

R-ISSR - Tool for Generation of a New Type of Products, Applied for the Identification of Putative Molecular Marker Linked to QTL Determined Tolerance to Nutrient Deprivation Stress in Rye (*Secale cereale* L.)

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Abstract

This study presents results confirming the possibility of the application of various combinations of RAPD and ISSR primers in one multiplex PCR for the rye genome and generating a new type of R-ISSR products. The plant material consisted of two inbred lines (Ot0-6 and Ot1-3), F₁ and two bulks (tolerant and susceptible), selected from a population of RILs (F₂) with different responses to stress caused by nutrient deprivation at the seedling stage. In one PCR reaction, the DNA of five genotypes was amplified separately, with RAPD, ISSR, and RAPD+ISSR primers. In total, 28 R-ISSR combinations were tested by combining 20 and 8 appropriately selected RAPD and ISSR primers. 567 loci were amplified, including 230 RAPD, 136 ISSR and 207 amplified R-ISSR loci, of which 114 were new. It was shown that only one ISSR product was identified amongst sequenced R-ISSR products with identical electrophoretic mobility as co-migrating RAPDs and ISSRs. The remaining ones had heteroamplicons (R-ISSR) sequences. A similar range of variability was observed both in R-ISSR profiles and in RAPD, as well as in ISSR. The correlation coefficient between the matrices of genetic similarity for five rye genotypes calculated by means of the Mantel test was highly significant $r_{ABC} = 0.964$.

Keywords: ISSR, molecular markers, nutrient deficiency, rye, R-ISSR, RAPD

Introduction

Identification of genes, factors and mechanisms regulating their expression enables their role in the functioning of organisms to be understood (Liu *et al.*, 2008; Moller *et al.*, 2011; Xu *et al.*, 2011). In organic plant cultivation, including cultivation of plants with decreased nutrient requirements, such information may significantly facilitate selection of desired genotypes (Wolfe *et al.*, 2008).

Such research provides a wealth of biological information and it is becoming increasingly easier to gain access to it. It is also increasingly easier to assign significance and sense to biological information obtained within experiments which are conducted based on information already collected using the synteny and collinearity of genomes (Devos and Gale, 2000).

Rye (*Secale cereale* L.) is an important cereal species grown in many countries of Central, Eastern and Northern Europe (RYE-BELT). Rye is used as a source of resistance genes for many cereal diseases, winter hardiness, tolerance to sprouting, drought, and nutrient deficiencies that can be transferred to closely related cereal crops (Geiger and Miedaner, 2009; Hillel and Rosenzweig, 2005; Wolfe *et al.*, 2008). Compared to wheat and barley, the data available on rye with various sequences collected at gene banks (primarily EST) remains modest (~10,000 sequences) (Bartoš *et al.*, 2008).

A large part of the rye genome (ca. 92%) is constituted of microsatellite sequences. Their structure and the mechanisms of their formation were presented in a study by Flavell *et al.* (1974), while their polymorphisms in studies by Bednarek *et al.* (2003), Bolibok *et al.* (2006), Bartoš *et al.* (2008) and Akhavan *et al.* (2010).

The essence of R-ISSR lies in the combination of decamer (Williams *et al.*, 1990) and microsatellite (Zitekiewicz *et al.*, 1994) primers in one multiplex PCR. Amplification is performed within new microsatellite and inter-microsatellite DNA regions, which are not amplified separately using RAPD (Ćwiklińska *et al.*, 2010; Mysłków *et al.*, 2001) or ISSR (Bolibok *et al.*, 2006; Saleh, 2011; Ye *et al.*, 2005).

This study demonstrates the possibility of the application of the R-ISSR technique as a screening tool to describe the new range of variability in the rye genome. It was devised for rye plant material prepared for research in response to nutrient deprivation stress aimed at: describing generated R-ISSR products, identifying R-ISSR loci for rye, examining the possibility of using them to identify a new type of putative molecular marker linked to different QTL (Quantitative Trait Loci), including those which determine rye tolerance to abiotic stress caused by nutrient deprivation at the seedling stage.

Materials and methods

Plant material

The plant material consisted of two rye inbred lines: Ot0-6 and Ot1-3, hybrid F_1 (Ot0-6 \times Ot1-3), two bulks (tolerant and susceptible to nutrient deprivation in medium assessed according to the method described by Rzepka-Plevneš and Kurek (1997), twenty tolerant and susceptible RILs (F_9) from two groups differing in their response to abiotic stress, selected in accordance with the methodology described by Micheltore *et al.* (1991) (unpublished results).

DNA

DNA was extracted from fresh leaves of 10-day-old seedlings using a DNeasy Plant Mini Kit (Qiagen) and was quantified using a GeneQuant Unit (Pharmacia LKB) to obtain 40-50 ng DNA pro 1 μ l of DNA template.

RAPD and ISSR primers

A set of twenty selected RAPD and eight selected ISSR primers were used (Tab. 1). Sequences of RAPD and ISSR primers were designed by the University of British Columbia, Canada [RAPD Primer Sets #1, 2, 3, 4 and 7; Primer Set #9 (microsatellite)] and synthesized (each in 0.025 nmol scale) by Sigma-Aldrich.

PCR conditions

A series of multiplex PCR reactions (in two replications) was conducted on them in order to select RAPD and ISSR primers cooperating in the same thermal profile, i.e. providing distinct fingerprints. In the following stage of the research, the experiment was repeated with the previously selected primers, adding two inbred lines (Ot0-6, Ot1-3) and hybrid F_1 (Ot0-6 \times Ot1-3) to the bulks. With five such chosen templates, reactions in the same thermal profile were carried out. Finally, electrophoresis was conducted for 15 accessions, five for each of the techniques applied.

DNA amplifications

DNA amplifications were conducted according to the method described by Williams *et al.* (1990). The reaction mixture (25 μ l) contained 10 \times PCR buffer with $(\text{NH}_4)_2\text{SO}_4$, 2 mM of dNTPs, 25 mM MgCl_2 , 2.5 μ M of primer (RAPD, ISSR, RAPD+ISSR, respectively), 1U of *Taq* DNA polymerase enzyme (Fermentas) and 50-70 ng of DNA template. The reactions were performed in a Mastercycler 5333 (Eppendorf). The program of thermal cycling was as follows: initial activation step at 95°C for 3 min followed by 32 cycles of 30 s at 94°C, 30 s at 36°C and 2 min at 72°C, with a final extension step at 72°C for 10 min.

Electrophoresis and data analysis

PCR products were analyzed by electrophoresis (Sub-Cell GT, Bio-Rad) in 1 \times TBE buffer in 1.5% agarose gel

(Basica LE GQT - Prona). An O'RangeRuler 200 bp DNA Ladder (3000-200 bp) and a GeneRuler 100 bp Plus DNA Ladder (3000-100 bp) (Fermentas) were used as size markers. PCR products were stained with ethidium bromide, visualized (MiniBIS Pro - DNR Bio-Imaging Systems) and scored for band presence or absence. The DNA fingerprints generated by each of the primers or primer pairs employed (Tab. 1) were used to assess genetic variability in the rye genotype tested. Presence (1) or absence (0) of each amplicon was scored for each genotype. Matrices of similarity were generated using PhylTool (Buntjer, 2001) and were compared by calculating the product-moment correlation (Pearson) between matrices and Mantel (Daniel's) test statistics (Z) of significance using XLSTAT. The relationships between the five rye genotypes were shown in the form of a dendrogram produced by cluster analysis using the UPGMA method. The strength of the internal branches from the resulting tree was tested by the TREE-CON bootstrap analysis application using 2,000 replications (Van de Peer and De Wachter, 1994).

PCR product extraction

PCR product extraction from the agarose gel was performed in order to allow sequencing using a Qiaquick Gel Extraction Kit (Qiagen, Germany). DNA was prepared from single bands selected from two R-ISSR fingerprints (Fig. 2A and B). DNA was extracted from R-ISSR products, which were assumed to be a putative molecular marker, or 'like'-RAPD or 'like'-ISSR products due to the identical electrophoretic mobility in agarose gels and from two PCR products, which were considered to be the new R-ISSR loci, according to Ye *et al.* (2005) and Saleh (2011). They were not observed in RAPD or ISSR profiles.

TOPO TA Cloning

Products were cloned in a pCR2.1 vector using a TOPO TA cloning[®] kit (Invitrogen), then transformed into TOP10 competent *E. coli* cells according to the manufacturer's instructions, and the correct transformants were selected according to the standard blue-white screening procedure. Plasmid DNA was purified from a 2 ml culture of individual colonies using a Plasmid Midi kit (A&A Biotechnology, Poland). Insert sizes were checked using 1% agarose electrophoresis after 5 min *EcoRI* fast digest restriction (Fermentas).

Sequencing and data analysis

Sequences were obtained using a Beckman Coulter TDCS kit and run on a CEQ 8000 capillary sequencer according to the manufacturer's instructions. The sequences were edited and assembled into a full-length insert sequence using the BioEdit tool (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) to investigate the presence of a combination of sequences of primers used for the amplifications and were analyzed for homology using the BLASTN and BLASTX algorithms in comparison with the GenBank database.

Results

Twelve ISSR and thirty RAPD primers were used in multiplex PCR. The reaction was performed for fifty varying combinations of RAPD and ISSR primers. Products visualized on the electrophoregrams with nineteen RAPD and eight ISSR were used for analysing the results.

It was shown that in the DNA fingerprints of the five rye genotypes there is a range of variability enabling the identification of monomorphic, polymorphic and genotype-specific loci. Their presence or absence in the genetic profile was described in various configurations for hybrid F₁, bulks (Fig. 1) and, in particular, forty RILs, and significant differences were found between them as regards their response to nutrient deprivation stress.

New genomic R-ISSR loci were amplified by combining RAPD and ISSR primers in one multiplex PCR (Fig. 2).

In general, 567 loci (3206 amplicons) were amplified in the experiment, out of which 230 and 136 loci were described as RAPDs and ISSRs respectively. 207 R-ISSR loci were identified, out of which 114 were described as new genomic loci (Tab. 1).

R-ISSR products of identical electrophoretic mobility were observed, both in the case of those separated on the left side of the gel - RAPD and on the right side - ISSR (Fig. 2). It was shown that 50 of this type of loci were characterized by the same length as RAPD and 43 as ISSR. 85 loci with the same electrophoretic mobility in RAPD, R-ISSR and ISSR profiles were not taken into account (Tab. 1).

It was shown that, despite the lack of specific anchored sequences at 3'-end, ISSR primers 876 [(GATA)₂(GACA)₂] and 872 (GATA)₄ generated repeatable profiles and cooperated well in R-ISSR reactions with selected RAPD primers (Tab. 1). The number of amplified R-ISSR products, includ-

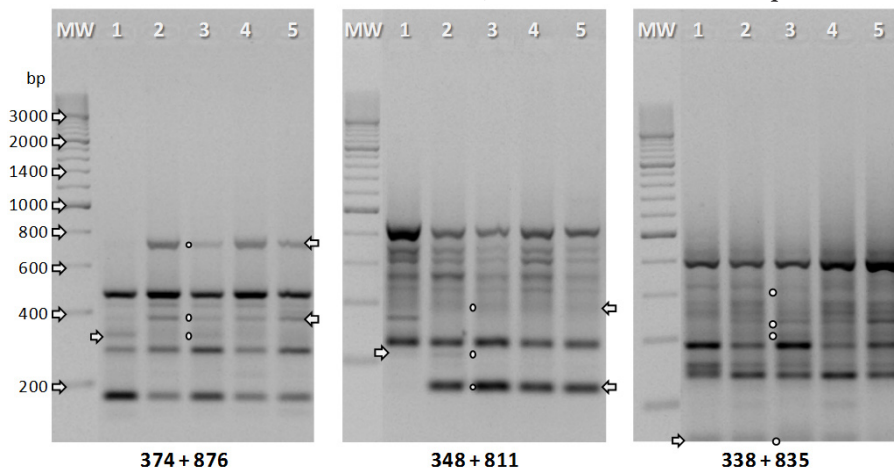


Fig. 1. Electrophoregrams of selected R-ISSR fingerprints, presented loci amplified for five different rye genotypes. White arrows and points mark selected amplicons observed in the F₁ profile as co-dominant markers

MW - molecular weight ladder; 1 - Ot0-6; 2 - bulk - RILs tolerant to nutrient deprivation, 3 - F₁ (Ot0-6 × Ot1-3); 4 - bulk - RILs susceptible to nutrient deprivation, 5 - Ot1-3. Primer - RAPD: 374, 348, 338; ISSR: 876, 811, 835.

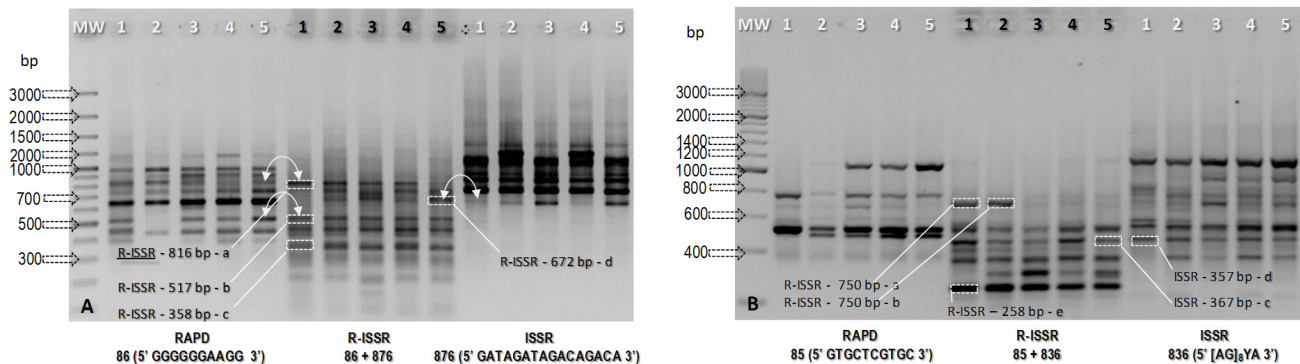


Fig. 2. Fingerprints of RAPD, R-ISSR and ISSR products amplified for five different rye genotypes. White rectangles mark selected amplicons (their length in bp) extracted from the gels, cloned into TOPO vectors and sequenced

MW - molecular weight ladder, 1 - Ot0-6; 2 - bulk - RILs tolerant to nutrient deprivation; 3 - F₁ (Ot0-6 × Ot1-3); 4 - bulk - RILs susceptible to nutrient derivation; 5 - Ot1-3

Tab. 1. Characteristics of RAPD, R-ISSR and ISSR loci amplified for selected genotypes of rye

RAPD	Primers and sequence		Range of amplified products (bp)	Total amplicons	Total loci amplified	Origin of loci amplified					³ Mono	
	Sequence 5' - 3'	ISSR				Sequence 5' - 3'	R ¹	R-I ¹	R-I new	R-I ²		I ²
81	GAGCACGGGG	835	(AG) ₈ YC	2180-260	102	20	10	2	3	3	6	1
81	CCTGGGTGGA	838	(TA) ₈ RC	1250-250	118	22	9	2	5	3	6	2
84	GGGCGCGAGT	811	(GA) ₈ C	1480-230	150	24	7	0	4	3	4	6
85	GTGCTCGTGC	838	(TA) ₈ RC	1130-210	114	23	4	1	7	0	8	4
86		836	(AG) ₈ YA	1210-220	93	17	7	2	5	0	3	2
86	GGGGGAAGG	811	(GA) ₈ C	1330-150	148	20	7	3	7	2	3	3
86		876	(GATA) ₂ (GACA) ₂	1340-210	172	25	9	5	8	0	5	3
89	GGGGGCTTGG	836	(AG) ₈ YA	1030-210	105	19	5	2	3	4	7	4
211	GAAGCGCGAT	876	(GATA) ₂ (GACA) ₂	1560-270	100	19	7	5	7	1	3	3
215	TCACACGTGC	872	(GATA) ₄	1890-280	100	17	7	2	3	2	5	2
226	GGGCCTCTAT	835	(AG) ₈ YC	1280-220	97	16	6	0	2	1	6	2
251		876	(GATA) ₂ (GACA) ₂	2050-350	102	26	12	3	8	0	3	3
251	CTTGACGGGG	844	(CT) ₈ RC	2030-380	135	22	11	2	3	2	4	5
284	CAGGCGCACA	835	(AG) ₈ YC	1520-280	103	17	8	0	2	2	5	2
302	CGGCCACGT	844	(CT) ₈ RC	1810-200	116	20	6	0	4	3	6	4
338	CTGTGGCGGT	835	(AG) ₈ YC	1200-220	105	19	6	1	4	1	3	6
345		886	VDV(CT) ₇	1010-270	124	14	4	0	2	0	3	5
345	GCGTGACCCG	811	(GA) ₈ C	1130-170	106	21	8	1	7	0	1	5
348		836	(AG) ₈ YA	1040-190	92	20	7	0	7	1	5	1
348	CACGGCTGCG	811	(GA) ₈ C	1410-150	115	20	11	3	3	2	4	2
349	GGAGCCCCCT	876	(GATA) ₂ (GACA) ₂	1830-190	118	21	10	5	3	1	6	2
374	GGTCAACCCT	876	(GATA) ₂ (GACA) ₂	1270-170	103	25	9	3	5	0	8	2
615		872	(GATA) ₄	2000-230	116	20	14	4	0	2	4	2
615	CGTCGAGCGG	836	(AG) ₈ YA	2070-280	129	20	10	2	0	3	7	2
615		844	(CT) ₈ RC	1960-320	120	24	6	0	5	0	6	7
648	GCACGCGAGA	876	(GATA) ₂ (GACA) ₂	1420-270	104	19	13	1	1	2	3	2
693		872	(GATA) ₄	2110-170	99	20	8	1	5	1	6	1
693	GACGAGACGG	811	(GA) ₈ C	1370-220	120	17	9	0	1	4	6	2
					3206	567	230	50	114	43	136	85
					207							

¹R - RAPD loci; ¹R-I - "like-RAPD loci" in R-ISSR profiles; new R-I - loci amplified; ²R-I - "like-ISSR loci" in R-ISSR profiles; ²I - ISSR loci.

Total number of loci amplified - is sum of ¹R + ²R-I (new) + I + ³Mono. ³Mono - loci observed in RAPD, R-ISSR and ISSR profiles with the same electrophoretic mobility, Y = C + T, R = A + G, V = G + A + C, D = G + A + T

ing the new genomic loci, depended on their combination with RAPD used in the research. The largest number of new R-ISSR loci were amplified using combinations of 876 (ISSR) and 251 (RAPD), while the fewest (one locus) using 876 (ISSR) + 648 (RAPD) and 811 (ISSR) + 693 (RAPD) primers (Tab. 1).

Sequential analysis of selected amplicons - marked with letters 'a', 'b' and 'd' in Fig. 2A - revealed that, despite identical electrophoretic mobility as 'reference' - RAPD or ISSR products, these constituted the R-ISSR products (Tab. 2A-a, -b, -d). Using the BLAST tool, alignments to sequences of products: 'a', 'b' and 'd' were identified among available *est_others* and listed in Tab. 2. It was found that the matched sequences were described in various studies as ESTs connected with the response to abiotic stress of various cereals caused by: drought, salinity and aluminium treatments (Tab. 2).

The sequence of the PCR product marked with the letter 'c' in Fig. 2A was described in the experiment as new genomic R-ISSR loci (Fig. 2A-c, Tab. 2A-c).

Analysis of the sequences of five subsequent amplicons (Fig. 2B, Tab. 2B), including four putative R-ISSRs and one ISSR, revealed that 'a' and 'b' amplicons were identical R-ISSRs (750 bp long). This product was presented as a molecular marker identified using the BSA method. Product 'a' was identified for the tolerant line - Ot0-6 and for bulk ('b') pool of the DNAs of twenty tolerant RILs (F₉). This product was not detected for inbred line Ot1-3, F₁ or in a susceptible bulk (Fig. 2).

Tab. 3 presents a contig created as a result of assembling sequences of a single DNA strand obtained for the 'a' and 'b' inserts (Tab. 2 and 3).

High e-values of alignments to sequences of the R-ISSR marker (*R-I_85+836_750*) for various ESTs were

Tab. 2. PCR product sequencing result of selected amplicons - described as results of R-ISSR or ISSR - and the result of their NCBI base sequencing alignments (EST)

Fig. 2	Product	Sequence		Type of product and length bp		
A	a	cap_MF30a	GATAGATAGA CAGACAAGAA GGTGGTTAAG	cap_MF30a	R-ISSR 816 bp
		cap_MF30e	GATAGATAGA CAGACAAGAA GGTGGTTAAG	cap_MF30e	
		cap_MR30a	cap_MR30a	CGCTC TTTGTGCAAT GTGCCCTTC CCCC	
		cap_MR30e	cap_MR30e	CGCTC TTTGTGCAAT GTGCCCTTC CCCC	
Accession E-value		treatments: drought and salinity (bluebunch wheatgrass FF341149.1 - salt and drought stressed roots, and salt and drought stressed shoots and crowns 2e-37); callus (barley: BU993554.1 - 2e-06 transcriptom analysis)				
b	align_MF32a	GGGGGAAGG CCGACGGGGA CGGGTATCAA	align_MF32a	TGTG CCATGTCATC TGTCTGTCTA TCTATC	R-ISSR 516 bp	
	align_MF32c	GGGGGAAGG CCGACGGGGA CGGGTATCAA	align_MF32c	TGTG CCATGTCATC TGTCTGTCTA TCTATC		
Accession E-value		callus: barley (BQ659305.1 - 2e-77, transcriptom analysis); identification of stress response genes (wheat: CJ523291.1 - 6e-77), genomics of abiotic stress (wheat and canola: CK207925.1 - 2e-23)				
c	align_MF33c	GATAGATAGA CAGACAAT TAGGCTGTCT	align_MF33c	TCACCG GGTTTCATCT GACTGCCTTC CCCC	R-ISSR 365 bp	
	align_MF33a	GATAGATAGA CAGACAAT TAGGCTGTCT	align_MF33a	TCACCG GGTTTCATCT GACTGCCTTC CCCC		
Accession E-value		not found				
d	d	cap_MF31d	GGGGGAAGG GCACACATCG CTGTCGCGCG	cap_MF31d	R-ISSR 672 bp
		cap_MF31b	GGGGGAAGG GCACACATCG CTGTCGCGCG	cap_MF31b	
		cap_MR31d	cap_MR31d	CTTTCCTC CTCGTATGTC TGTCTATCTA TC	
		cap_MR31B	cap_MR31B	CTTTCCTC CTCGTATGTC TGTCTATCTA TC	
Accession E-value		treatments: aluminium (wheat (root): CJ870080.1, CJ865897.1, CJ864508.1, CJ853125.1 - 2e-14)				
Accession E-value		ISSR - (5' GATA GATA GACA GACA 3') + RAPD - (3' GGAAGGGGGG 5') or RAPD - (5' GGGGGGAAGG 3') + ISSR - (3' ACAG ACAG ATAG ATAG 5')				
B	a	cap_MF3a	GTGCTCGTGC CATAAGCCAG GTACTTATCA	cap_MF3a	R-ISSR 750 bp
		cap_MF3b	GTGCTCGTGC CATAAGCCAG GTACTTATCA	cap_MF3b	
		cap_MR3b	cap_MR3b	TCT TCTCTCTCT GCTCTCTCTC TCTCTCT	
		cap_MR3a	cap_MR3a	TCT TCTCTCTCT ACTCTCTCTC TCTCTCT	
Accession E-value		treatments: aluminium (wheat: CJ808093.1 - 2e-45 - unpublished), cold (rye: BE704490.1 - 4e-11, unpublished), drought (barley: BG300360.1 - 7e-27, CD662726.1 - 1e-18 - unpublished), salinity (wheat: CK197361.1 - 1e-18 - unpublished). Project 'Genoplante' (wheat: CD877496.1 - 6e-66, CD881787.1 - 1e-42, CD891252.1 - 3e-18, CD875216.1 - 3e-18 - unpublished) - "Validation of gene function to increase the agricultural productivity of wheat and their tolerance to i.a. climatic variation". SCARECROW-like GRAS-family - transcription factor (TF) - candidate gene - Leymus sp. - wild rye: EG393386.1 - 1e-35, EG377967.1 - 3e-32 - Kaur et al. 2008); screening of stress response genes in wheat (CJ682864.1-2e-14, CJ576180.1 - 93-13 - Mochida et al. 2006)				
b	cap_MF4f	GTGCTCGTGC CATAAGCCAG GTACTTATCA	cap_MF4f	TCTTCTC TCTCTACTC TCTCTCTCTC TCT	R-ISSR 750 bp	
	cap_MF4g	GTGCTCGTGC CATAAGCCAG GTACTTATCA	cap_MF4g	TCTTCTC TCTCTACTC TCTCTCTCTC TCT		
Accession E-value		treatments: aluminium (wheat: CJ808093.1 - 2e-45 - unpublished), cold (rye: BE704490.1 - 4e-11, unpublished), drought (barley: BG300360.1 - 7e-27, CD662726.1 - 1e-18 - unpublished), salinity (wheat: CK197361.1 - 1e-18 - unpublished). Project 'Genoplante' (wheat: CD877496.1 - 6e-66, CD881787.1 - 1e-42, CD891252.1 - 3e-18, CD875216.1 - 3e-18 - unpublished) - "Validation of gene function to increase the agricultural productivity of wheat and their tolerance to i.a. climatic variation". SCARECROW-like GRAS-family - transcription factor (TF) - candidate gene - Leymus sp. - wild rye: EG393386.1 - 1e-35, EG377967.1 - 3e-32 - Kaur et al. 2008); screening of stress response genes in wheat (CJ682864.1-2e-14, CJ576180.1 - 93-13 - Mochida et al. 2006)				
c	cap_MR5d	AGAGAGAGAG AGAGAGCACA CGTATGCGCA	cap_MR5d	GCAC TTCAGGATTA CTCTCTCTCT CTCTCT	ISSR 357 bp	
	cap_MF5d	cap_MF5d	GCAC TTCAGGATTA CTCTCTCTCT CTCTCT		
Accession E-value		not found				
d	cap_MF6a	AGAGAGAGAG AGAGAGTACA GTCTGTTTCG	cap_MF6a	CTACT GCTGCTTGGT GCTCTCTCTC TCTCTCT	ISSR 367 bp	
	cap_MR6a	AGAGAGAGAG AGAGAGTACA GTCTGTTTCG	cap_MR6a	CTACT GCTGCTTGGT GCTCTCTCTC TCTCTCT		
Accession E-value		expression profile of storage proteins gene families (wheat: BJ273720.1); wheat (anthers ungergoing meiosis) CA599965.1				
e	e	cap_MF7c	AGAGAGAGAG AGAGAGCACC TGCTCGTCTG	cap_MF7c	CT CTAGCCAACC CTACGTGAGC ACGAGCAC	R-ISSR 258 bp
		cap_MF7d	AGAGAGAGAG AGAGAGCACC TGCTCGTCTG	cap_MF7d	CT CTAGCCAACC CTACGTGAGC ACGAGCAC	
		cap_MR7c	AGAGAGAGAG AGAGAGCACC TGCTCGTCTG	cap_MR7c	CT CTAGCCAACC CTACGTGAGC ACGAGCAC	
		cap_MR7d	AGAGAGAGAG AGAGAGCACC TGCTCGTCTG	cap_MR7d	CT CTAGCCAACC CTACGTGAGC ACGAGCAC	
Accession E-value		treatments: drought - screening of stress response genes (wheat: CJ717079.1, CJ612834.1)				
Accession E-value		ISSR - 5' GATA GATA GACA GACA 3' + RAPD (3' GGAAGGGGGG 5') or RAPD - (5' GTGCTCTGTC 3') + ISSR - (3' AY8[GA] 5')				

Tab. 3. Sequences and fingerprints of PCR product (R-ISSR marker) identified for inbred line and bulk tolerant to nutrient deficiencies

Sequence of PCR product (R-ISSR marker) identified for inbred line and bulk tolerant to nutrient deficiencies		Type of product amplified
<pre> 5 15 25 35 45 55 cap_MF3a GTGCTCGTGC CATA CGCCAG G TACTTATCA GGTCCAAGA ATCCCACCCC -GTCCGTATT cap_MF3b GTGCTCGTGC CATA CGCCAG G TACTTATCA GGTCCA-A ATCCCACCCC CGTCGGTATT cap_MR4f GTGCTCGTGC CATA CGCCAG G TACTTATCA GGTCCA-A ATCCCACCCC CGTCGGTATT cap_MR4g GTGCTCGTGC CATA CGCCAG G TACTTATCA GGTCCA-A ATCCCACCCC CGTCGGTATT cap_MF4f cap_MR3b cap_MR3a contig-0 GTGCTCGTGC CATA CGCCAG G TACTTATCA GGTCCA-A ATCCCACCCC CGTCGGTATT </pre>		<p>example of new type of molecular marker (R-ISSR) observed in tolerant bulk</p> <p>tolerant inbred line → a → bulk-tolerant → b → c</p> <p>1 – 153/79-1; 2 – bulk – tolerant, 3 – F₁; 4 – bulk – susceptible; 5 – Ot1-3; a – product specific for line 153/79-1, b – monomorphic products, c – PCR product specific for line Ot1-3, observed in fingerprints of F₁ and both bulks</p>
Figure 2B	<p>Products: a and b</p> <p>RAPD (85) – (5' GTGCTCGTGC 3')</p> <pre> 695 705 715 725 735 745 cap_MF3a cap_MF3b cap_MR4f GAGCCGGCTA CTAACGAAGA GCCCGTCTCT CTCTCTCTCT CTACTCTCTC TCTCTCTCT cap_MR4g AAGCCGGCTA CTAACGAAGA GCCCGTCTCT CTCTCTCTCT CTACTCTCTC TCTCTCTCT cap_MF4f GAGCCGGCTA CTAACGAAGA GCCCGTCTCT CTCTCTCTCT CTACTCTCTC TCTCTCTCT cap_MR4g GAGCCGGCTA CTAACGAAGA GCCCGTCTCT CTCTCTCTCT CTACTCTCTC TCTCTCTCT cap_MR3b GAGCCGGCTA CTAACGAAGA GCCCGTCTCT CTCTCTCTCT CTACTCTCTC TCTCTCTCT cap_MR3a GAGCCGGCTA CTAACGAAGA GCCCGTCTCT CTCTCTCTCT CTACTCTCTC TCTCTCTCT contig-0 GAGCCGGCTA CTAACGAAGA GCCCGTCTCT CTCTCTCTCT CTACTCTCTC TCTCTCTCT </pre>	
ISSR (836) – (3' AY ₈ [GA] 5')		R-ISSR - 750 bp

confirmed, including: wheat, barley, *Leymus* sp. and rye, which were obtained during research on the construction of cDNA libraries, in which mRNA was harvested from different plant organs affected by stress induced by various treatments, i.e. drought, aluminium, salinity and cold. Selected records are presented in Tab. 2.

The amplification of 20 tolerant (RILs) and 20 susceptible DNA samples revealed that the R-ISSR product

showed frequency deviated from the expected 1:1 ratio established on the basis of the χ^2 test. Frequency within the group tolerant to nutrient deficiency stress (band:null) was 17:3 (9.80"), whereas within the group of susceptible RILs (band:null) it amounted to 7:13 (0.20) (Fig. 3).

The 'e' product was described as a new genomic R-ISSR locus, whereas 'c' as ISSR (Tab. 2, Fig. 2B, Tab. 2B-c).

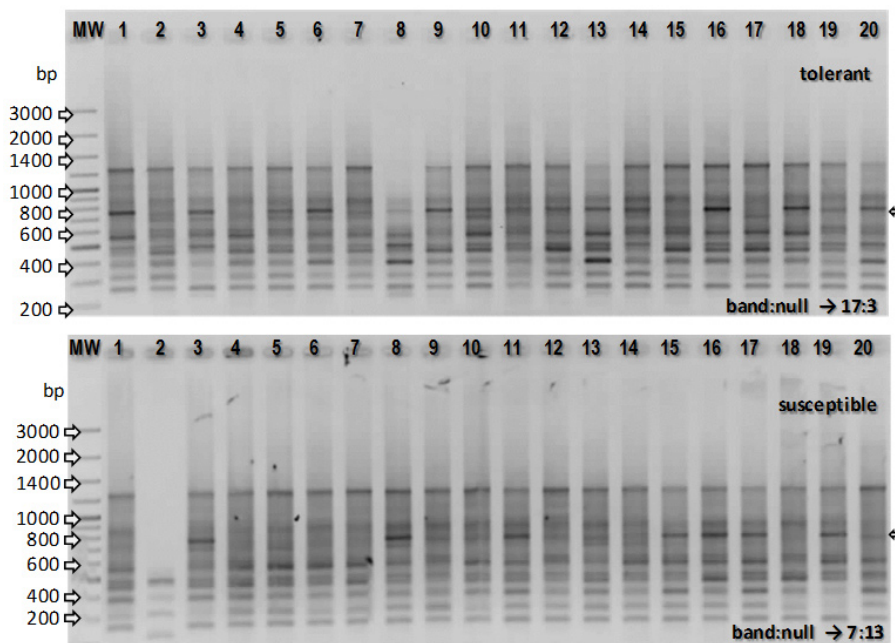


Fig. 3. Electrophoregrams of R-ISSR fingerprints presenting segregation of the R-ISSR marker (*R-I_85+836_750*) in two groups of RILs of rye differing in response to nutrient deprivation stress. Differences in segregation (band:null ratio) were established on the basis of the χ^2 test

Assessment of genetic similarity between rye genotypes was presented in the form of a dendrogram (Fig. 4).

Each of these groups shows genetic relationships between rye genotypes analyzed using one of three different methods. Genotypes characterized according to R-ISSR fingerprints were included in group 'a', ISSR in group 'b', whereas RAPD in group 'c'. A significant correlation ($r_{ABC} = 0.964$), calculated by means of the Mantel test, indicated congruence between the compared matrices and, in this case, molecular marker methods.

Discussion

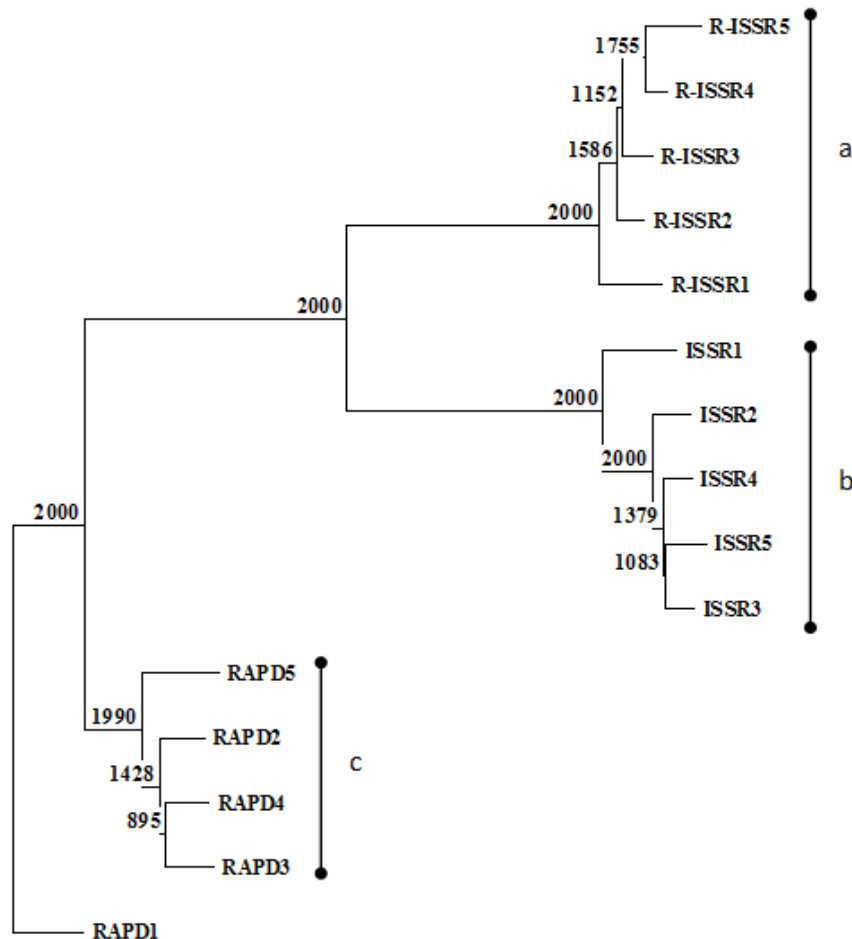
The results of this study confirm the possibility of using the R-ISSR tool for exploring the new scope of variability in the rye genome occurring in informative and poorly known regions, and microsatellite and inter-simple sequence repeat sequences, which were not often amplified due to limitations, e.g. primer hybridization sites, screening methods, i.e. RAPD (Ćwiklińska *et al.*, 2010; Myśków

et al., 2001) and ISSR, as well as SSR and AFLP (Lawson and Zhang, 2006; Rakoczy-Trojanowska and Bolibok, 2004; van der Nest *et al.*, 2000; Ye *et al.*, 2005).

As opposed to Ye *et al.* (2005) who first used 13-14 bps long ISSR primers in R-ISSR, 16-18 bps long ISSR were used in this research, in a manner similar to Saleh (2011). Those with low T_m were selected from amongst them and were combined with selected RAPD primers in multiplex PCR.

R-ISSR products, as in the studies of Ye *et al.* (2005) and Saleh (2011), were separated in 1.5% agarose gel. R-ISSR fingerprints obtained for two hybrid lines, F_1 and for two bulks were described as repeatable and co-dominant R-ISSR products. Both features, as opposed to RAPD, are characteristic of ISSR (Pradeep Reddy *et al.*, 2002; Rakoczy-Trojanowska and Bolibok, 2004).

A set of R-ISSR products was amplified and described in the experiment, in a manner similar to Ye *et al.* (2005) and Saleh (2011). As shown in Tab. 1, 50 products were described as 'like'-RAPD, 43 as 'like'-ISSR, and 114 as new



a, b and c - groups of similarity

1 - Ot0-6; 2 - bulk - RILs tolerant to nutrient deprivation; 3 - F_1 (Ot0-6 \times Ot1-3); 4 - bulk - RILs susceptible to nutrient deprivation; 5 - Ot1-3

Fig. 4. UPGMA dendrogram presenting genetic relationships among five rye genotypes investigated *via* three different techniques (RAPD, R-ISSR and ISSR). Numbers above branches indicate the real values of bootstraps in which the branch was observed in 2,000 pseudoreplications

R-ISSR. In sequential analysis, it was shown that three R-ISSR products, which, following Ye *et al.* (2005) were regarded as 'identically' migrating as RAPD and ISSR constituted R-ISSR products (Fig. 2A, a, b, d). Two out of the selected R-ISSR amplicons were sequenced. It was shown that they were R-ISSR products (Fig. 2A-c, B-e).

These results modify the opinion presented by Ye *et al.* (2005), which was later adopted by Saleh (2011). The results provide new information concerning the essence of R-ISSR. It makes it possible to conclude that three types of products should be obtained in multiplex PCR while using RAPD and ISSR primers: ISSR, RAPD and heteroamplicons - R-ISSR. It was confirmed that 'typical' R-ISSRs have a heteroamplicon character; however, R-ISSR products co-migrating as RAPD and ISSR are rarely RAPD and ISSR. They are usually R-ISSR with lengths differing by several base pairs. It was shown that one R-ISSR product (Fig. 2B-c) was an ISSR with a length similar to that 'referentially' sequenced.

Not all products present in the R-ISSR profiles were sequenced; hence, conclusions are limited only to the results obtained for sequences of seven amplicons selected from two different R-ISSR fingerprints which presented the essence of R-ISSR. The sequential character of 85 R-ISSR products migrating in agarose gel with the same mobility as RAPDs and ISSRs was not described (Tab. 1). They were found in R-ISSR fingerprints and described by Ye *et al.* (2005) and Saleh (2011). It can only be supposed that these are heteroamplicons of a similar length.

In the profiles of the two bulks presented in this study (Fig. 2B - R-ISSR), one R-ISSR product specific for tolerance to nutrient deprivation inbred lines - Ot0-6 was identified. This product was considered to be a molecular marker putatively linked to one of the loci determining rye tolerance to nutrient deficiencies. Sequential analysis and sequence alignment performed using BLAST has shown its high homology to various ESTs expressed in response to abiotic stress caused by different treatments: aluminium, drought, salinity and cold, where the criterion of morphological assessment of response to stress is, amongst others, root morphology (Tab. 2). One of the EST sequences aligned to the sequence of the R-ISSR marker was the locus of the *SCARECROW-like GRAS-family* transcription factor (TF) (BG01_2.3579.C1.Contig4507) (1e-35) identified amongst wild rye ESTs (*Leymus* sp. - *Triticeae*) by Kaur *et al.* (2008). This transcription factor is involved in cell division, but is not sufficient for cell differentiation. The *SCR* mRNA was localized in the root within the cortex/endodermis zone in *Arabidopsis* (Di Laurenzio *et al.*, 1996). EST no. CJ808093.1 (2e-45) is another interesting sequence. It was identified during a project by Ogihara *et al.*, (unpublished - deposited in 2007 in GenBank) as results of a transcriptome analysis (cDNA) of wheat roots growing in aluminium treated soil. This response leads to a dramatic reduction in root growth (Anioł, 1990; Milla *et al.*, 2002).

Without a comprehensive analysis of data obtained from research on the rye transcriptome (ESTs, microar-

rays), it is difficult to match the biological functions to an individual marker (sequence). Information about the sequence of one (or more) molecular marker(s) occur within bulks, in accordance with one possible pattern presented by Michelmore *et al.* (1991), Masojć *et al.* (2009), which may confirm the usefulness of R-ISSR in the identification of pools of putative molecular markers. These experiments need to be continued with an attempt at determination of their chromosomal localization and thereby confirmation of their usefulness as an additional type of markers used for construction and saturation of genetic maps.

The correlation coefficient calculated using the Mantel test for RAPD, R-ISSR and ISSR matrices was high ($r_{ABC} = 0.964$) and may indicate congruence between the compared molecular marker methods (Sneath and Sokal, 1973).

Conclusions

R-ISSR is a cost-efficient research tool and can be used, alongside RAPD, ISSR or other tools like IRAP REMAP (Kalendar and Schulman, 2006; Smykal, 2006) for evaluation of genetic variability, fingerprinting, identification of molecular markers, and mapping for gene tagging not only for rye. It has been demonstrated that already a few ISSR primers are capable, together with various RAPDs, to generate informative R-ISSR products providing a new set of biological information.

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