands amplified from plants regenerated from the D, F and O lines (one C20a and one C20b from each line), were sequenced. No differences were found between the nucleotide sequence obtained in the three replicates. The comparison between the sequence data of both bands indicated that the longer one showed the same sequence as the shorter one, but was interrupted by an insertion of 898 bp (Fig. 2). Several features of these sequences were relevant:

- The two borders of the internal sequence showed a direct repeat of the sequence 5′-ATAAATTAT-3′. This motif appears only once in the short band (position 277–285).
- The internal sequence has potential to form a secondary structure as predicted by the fold program of UWGCG package (Fig. 3). Several copies of short repeating units are situated along the sequence (Table 2).
- The internal sequence is highly represented in the genome (Fig. 4), whereas the C20a band is a single-copy sequence (Linacero et al. 2000).
- This internal sequence does not contain any obvious open reading frame.Two specific primers, $C20_1$ and $C20_2$, located near the borders of the C20a and C20b sequences (Fig. 2), were used to amplify the DNA of the previously studied plants from the variable lines. In each case the expected bands were obtained, the 376 bp band in the plants possessing the C20a sequence and the band of 1,293 bp in those with the C20b sequence (Fig. 1).

The presence or absence of these bands in the in vivo grown plants of the cultivars Merced and Ailes was also studied. The RAPD analysis of the DNA from these plants was performed using the OpC20 oligonucleotide as primer. Out of 54 plants of cultivar Merced, 30 presented the C20a band; eight, the C20b band; two, both bands; and 14, neither of them. In the case of cultivar

Table 2 Repeat motifs within the *RYS1* sequence

Motif (consensus)	Number of repetitions
GCTACAACC	28
TAGAAGCTTTT	18
CCGGCGGGA	16

Motifs represented in a high number of repetitions. Other motifs with a lower number of copies are not indicated

Ailes, of 26 plants studied, 12 had the C20a band; four, the C20b; and ten neither of them.

Discussion

The *RYS1* transposon element is present within the C20b sequence

We found that some of the clones showed different RAPD patterns: plants with the C20a and others with the C20b bands were regenerated from the same cell line. In order to confirm that in both cases we were observing the same locus, and to avoid any other possible interpretation, we performed direct PCR using locus-specific primers $C20_1$ and $C20_2$. The C20a is a single-copy sequence, as Southern hybridisation indicates. The specific primers, designed within this unique sequence, amplified only one band of the expected size in the variable plants. Even though the internal sequence in C20b is repetitive, both C20a and C20b corresponded to the same rye locus. The sequence data of C20a and C20b indicate that the sequence interrupting C20b represents a mobile element, the insertion of which has caused the C20a sequence to lengthen. We have called this element *RYS1*. *RYS1* shows some characteristics of the FB transposons, which are present in animals and plants, although they have been rarely referred to in plants. The FB transposons exist as structurally diverse families within the same species. These elements, with high copy numbers in the genomes, may form an extensive secondary structure, most of them do not contain protein-coding sequences, they are flanked by short repeats (target-site duplications), of approximately 7–9 bp, and their size ranges from 0.5 to several kilobases (Rebatchouk and Narita 1997; Windsor and Waddell 2000). All these features are met by *RYS1*, although its modular structure is more degenerate than in other FB elements.

We found a partial similarity between the internal sequence of C20b and the *WIS1* sequence of wheat. *WIS1* (Martienssen and Baulcombe 1989), was described as a stem-loop sequence with features of transposable elements, including target site duplications (TSDs) and terminal inverted repeats. In this case the repeat found in the target site was 9 bp long, equal to the length of *RYS1* repetition. *WIS1* also presented a modular organisation, and it is able to form a secondary structure. We propose that *WIS1* represents a FB transposon belonging to a wheat FB family and *RYS1* might probably be a member of the corresponding homologue family in rye.

Similar to other FB elements, *RYS1* has no coding capacity, but we observed that it was able to transpose because one of its copies was located in not the same position in plants regenerated from the same cell line. Because it is not possible to account for an endogenous enzymatic activity involved in their mobility, this must be explained by the use of *trans*-acting factors. Two possibilities have been pointed out (Rebatchouk and Narita 1997): FB families contain a small number of 'complete' transposable elements which support transposition of 'defective' elements, or they could take advantage of a transposase encoded by a non-FB element. In any case, the mechanism of transposition must be different from these postulated for other transposons, because no perfect inverted repeats, the TIRs, are present at the termini of this element, and also, we did not find the dyad symetry observed in the *FARE* elements, which like *RYS1* do not present TIRs at their ends. In any case little is known about the mechanism of transposition of FB elements (Windsor and Waddell 2000).

In the case of other FB elements described in plants, no reference to their activation has been provided, our data being the first reported, but the presence or absence at specific loci in different genotypes of the same species, such as *FARE* in *Arabidopsis thaliana* (Windsor and Waddell 2000), or related species, as *SoFT* in Solanaceae (Rebatchouk and Narita 1997), suggested that they were recently capable of transposition. Another indication of a recent transposition is the presence of a perfect target duplication. In our case the duplication found at the borders of the *RYS1* element was perfect.

We do not know if the element entered or were excised, because we could not ascertain its original position before tissue culture. Rye is an allogamous species

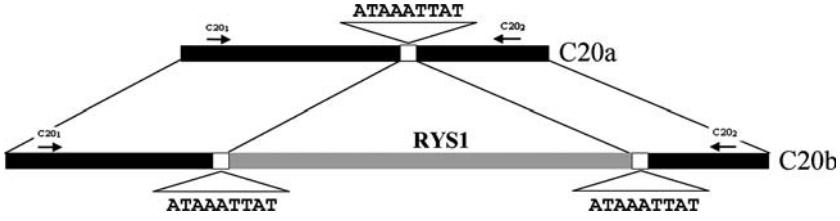

Fig. 2 Schematic diagram of the insertion of the *RYS1* element (*grey box*). Identical nucleotide sequences are indicated by *black boxes*. The insertion and the duplication of its target site are indicated by *white boxes*. The *arrows* indicate the primer's positions in both sequences

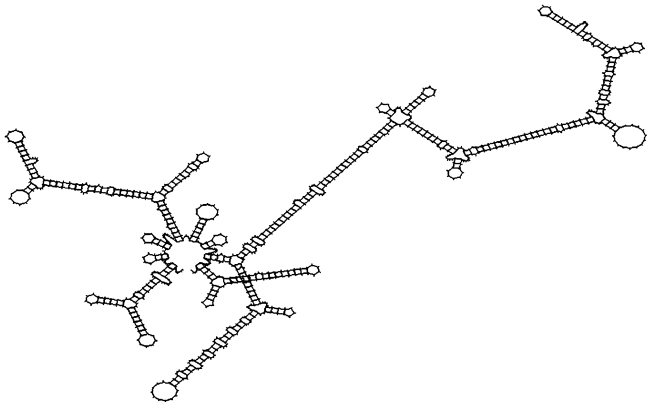

Fig. 3 Prediction of the secondary structure within the *RYS1* element using the Fold program (UWGCG). The free-energy value is –148 kcal mol^{-1}

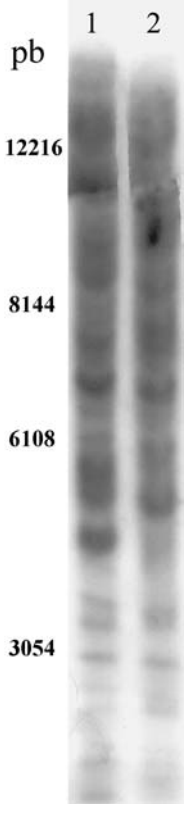

Fig. 4 Hybridisation pattern of the *RYS1* element. DNA isolated from plants of the in vivo population of the Ailes (*1*) and Merced (*2*) cultivars was cut with *Eco*RI, run in a gel and transferred to a membrane to perform the Southern blot hybridisation. The cloned *RYS1* element was used as probe

and highly variable, and therefore each embryo has a different genotype. The embryos generating the embryogenic cell lines in rye are quite small, less than 0.5 mm, so no tissue was left with which to study the element position prior to tissue culture. If the element were excised during tissue culture, the original sequence present in the embryos was C20b, and the C20a sequence appeared. However, if the initial embryo presented the C20a sequence the element was inserted and the new sequence was C20b. It is known that transposons excise by a slightly imprecise mechanism, leaving small sequence changes at the former position, the so-called 'transposon footprints' (Scott et al. 1996). The comparison between the C20a and C20b sequences indicate that C20b includes a perfect duplication of the target sequence in the region flanking the element, a sequence which is present only once within the C20a sequence. If the modification were the elimination of the element, we would be dealing with one of the rare occasions where transposons excise very precisely not leaving a footprint. Sequence data of bands, in plants derived from three different lines (D, F and H), show that three independent excision events would have left the original sequence unaltered. Precise excisions have been demonstrated for FB elements of *Drosophila* which have a high frequency of such excisions, more than 1 in 10^3 chromosomes (Collins and Rubin 1983). It is noticeable that, as in our case, the TSDs of these FB elements comprise 9 bp, and these authors refer to two prokaryotic transposable elements, which are structurally reminiscent of FB elements, and which also create 9-bp duplications on insertion. Accordingly, it is possible that *RYS1* could be excised in our cultures, leaving no footprint, but we cannot eliminate the possibility of a new insertion.

Of particular interest are the high frequency of *RYS1* activation and its preferential locus of excision/insertion. Out of 97 regenerated plants obtained from 20 cell lines, derived from 20 different embryos, four showed activation of *RYS1*; therefore in our conditions this element was quite active. Remarkably, in all plants in which *RYS1* was excised, or inserted, the same locus was involved because the sequences flanking the element were identical. Since C20a band is a single-copy sequence, this indicates a preferential point of insertion or a particularly high mobility of the *RYS1* element present at this point. Obviously we are not excluding the possibility of activation of other *RYS1* elements present in other points of the genome, because due to our experimental approach we only know the behaviour of the element which is inserted at a particular point, the C20a sequence. Rebatchouk and Narita (1997) suggested that phenomena similar to those described in *Drosophila* may exist in plants, and an FB element might flag a particular locus as a potential mutational hot spot. For other plant transposons there is a strong preference to insert into low-copy sequences (Raizada et al. 2001; Jiang et al. 2003). Dietrich et al. (2002) reported non-random insertion of *Mu* in several positions in the maize genome, and especially those situated within the *gl8* locus in which the nucleotides at positions directly flanking the 9-bp TSDs were conserved. Also the *PIF* elements of maize (Walker et al. 1997) have a strong target site preference for the *r* locus in maize in which they are inserted into a conserved target sequence. In rice, a MITE (miniature inverted-repeat transposable element), the *mPing* element, was

active in the slender glume (*slg*) mutant, and the excision of *mPing* from the *slg* plants resulted in reversion to a wild phenotype (Nakazaki et al. 2003). However, in most of the cases the excision produced a footprint at the deletion site. *RYS1* behaves in a similar way at the studied locus, but its precise insertion or deletion, exactly between the same two nucleotides, makes its behaviour quite unique and only partially similar to the cases of *Mu*, *PIF* and *mPing* elements mentioned above.

RYS1 is also present in the studied loci in the in vivo cultivated populations of rye

Plants from in vivo grown populations of cultivars Merced and Ailes were studied to find out if the *RYS1* element is present or not at the same genomic position at which the activated elements were found. We observed plants with the C20a band, with the C20b band, with both bands or without either of them indicating a high variability within the cultivars.

The criteria for a recent mobility of an element are the presence or absence at different loci in different cultivars or ecotypes within a species. Hence we assume that *RYS1* became active very recently. The high variability within the studied locus in the in vivo and in regenerated populations suggest that probably the same mechanisms operating in nature which promote genetic variability are the same as those inducing the variation found in vitro. Thus, in vitro stress, as we have already pointed out (Linacero et al. 2000), only increases the rate of mutation, and the types of variations observed are the same ones that appear naturally.

A more extensive study, now underway, of the new insertions of this element could provide us with valuable information concerning the mechanism(s) of FB activation, and its implication for rye genome evolution.

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