DOI: 10.1007/s10535-015-0531-0 BIOLOGIA PLANTARUM 59 (4): 637-644, 2015

Analysis of the flanking sequences of the heterochromatic JNK region in *Secale vavilovii* chromosomes

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Abstract

The JNK motif is repeated 4 000 times in an additional heterochromatin band. Flanking sequences of a JNK region, which form extra heterochromatin in 2R rye (*Secale vavilovii* Grossh.) chromosomes, were studied using a genome walking technique. The results clearly indicate that there were blocks of JNK sequences adjacent to the R173 family of repeated sequences. Moreover, it appears that the R173 are sequences flanking in both directions, *i.e.*, upstream and downstream. Downstream, the R173 is adjacent to the JNKs in an anti-parallel orientation, whereas upstream it is adjacent in a parallel orientation. In order to confirm the presence of the R173 sequence, fluorescence *in situ* hybridization was carried out. Using both JNK and R173 molecular probes, overlapping hybridization signals in the 2RL pair of chromosomes were observed, indicating an identical location of the two sequence elements.

Additional key words: FISH, genome walking, repeated sequences, rye.

Introduction

A detailed cytogenetic analysis of *Secale vavilovii* chromosomes revealed, that in some plants, an additional heterochromatic band on the 2R chromosomes is present. Further studies demonstrated that this band is made of JNK sequences which were previously described by Nagaki *et al*. (1999) in *Secale cereale*. A JNK region is made up of highly methylated tandem repeats consisting of 1 200 bp repeated 4 000 times in an additional heterochromatin band (Nagaki *et al*. 1999, Rogalska *et al.* 2002a). In plants with no additional heterochromatin band on the 2R chromosomes, the JNK sequence is dispersed throughout the genome, where it occurs in 20-fold repeats (Nagaki *et al.* 1999). Recent studies have revealed a high similarity of the JNK sequence to a part of the nucleotide 5S rRNA sequence derived from *Hordeum chilense* and *H. marinum,* and also to the *Angela speltoides* retrotransposon (Achrem *et al.* 2010). These results may indicate the presence of an element, initially mobile, which caught the 5S rRNA nucleotide sequence and then lost its ability to transpose. Such additional 5S rDNA and 45S rDNA loci have also been observed in *A. speltoides* in both the somatic and generative tissues; these additional loci occur in conjunction with clusters of *En/SPM* retrotransposons. In addition, a reduction in the "normal" 45S rDNA block, near which an *En/SPM* cluster is detected, is observed (Raskina *et al.* 2004). It is believed that 5S rRNA genes can be carried in the genome, although the process of transposition and spreading these genes is not fully understood. Nevertheless, their presence can result in the formation of pseudogenes (Martins and Wasko 2004). The majority of pseudogenes are formed as a result of the loss of part of a gene sequence. The 5S rDNA pseudogenes and their variants frequently appear in both animals (*e.g.*, Martins *et al.* 2002) and plants (Van Ryk *et al.* 1990, Gniadkowski *et al.* 1991). This gives rise to the question of how many non-functional genes an organism can tolerate and whether such genes affect its vitality. Therefore, the area of an atypical heterochromatic band can also comprise a complex of defective genes or mobile elements that have collected at one location on the 2RL chromosome (Achrem *et al.* 2010).

Studies involving various species of the genus *Secale,* including *S. cereale* and *S. vavilovii,* have revealed that certain types of plants tend to be eliminated from the studied population. There is no indication that these plants are infected by any disease or infested by pests. Cytogenetic observations during these studies revealed

Submitted 10 October 2014, *last revision* 8 April 2015, *accepted* 28 April 2015.

Abbreviations: DAPI - 4′,6-diamidino-2-phenylindole; FISH - fluorescence *in situ* hybridization; SSC - saline sodium citrate; TBE - Tris-borate-EDTA.

Acknowledgments: This work was financially supported by the National Science Centre, Poland (Grant No. NN 310 436038).

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telomere and intercalary heterochromatin polymorphisms and the presence of an additional heterochromatin band on the long arm of one or both of the 2R pair of chromosomes (Nagaki *et al.* 1999, Rogalska *et al.* 2001). This additional band is located at a distance ranging from 1.95 to 2.08 μm from the centromere, and it is approximately 0.65 μm in size (Rogalska *et al.* 2002a). To obtain seeds from plants with an additional heterochromatin band on both 2R chromosomes is difficult: such plants are weak, lack vitality, and have a substantially lower fertility, and thus this type of a plant tends to die out quickly. Investigation of meiosis in plants, in which an additional heterochromatin band is observed, does not reveal any abnormalities in chromosome pairing (Rogalska *et al.* 2002b). However, study of the mitochondrial genome of *S. vavilovii,* which contains an extra heterochromatin band, revealed a change in *atp1* gene. This gene encodes α F1 subunit of ATPase which is involved in ATP synthesis. Mutation in this gene may be reflected in the conformation of the subunit, and thus in the quantity of synthesized ATP (Skuza and Rogalska 2012).

Heterochromatin has the capacity to spread to adjacent chromatin regions unless prevented by barrier sequences acting as insulators. If there are no chromatin barriers, heterochromatin can exert a repressive effect on adjacent genes and thus block their activity (Eissenberg and Elgin 2000, Grewal and Elgin 2002).

The analysis of the sequences adjacent to the additional band can verify a hypothesis that the expansion of heterochromatin may be responsible for silencing certain genes, which then leads to reduced vitality and fertility in particular plant specimens. To become acquainted with the JNK region flanking sequences, we used a genome walking technique. This technique is carried out on uncloned DNA and can identify fragments of up to approximately 4 kbp in a single experiment, and sequences can be determined in both the 3' and 5' regions of the specific DNA fragment (Xu *et al.* 2013). The genome walking technique is used to identify regulatory regions and introns and to map regions within which sequences have been inserted by retroviruses and transposons (Huang *et al.* 2000, Terauchi and Kahl 2000, Chawla and DeMason 2003, Yan *et al.* 2003, Levano-Garcia *et al.* 2005, Leoni *et al.* 2008). Furthermore, in order to verify the co-localization of the identified sequences and the JNK repeats, we carried out fluorescence *in situ* hybridization with these sequences as probes.

Materials and methods

Plants: The flanking sequences of a JNK region were in the chromosomes of inbred lines of rye *Secale vavilovii* Grossh. cv. 52 and 225 zcz. In this rye populations, there were atypical characteristics in some plants, *i.e.*, mosaic coloration of anthers and caryopses. By self-pollination of these plants, inbred lines were created. Seeds were germinated in darkness for 5 d at room temperature on wet filter paper in Petri dishes.

DNA isolation: The coleoptiles of etiolated rye seedlings were thoroughly ground in a mortar and pestle in liquid nitrogen. The ground tissue was processed for total DNA extraction using a *DNeasy* plant mini kit (*Qiagen*, Valencia, USA) following the manufacturer's instructions. The purity and quantity of the extracted DNA was evaluated using a *NanoDrop 2000c* spectrophotometer (*Thermo Scientific*, Vilnius, Lithuania). The size of the genomic DNA was assessed by 0.8 % (m/v) agarose gel (*Sigma-Aldrich*, St. Louis, USA) electrophoresis in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3).

Genome walking procedure was performed according to the *Clontech* (Mountain View, USA) standard protocol. Briefly, in order to construct genomic libraries, DNA was digested with four restriction enzymes (*Dra* I, *Eco*R V, *Pvu* II, and *Stu* I) which were included in a *Genome Walker*™ universal kit (*Clontech*) and two other enzymes (*Smi* I and *Mss* I) (*Thermo Scientific)*. The next step involved DNA purification followed by ligation of adaptors (included in the kit).

For PCR reactions, six JNK-specific primers were designed (Table 1). Each primer was oriented in the reverse direction in order to amplify a region of an adjacent unknown sequence - both upstream and downstream from the JNK sequence. Primers were

Table 1. JNK-specific primers used in genome walking procedure.

Name	Sequence (5'→3')	Region in JNK	Direction of amplification
revGW1	TCCGCCCAGATCGAGGCTCGCCTGAATGAA	488 - 517	upstream
revGW2	CGACAAGTGGCAGTGCGAATCTTGGCGCA	161 - 189	upstream
revGW3	TCAGAGAGTGGCGTTTTGGCACACGGATGC	18 - 47	upstream
forGW1	TTCATTCAGGCGAGCCTCGATCTGGGCGGA	488 - 517	downstream
forGW2	TTCCTTGGTTGCCACTTGCCAGGATGGGAG	611 - 640	downstream
forGW3	GTCGGCATCCACAGCTCCGGCTTCAGAGAT	816 - 845	downstream

Table 2. The combinations of JNK-specific primers (JNK) and adaptor-specific primers (AP) used in primary and secondary PCRs.

No.	Primary PCR		Secondary PCR	
	JNK	AP	JNK	AP
1	revGW1	AP-1	revGW2	AP-2
2	revGW2	AP-1	revGW3	AP-2
3	forGW1	AP-1	forGW2	AP-2
4	forGW2	AP-1	forGW3	AP-2

designed with the *FastPCR* software *v. 6.2.89* (Kalendar and Schulman 2011), using an inverted PCR option, for a JNK sequence (clone pScJNK1 - AB008922.1; the length of the sequence 1 192 bp). All six libraries were amplified using a set of JNK-specific primers and adaptor-specific primers (AP-1 and AP-2 which were included in the kit). The combinations of primers used in primary and secondary PCRs are shown in Table 2. The final concentration of reagents in a 0.05 cm^3 reaction mix was: 1× *Advantage 2* PCR buffer, 0.005 M dNTP, 5 µM adaptor-specific primer, 5 µM JNK-specific primer, and 1× *Advantage 2* polymerase mix (*Clontech*). The master mix (0.049 cm^3) was added to 0.001 cm^3 of the DNA library. The temperature profile of PCR is shown in Table 3. The concentration of reagents in the secondary PCR was the same as in the primary PCR, and 0.049 cm^3 of the master mix was added to 0.001 cm^3 of a 50 × diluted primary PCR product. Positive and negative controls were included in each experiment.

Table 3. Primary and secondary PCR parameters used in the genome walking protocol.

Primary PCR			Secondary PCR		
number of cycles	temp. [°C]	time [s]	number of cycles	temp. [°C]	time [s]
7	94	25	5	94	25
	72	180		72	180
32	94	25	20	94	25
	67	180		67	180
1	67	420	1	67	420

For gel electrophoresis, 0.020 cm^3 of each sample was loaded into the agarose gel well. The samples were electrophoresed at 80 V in a 1.5 % (m/v) agarose gel in 1× TBE for 3 h. The gel contained a *Midori Green* DNA stain (*Nippon Genetics*, Duren, Germany) at a concentration suggested by the manufacturer. The PCR products were visualized under UV radiation using a *GelDoc XR* system (*BioRad*, Hercules, USA). The sizes of PCR products were determined by comparing them with a *Massruler*™ DNA ladder mix (*Thermo Scientific*) using the *Quantity One*® software (*BioRad*).

Appropriate DNA fragments were excised from the gel and purified using a *PrepEase*® gel extraction kit (*Affymetrix*, Cleveland, USA) according to the manufacturer's instructions.

Sequencing was performed at *Macrogen* (Amsterdam, Netherlands) with each reaction being repeated three times. The sequences were analyzed using the *BLASTN 2.2.26+* (Zhang *et al.* 2000) software.

Fluorescence *in situ* hybridization (FISH): Mitotic chromosome preparations were obtained from root tips of germinating seeds. Roots were treated with 0.05 % (m/v) colchicine (*Sigma-Aldrich*) at 16 °C for 3 h and then fixed in 99.8 % ethanol and 99.5 % acetic acid mixture (3:1, v/v, 4 °C) for 24 h. The roots were macerated in a mixture of 4 % (m/v) pectinase, 3 % (m/v) hemicelullase (both from *Aspergillus niger*, *Sigma-Aldrich*), and 1 % (m/v) cellulase (from *Trichoderma viride*, *Sigma-Aldrich*) in a 0.01 M citric acid - sodium citrate buffer (pH 4.8), at 37 °C for 3 h. Each root tip was squashed in a drop of 45 % (m/m) acetic acid on a glass slide. The cover slip was removed after freezing over dry ice. The slides were air-dried overnight. The preparations were incubated with RNase (DNase-free, 10 µg cm^{-3}) (*Sigma-Aldrich*) in a 2× SSC buffer (0.03 M Na citrate, 0.3 M NaCl, pH 7.0) at 37 °C for 1 h. The slides were then washed twice in 2× SSC buffer at 37 °C for 5 min. The slides were incubated in 0.12 % (m/v) pepsin (*Carl Roth*, Karlsruhe, Germany) at 37 °C for 15 min and washed twice in a 2× SSC buffer for 5 min. The preparations were dehydrated in a graded ethanol series (70, 90, and 99.8 %, v/v) at room temperature.

JNK and R173 probes were both labelled using a PCR method. The JNK primers were designed (the *Primer3* software; Rozen and Skaletsky 2000) using a sequence from *S. cereale* deposited in GenBank (AB008922.1). A pair of primers (JNKA: CACAGACCTTGGAATCG TGA, and JNKB: TCCGAGTTCGTATGCAAAAGT) were used to amplify a 934 bp product. PCR labelling was carried out in a total volume of 0.025 cm^3, containing 0.2 µg of *S. vavilovii* total DNA, 1.2 µM each oligonucleotide primer, 240 µM dATP, dCTP, and dGTP, 150 µM dTTP, 40 µM biotin-11-dUTP (*Thermo Scientific*), a 1× buffer, 3.5 mM $MgCl_2$, 4 µg of bovine serum albumin (BSA), and 5 U *Taq* DNA polymerase (native, *Thermo Scientific*). The PCR conditions were: an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. For R173 probe, a set of primers was designed (the *FastPCR* software *v. 6.2.89*, Kalendar *et al.* 2011) using a consensus sequence (3 879 bp, Fig. 1 Suppl.) composed of *S. cereale* R173-2 (GenBank: X64101.1) and R173-3 (GenBank: X64102.1) sequences. The consensus sequence was generated using the *CLC* sequence viewer *6.6.2* (*CLC Bio*, *Qiagen*). Fourteen different primers were designed using the *FastPCR* software. After testing different combinations, four pairs were chosen for labelling (Table 4). All four probes were used in FISH because the R173 sequence is long and dispersed and thus difficult to visualize with a single short probe. PCR

labelling was carried out in a total volume of 0.05 cm^3, containing 0.2 μg of *S. vavilovii* total DNA, 1 μmol of each oligonucleotide primer, 200 μM dATP, dCTP, and dGTP, 130 μM dTTP, 20 μM *Texas red®-5-dUTP* (*PerkinElmer*, Boston, USA), a 1× buffer, 2.5 mM $MgCl_2$, 4 μg of BSA, and 2 U *Taq* DNA polymerase (native, *Thermo Scientific*). The PCR conditions were: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 52 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 7 min. The probes were mixed with a hybridization mixture containing: 50 % (v/v) formamide, 10 % (m/v) dextran sulfate (*Sigma-Aldrich*), 0.1 % (m/v) sodium dodecyl-sulphate, a 2× SSC buffer, and 0.3 μg cm^{-3} salmon sperm DNA (*Roche Diagnostics*). The final concentration of each probe was 1 ng cm^{-3}.

The probes and the target chromosomal DNAs were denatured simultaneously on a slide at 83 °C for 5 min. Hybridization was carried out overnight at 37 °C. Post-hybridization washes were performed at 43 °C for 5 min in 50 % formamide (three times) and in a 2× SSC buffer (three times). The slides were incubated in a DB buffer (4× SSC, 0.2 %, v/v, *Tween 20*) at 37 °C for 5 min. Then, the slides were incubated in 1 × *Animal-free blocker*™ (*Vector Laboratories*, Burlingame, USA) at room temperature for 30 min. JNK probe detection was carried out using 5 μg cm^{-3} *Avidin-FITC* (*Sigma-Aldrich*). Signal amplification was carried out by incubation in a 5 μg cm^{-3} *Anti-avidin* antibody (*Vector Laboratories*) and unconjugated in 10 μg cm^{-3} *Anti-rhodamine* (*Vector Laboratories*), followed by incubation in 5 μg cm^{-3} *Avidin-FITC* and 20 μg cm^{-3} *Texas red®* (*Vector Laboratories*). Signal amplification was repeated twice. After incubation, the slides were washed three times in a DB buffer at 37 °C for 5 min. Chromosomes were counterstained with 1 μg cm^{-3} 4′,6-diamidino-2-phenylindole (DAPI; *Sigma-Aldrich*) for 15 min. The slides were mounted in a *Vectashield® Hard Set* (*Vector Laboratories*) medium and the preparations were analyzed with a epifluorescence microscope *Axio Imager Z2* (Carl Zeiss, Oberkochen, Germany). The resulting images were captured and analyzed using the *GenASIs* software (*Applied Spectral Imaging*, Yokneam, Israel).

Table 4. R173-specific primer pairs used in probe labeling (F - forward, R - reverse).

No.	Name	Region in R173	Sequence (5'→3')	Length of the probe
1	F1	215 - 236	GGGAGTTTTCAAGTGTGCGAGG	504
	R72	700 - 719	TTAACCCATGCAACGGAAGC	
2	F10	892 - 912	ATTGACTTGGCGTATGCTCAC	321
	R62	1191 - 1213	CCCCAGAAACATGGTTACCGCTC	
3	F16	1194 - 1216	CGGTAACCATGTTTCTGGGGACG	281
	R55	1456 - 1475	TGAGGACATCCATACCGGGA	
4	F48	2735 - 2756	AGCTTCTCCACCACCATGGACG	568
	R16	3284 - 3303	ATCCAGAACATCTCGCTGGT	

Results

In order to determine which sequences are adjacent to JNK tandem repeats, both forward and reverse primers were designed to be used in the genome walking procedure. We were able to amplify many fragments of different lengths from six libraries constructed using various restriction enzymes. The most distinctive of these bands were extracted from gels, purified and sequenced. In total, 12 different fragments were analyzed, and the length of all these fragments was between 500 and 1 970 bp (Table 5). Only good quality sequence data were included in the study. We sequenced only the products obtained by PCRs using forward GW3 or reverse GW3 nested primers. Control reactions consisting of secondary PCR using forward GW2 or reverse GW2 were carried out in order to verify the specifity of the amplified fragments. An appropriate fragment would appear in the reactions using both forward GW3 and reverse GW3 and those using forward GW2 and reverse GW2, but in the case of the reactions using the GW2 primers, the product would be proportionally longer.

The sequencing results clearly show that blocks of the JNK sequences were adjacent to sequences from the R173 family. Moreover, it appears that R173 sequences were the flanking sequences in both upstream and downstream directions (Fig. 1 Suppl.). The R173 sequences directed downstream were adjacent to JNK in an antiparallel orientation (Fig. 1*A* Suppl.), whereas upstream they were adjacent in a parallel orientation (Fig. 1*B* Suppl.). Almost all the PCR products which were sequenced were found to consist of a sequence which closely resembled some part of the R173 sequence; either R173-2 or R173-3 members of the R173 family. In addition, it was found that at the 5' ends of the JNK blocks, the sequence was truncated at the 519 nucleotide. In order to define which of the R173 sequences borders the blocks of JNK, we aligned the R173-2 and R173-3 sequences and thus determined their similarity. The R173-2 was 4 247 bp long and R173-3 was 4 649 bp

long; the region of a high similarity was between 246 and 4 111 in R173-2 and between 614 and 4 450 in R173-3. We were not able to define which of R173-2 or R173-3 borders the JNK motifs as, quite interestingly, we found that 11 out of the 12 PCR products were aligned at exactly the same points on R173-2 and R173-3 (4 111 nt in R173-2 and 4 450 nt in R173-3) – the last nucleotide of the region of their similarity (Fig. 1). As the 3' ends of R173 were directed towards the blocks of JNK, we did not obtain any information about the 5' ends of R173. For clarity, only the results showing comparisons between amplified fragments and the R173-3 sequence have been presented here. We have also done this for the alignment between the amplified fragments and the JNK sequence, and have presented only comparisons between the fragments and the pScJNK1 clone from the JNK family, but not comparisons with other clones from the same family (pScJNK2, pScJNK3, and pScJNK4).

The R173 sequence was long, and there were restriction sites located along its entire length. We checked the position of the restriction sites for the enzymes which were used in this study in both R173-3 and R173-2. There were cut positions in R173-3 for *Dra* I at 1 196 and 3 172 bp, for *Eco*R V at 2 453, 2 549, and 3 884 bp, and for *Pvu* II at 4 024 bp; there were no restriction sites in this sequence for *Stu* I, *Smi* I, or *Mss* I. There were cut positions in R173-2 for *Eco*R V at 2 110, 2 206, and 3 549 bp, for *Pvu* II at 3 689 bp, and for *Stu* I at 4 225 bp; there were no restriction sites in this sequence for *Dra* I, *Smi* I, or *Mss* I. Based on all these sets of data (the cut position, length of adaptor, and primer position), we could, in theory, obtain fragments of the following length in the forward direction: ~850 bp in the *Pvu* II library, ~950 bp or above in the *Eco*R V library, and ~1 650 bp or above in the *Dra* I library. As the JNK sequence was truncated at 519 nt in the reverse direction, the part from 519 to 1 190 nt had to be included. The revGW3 annealing site was located at 18 - 47 nt on the next JNK copy. We could, therefore, in theory expect fragments in the reverse direction to have the following lengths: ~2 050 bp in the *Dra* I library, ~1 300 bp or above in the *Eco*R V library, and ~1 150 in the *Pvu* II library. However, in practice we also amplified fragments of other lengths (Table 5). One possible cause for this result is a point mutation which caused the appearance or disappearance of particular restriction sites. Since the R173 sequence family is thought to be non-coding, such mutations are not a rare phenomenon. Another potential reason for the occurrence of fragments, in addition to those expected, might be the fact that JNK can sometimes adjoin other sequences. However, only one of the PCR products (from *Pvu* II library, 550 bp; Table 5) indicated that there might be JNK adjacent to another sequence. In that case, the JNK sequence was also truncated, but at the 959 nucleotide. The sequence which bordered that motif resembled the LTR of retro-transposon *Laura* from *Triticum aestivum* (FN564427.1). As mentioned above, JNK sequences may also be present in loci other than their main locus in the 2R chromosomes. By means of this study, we were not able to affirm whether this sequence similar to Laura was located near the main locus or the other one. The fact that JNK may also adjoin to sequences other than JNK sequences revise the hypothesis that JNK is inserted into specific sequences. However, it is possible that these other JNK loci appear as a result of retrotransposon activity. The 282 bp fragment which resembled LTR of the Laura retrotransposon was aligned from 165 971 bp to 166 246 bp on LTR of the Laura retrotransposon, and 166 245 nt is the point at the end of this LTR sequence. The JNK might thus adjoin to LTR of the Laura retrotransposon, which would make plausible a hypothesis that through the process of retrotransposition, JNK sequences or their fragments or even parts of blocks of JNK may be transferred to other loci.

In order to establish the number of the loci of the JNK sequences and to address the question of whether JNK colocalizes with the R173 sequence family, we performed FISH. The results clearly show that the JNK sequences were clustered on the long arms of the 2R chromosomes (Fig. 1). There is no doubt that R173 sequences were present alongside the JNK sequences in this particular locus (Fig. 1). We were not able to exclude for certain the possibility that the JNK sequences were somehow interspersed with the R173 sequences using either the genome walking technique or FISH. However, we have already performed some initial analyses to determine whether any kinds of additional sequences disunite some particular JNK repeats. We discovered that such a possibility exists, but that JNK sequences were certainly not interspersed with R173 sequences. It is interesting to note that in some plants, an additional JNK locus lacking an R173 sequence element was observed in the 2R chromosome (Fig. 1). These results support the findings we obtained using genome walking.

Table 5. The length [bp] of secondary PCR products in the genome walking procedure (F - forward GW3 specific primer, R - reverse GW3 specific primer).

Dra I		*Pvu* II		*Eco*R V		*Stu* I		*Smi* I		*Mss* I	
F	R	F	R	F	R	F	R	F	R	F	R
670	-	1100, 820	1120, 550	1970, 960	1320	1050, 500	1540	-	-	-	1260

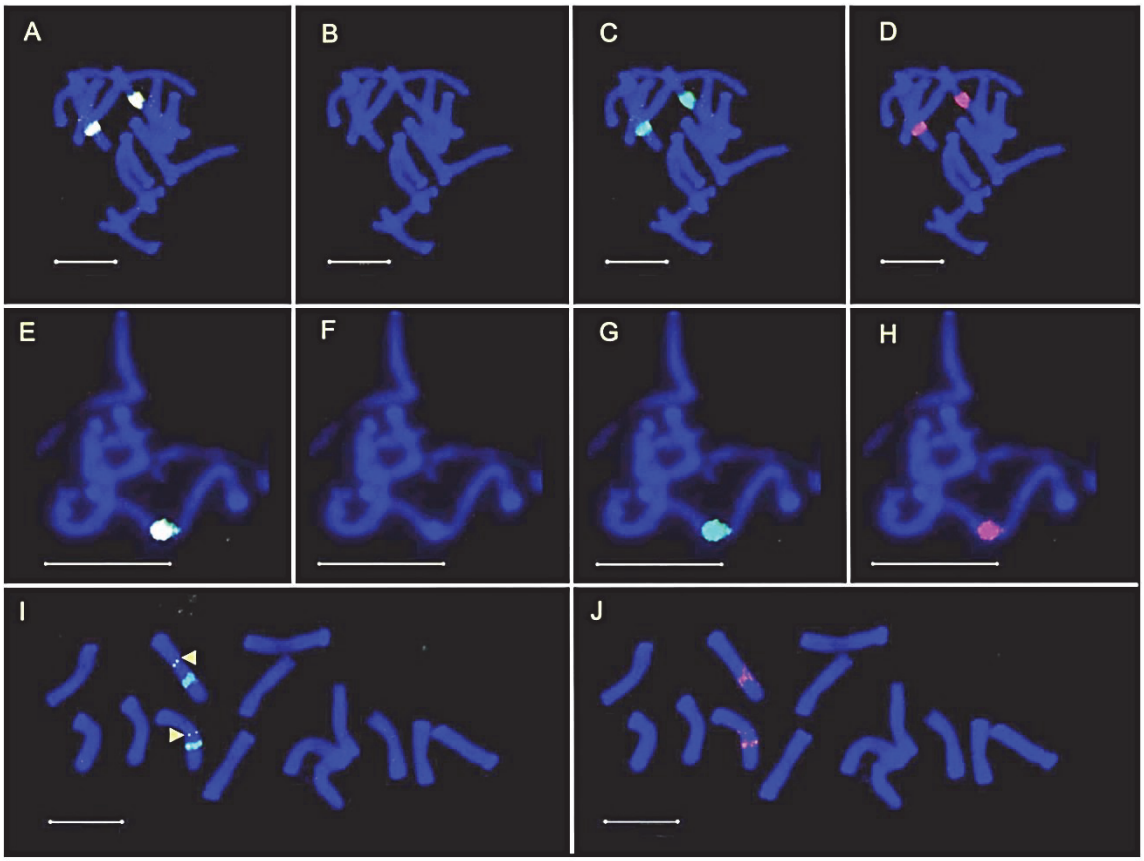

Fig. 1. The fluorescent *in situ* hybridization analysis using JNK (*green*) and R173 (*red)* probes on chromosomes *S. vavilovii* lines 225zcz (*A-D,I,J*) and 52 (*E-H*). Both sequences were localized on the long arm of both 2R chromosomes (*A,E*). The *arrows* in (*I*) show the additional positions of the JNK sequences on chromosome 2R. The chromosomes were counterstained with DAPI (*blue*). The *scale bars* 10 μm.

Discussion

The JNK sequences in the *S. vavilovii* genome are located in a highly specific region of the 2R chromosomes (Nagaki *et al.* 1999). It is not known whether the location of JNK is random, or whether the JNK sequence is inserted into certain other sequences and thus may play either a regulatory role or a structural role. It is also possible that JNK, owing to its heterochromatic nature, may influence sequences adjacent to it. The genome walking technique was used to identify the regions flanking the JNK sequence in *S. vavilovii.* This technique has already been successfully used in identifying promoter sequences in various plant species (Trindade *et al.* 2003). It makes possible the identification of flanking regions, *e.g.,* that of the Lhcb1.1 gene present in the genus *Spinacia* (Leoni *et al.* 2008). The sequence obtained using genome walking in the present study demonstrates the highest degree of similarity (90 %) to two clones: R173-2 and R-173-3 of the R173 repeated sequence family that is characteristic of the genus *Secale* (Guidet *et al.* 1991, Rogowsky *et al.* 1991, 1992). This study demonstrates that in inbred lines of *S. vavilovii*, the R173-2/173-3 sequence was present only in chromosomes in which an atypical heterochromatin was located, whereas Rogowsky *et al.* (1992) found that the R173 sequence is dispersed over the entire length of all *Secale cereale* chromosomes except for the centromere. This pattern of distribution was confirmed by González-García *et al.* (2011). They designed a probe named *UCM600* which is composed of a 592 bp fragment of the R173 sequence and used it in FISH experiments on rye chromosomes. In our study, we obtained a distinct pattern of R173 distribution, which was quite unexpected. It is difficult to explain this significant difference in localization. Rogowsky *et al.* (1992) analyzed the localization of this sequence in *S. cereal*e, thus the localization may be different in *S. vavilovii*. However, González-García *et al.* (2011) studied distributions in *S. cereale* and *S. vavilovii*, and the localization of R173 in both the species is similar. Another explanation of observed differences are probes which were used in the study. We used a blend of four probes designed to the R173-2 and R173-3 consensus sequence. González-García *et al.* (2011) used a different fragment of R173-3 (from 1655 to 2247 nt) as a probe. There is a possibility

that the R173 family is heterogenic, and they had been able to detect all members of the family by means of *UCM600*, whereas our probes detected only some of them. Then, it would be very interesting, if some members of the R173 family existed and acted as insulators, preventing heterochromatin spreading. From another point of view, there is always a risk of unspecific hybridization. As *UCM600* shows a partial homology to other repeated sequences (*e.g.*, *Camila* and *Lotte* retrotransposons) it can give more FISH signals. Especially, González-García *et al.* (2011) labeled their probe with nick translation which creates a number of small fragments. It is striking that according to Rogowsky *et al.* (1992), there are only about 15 000 copies of R173 per diploid rye genome, whereas the chromosomes in the González-García *et al.* (2011) report are whole covered with FISH signals. Unfortunately, Rogowsky *et al.* (1992) did not insert any FISH image in their paper so we cannot compare the results. As we are not able to clarify these differences, further studies should be performed. Many examples of changes in the distribution of repeated sequences in related species or even cultivars can be found in the literature, such as differences in the distribution of four types of tandemly repeated sequences which are characteristic of *Cucumis* and of 45S rDNA in three cultivars of *Cucumis sativus* (Zhao *et al.* 2011).

These results indicate that the JNK sequence was located adjacent to the R173 repeat sequence. The R173 does not encode for any known protein; therefore, the influence of heterochromatin on this area would not cause any changes to the vitality of a plant specimen which contains an additional band consisting of sequences belonging to the JNK family. The area is directly adjacent to an area of heterochromatin, on both sides. It is possible that this configuration acts as an insulator which prevents the heterochromatin from spreading to the adjacent genes; such barriers, separating heterochromatic from euchromatic regions, are common in both plants and animals (Kellum and Schedl 1991, Sun and Elgin 1999). A good example of this is yeast in which silencing barriers of tDNAs have been observed in the locus TRT2 near the STE6 gene, and in the locus tRNAAla near the centromere (Simms *et al.* 2004, Scott *et al.* 2006). What makes this hypothesis more likely is the fact, that in both types of repeated sequences, namely R173 and JNK, CTCF binding sites are localized. These motifs can be either positive or negative transcription regulatory sequences (Filippova *et al.* 2008, Zlatanova and Caiafa 2009, Herold *et al.* 2012).

Although there is a sizeable body of data documenting the evolution of repeated sequences (Cuadrado and Jouve 2002, Schindelhauer and Schwarz 2002, Sharma *et al.* 2013), the origin of the new types of repeated sequences has not been elucidated yet. In many plant species, newly created satellite sequences display a high homology with retrotransposons (Cheng and Murata 2003, Tek *et al.* 2005). The results also show that the JNK sequence can also occur adjacent to another sequence. The R173 FISH signals were observed only near the main JNK locus in the 2RL and were absent from the additional one. Hence, we can conclude that in the other locus, the JNK sequences are flanked by other than R173 sequences. This would refute the hypothesis that the JNK sequence is inserted into specific sequences at a single location in the genome. It is possible, however, that these other JNK loci occur as a result of retrotransposon activity, evidence for which includes the fact that their fragments resemble the Laura LTR retrotransposon. Such results indicate that the JNK sequence or its fragments can be transported by the process of retrotransposition, and thus, the presence of retrotransposon copies would be expected. In the case of the additional heterochromatic bands, this was not observed. FISH using probes constructed from reverse transcriptases (RT) of the Ty1-*copia* and Ty3-*gypsy* retrotransposons gave no hybridization signals in the area of the band, but did so at a different location in the chromosome 2R. The RT of both groups of retro-transposons was distributed along the entirety of their arms with the exception of an additional band and the telomeric heterochromatin (Achrem *et al.* 2009).

The presence of JNK sequences, which closely resemble 5S rRNA sequences, may attest to the intense dynamics of these repeat elements in the rye genome. It is probable that these sequences underwent heterochromatinization and were surrounded by R173 sequences, thus preventing the heterochromatin from spreading to adjacent regions.

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