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Application of ISSR-PCR, IRAP-PCR, REMAP-PCR, and ITAP-PCR in the assessment of genomic changes in the early generation of triticale

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Abstract

Analysis of structural changes of octoploid triticale genomes was conducted in F_2 and F_3 generations. The plants were derived from crosses of five cultivars and breeding lines of hexaploid wheat (*Triticum aestivum* L.) with one cultivar of rye (*Secale cereale* L). The study used four marker systems: inter-simple sequence repeat (ISSR), inter-retrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite amplified polymorphism (REMAP), and a technique named inter-transposon amplified polymorphism (ITAP) developed by the authors. Most frequently, elimination of specific bands was observed, especially of rye bands. Depending on the cross combination, the percentage of eliminated rye bands ranged from 73.6 to 80.6 %. A lower percentage of wheat bands was eliminated, *i.e.*, from 57.6 to 76.48 %, depending on the combination of crosses. The emergence of new types of bands in hybrids absent in the parental forms was the rarest phenomenon (14.5 - 17.9 %). The results indicate the ongoing process of genome rearrangements at the molecular level in the early generations of plant crosses that also involve repeated nucleotide sequences of DNA.

Additional key words: dominant DNA markers, mobile elements, retrotransposons, transposons.

Introduction

Repetitive DNA nucleotide sequences constitute a significant fraction of all eukaryotic genomes, especially in plant genomes. Some of these sequences are genetic mobile elements and others are tandem repeats exhibiting no mobility (Liu and Somerville 1996). Mobile elements typically constitute 50 - 80 % of the genome of plants, and in grass species, they can comprise as much as 90 %. Most of them are "latent" and could be activated under stress and the so called genomic shock (a programmed response to stress caused by hybridization; Chen and Ni 2006). Many reports have indicated that mobile elements play a key role in the regulation and speciation of a genome. They are also considered to be a driving force behind the evolution of plant genomes by modifying the structure and size of a genome (Bento *et al*. 2008, 2010, Kraithstein *et al.* 2010, Jiang *et al*. 2011). Retrotransposons can be distinguished as one type of mobile genetic elements that form the largest group of these elements in eukaryotic genomes (Kraithstein *et al.* 2010). Voytas and Naylor (1998) suggested that retrotransposons may play a significant role in genomic changes in allopolyploids. Hence, it seems extremely important to track such changes in hybrids derived from crossing wheat with rye. The second group of mobile elements are transposons which are found in most eukaryotes mainly as moderately repetitive sequences dispersed throughout genomes. Non-mobile repetitive sequences are of different sizes, they are repeated several times in a genome and are particularly susceptible to various changes such as insertions, deletions, or inversions. Microsatellite markers are an example of sequences that belong to this group. They are found in both eukaryotes and prokaryotes (Li *et al*. 2004, Sharma *et al.* 2008). Microsatellites can be uniformly dispersed in chromosomes, or they can show preference for pericentromeric regions. They can occur either within genes as well as within intergenic regions (in plants, dinucleotide sequences are more common in introns whereas trinucleotides are mainly found in exons); both types are more numerous in sequences flanking

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Abbreviations: AFLP - amplified fragment length polymorphism; ISSR - inter-simple sequence repeat; IRAP - inter-retrotransposon amplified polymorphism; ITAP - inter-transposon amplified polymorphism; LTR - long terminal repeat; PCR - polymerase chain reaction; PIC - polymorphic information content; REMAP - retrotransposon-microsatellite polymorphism; RFLP - restriction fragment length polymorphism; SSR - single sequence repeat; T_a - annealing temperature; TIR - terminal inverted repeat.
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5' rather than 3' genic regions (Achrem *et al*. 2014).

Tests for the presence of molecular markers are very useful in the assessment of changes in plant genomes. This study presents the evaluation of changes in DNA nucleotide sequences in early generations of triticale using molecular markers. A set of four marker systems were used (ISSR, IRAP, REMAP, and ITAP) to evaluate changes in the genomes of these plants.

Inter-simple sequence repeat (ISSR) analysis is based on the amplification of DNA fragments located between two microsatellites oriented in opposite directions (Ziętkiewicz *et al.* 1994). Changes in these regions result in the amplification of the product or lack of it, which is visualized on an electrophoretic gel (Vaillancourt *et al*. 2008). With the implementation of inter-retrotranposon polymorphism (IRAP), it become possible to replicate the sequence contained between two retrotransposons, belonging to the same family, located at a distance of a few dozen to several thousand base pairs. The use of one primer in the IRAP reaction allows obtaining a multilocus pattern (Kalendar *et al.* 2011, Achrem *et al.* 2014). Retrotranposon-microsatellite polymorphism (REMAP) is a method similar to the IRAP involving the amplification of DNA regions between long terminal repeat (LTR) and microsatellite sequences. This technique also gives a pattern of multiple bands. It makes use of the fact that the genome of plants is rich in microsatellite sequences associated with retrotransposons (Kalendar *et al.* 1999). For the purpose of this study, aninter-transposon amplified polymorphism (ITAP) system has been developed to allow amplification of sequences located between transposons belonging to the same family. It was designed on the basis of a marker system used in the analysis of plant and animal genomes called inter-MITE polymorphism (IMP) in which an amplified sequence is located between two *MITE* transposons (Chang *et al.* 2001). The ITAP is a technique similar to the IRAP as it also enables obtaining a pattern of multiple bands and is based on the fact that transposons are a significant part of plant genomes.

Triticale (× *Triticosecale* Witt.) is an example of an allopolyploid organism which contains two or more distinct genomes derived from closely related species (wheat and rye). The early generations of triticale polyploid plants provide an excellent genetic material for comparative analysis of gene expression and changes in genomic structure, as the parental forms are known. The oldest synthetic form of octoploid triticale shows preferable traits affecting adaptation to a new environment because its genome contains hexaploid wheat germplasm. Cytogenetic instability in triticale has been observed and manifested by the formation of aneuploids. This is probably due to meiotic dysfunctions which might lead to changes in the number of chromosomes. Meiotic dysfunctions are associated with impaired chromosome segregation, chromatin degradation, or fragmentation of chromosomes in subsequent generations of plants. Researchers evaluated cytogenetic instability of the triticale genome, and they most frequently observed elimination of a rye chromosome from the genome of the hybrid (Rogalska *et al.* 1991). Therefore, it is still relevant and worthwhile to conduct research on this form of hybrid. Studies in other polyploids, such as wheat (*Triticum* spp), *Spartina*, or *Arabidopsis* showed that genomic changes that occur during their formation are highly reproducible and non-random (Liu *et al* 2001, Kashkush 2002, Ozkan *et al.* 2003, Madlung *et al* 2005, Bento *et al* 2010, 2011).

The use of these four techniques in the current study allowed to assess changes on the genomic scale and to determine which of the parental genomes in early generations of octoploid triticale was more reorganized.

Materials and methods

The plant material consisted of two early F_2 and F_3 triticale generations. The first generation of triticale was derived from the Plant Breeding Company Strzelce, the agency Małyszyn. They were obtained by crossing three hexaploid wheat (*Triticum vulgare* L.) cultivars Zyta, Tonacja, and Ostka Strzelecka and two breeding strains (STHN 5067 and STHN 1002/1003) with a widely planted rye (*Secale cereale* L.) cv. Dańkowskie złote (Table 2). The next generation was propagated in a greenhouse that belonged to the West Pomeranian University of Technology in Szczecin.

Genomic DNA was isolated from coleoptiles (0.2 g per sample) of etiolated seedlings by a *GeneMATRIX Plant&Fungi* DNA purfication kit (*EURx*, Gdańsk, Poland). The isolated DNA was subjected to quantitative and qualitative evaluation by measuring the absorbances at 230, 260, and 280 nm using a *NanoDrop 2000c* spectrophotometer (*Thermo Scientific*, Madison, USA).

Thirty ISSRs, 10 IRAPs, 20 REMAPs, and 10 ITAPs primers were tested, of which 16 ISSR, 6 IRAP, 15 REMAP, and 5 ITAP primers were selected for further study (Tables 1 Suppl. and 2 Suppl.). For the IRAP, primers for Angela (IR1), Bilby (IR2), Cassandra (IR3), and Sukkula (IR6) retrotransposons were used according to Kalinka (2010). These retrotransposon sequences were derived from the genomes of the genera *Secale* (Bilby, Cassandra, and Sukkula) and *Triticum* (Angela). Nikita (IR4) and Sabrina (IR5) primers were designed for LTR regions at the 5'end of barley retrotransposons according to Bento *et al*. (2010).

Primers for ITAP were designed to amplify TIR regions of the 5'end of a transposon. An IT1 primer flanked the sequence of a transposon Caspar (HE 774675.1), IT2 - Jude (HE 774675.1), IT3 - Mutator (JF 701619.1), IT4 - Revolver (AB 646254.1), and IT5 - Sherlock (HE 774675.1). Transposon sequences were derived from the genomes of the genera *Secale* (IT4), and *Triticum* (IT5, IT3, IT2, and IT1). All primers were

synthesized at the Institute of Biochemistry and Biophysics (the Polish Academy of Sciences, Warszawa, Poland) and at *Genomed S.A* (Warszawa, Poland).

Polymerase chain reactions (PCRs) were performed in 0.02 cm^3 of a reaction mixture containing 10 - 100 ng of DNA, a 1× standard Taq polymerase buffer (*Fermentas*, Vilnius, Lithuania), 1.8 - 4.4 mM $MgCl_2$, 0.2 - 0.4 mM *dNTP* (*Fermentas*), 1.25 - 2 μM primer, and 1 - 2 U of polymerase (*Fermentas*); concentrations were optimized individually for each primer. The PCR program was as follows: 94 °C for 3 min; 40 cycles with 94 °C for 60 s, 42 °C to 60 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. An annealing temperature was based on a primer melting point (Tables 1 Suppl. and 2 Suppl.). The PCR was performed using an *MJMini*™ personal thermal cycler (*Bio-Rad*, Hercules, USA) and a *T100*™ thermal cycler (*Bio-Rad*). The products were separated on a 2 % (m/v) agarose gel in a 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA, pH 8.3). Ethidium bromide was added to the gel to a final concentration of 0.1 μg cm^{-3}. Electrophoresis was carried out in a *Sub-Cell Model 192 PowerPac HV* electrophoresis system (*Bio-Rad*) in a 1× TBE buffer at 85 V for approximately 5 - 7 h. The gel images were captured with a *Gel Doc XR* system (*Bio-Rad*). The bands were scored and analyzed with the *Quantity One* software (*Bio-Rad*). The size of the products was determined by comparison with a DNA ladder (*MassRuler*, *Fermentas*).

The bands were converted into a binary character matrix with "1" for the presence and "0" for the absence of a band at a particular position. Polymorphic information content (PIC) coefficients were calculated according to Ghishlain *et al.* (1999) for dominant marker systems, *i.e.*, PIC = 1 - p^2 - q^2, where p is the band frequency and q is no band frequency.

Results and discussion

Newly synthesized allopolyploids, which parental forms are known, are a very useful material to study both genetic and epigenetic changes emerging in the early stages of their evolution. Intraspecific crossing and polyploidization result in the formation of new allopolyploids with extensive changes, such as genomic rearrangements, changes in the regulation of gene expression, activation of mobile elements, deletion or amplification of highly repeated or unique sequences, as well as various epigenetic modifications (Kashkush *et al.* 2002, Adams and Wendel 2005, Wang *et al.* 2006, Liu *et al.* 2009, Kraithstein *et al.* 2010, Jiang *et al.* 2011). Many researchers have analyzed changes occurring in the nucleotide sequence of triticale using different molecular techniques, such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) (Ma *et al.* 2004, Ma and Gustafson 2008, Bento *et al.* 2011), single sequence repeat (SSR) (Vaillancourt *et al.* 2008), the IRAP, REMAP, and ISSR (Bento *et al.* 2008, Kalinka 2010).

In triticale, rearrangements of nucleotide sequences typically involve microsatellite regions as well as regions rich in mobile elements (Bento *et al.* 2010). For this reason, we applied four different marker systems: the ISSR, IRAP, REMAP, and ITAP to be able to detect various changes occurring in the early generations of primary octoploid triticale. A total of 42 different reactions have been conducted, of which 16 were ISSRs, 6 IRAPs, 15 REMAPs, and 5 ITAPs.

In 16 reactions carried out with the ISSR marker system, 341 different types of bands have been generated for all plants tested, of which 311 were polymorphic. It should be noted that the percentage of polymorphic bands in any reaction was not lower than 80 %, and the average value for all reactions was 91.25 %. The PIC value ranged between 0.33 and 0.42, and was highest for the IS7 primer. The products obtained in the reactions were in a size range of 98 - 2 213 bp (Table 1).

Six different reactions were carried out using the IRAP marker system, and 128 bands were acquired in all plants tested, of which 110 were polymorphic. In total, for all primers used in IRAP reactions, the percentage of polymorphic bands in any of the reactions was not lower than 71 %, and the average of all reactions was 85.33 %. The reaction products were in a size range from 91 to 1 416 bp (Table 1).

Fifteen various REMAP reactions have been carried out, which resulted in 164 bands, of which 123 were polymorphic. In all IRAP reactions, 4 to 17 different types of bands were detected, of which between 2 and 16 were polymorphic. An overall percentage of polymorphic bands in all IRAP reactions was not lower than 50 %, and an average for all reactions was 72.13 %. The PIC value for particular reactions was between 0.19 and 0.39 with the highest value observed for IR5/IS12 primers. The reaction products ranged from 84 to 2 007 bp (Table 1).

Five ITAP reactions generated 66 bands in all forms analyzed, of which 52 were polymorphic. On average, 78.2 % of the band types observed were polymorphic. In general, 10 to 19 bands were observed in all ITAP reactions, of which 7 - 15 were polymorphic. The PIC value for particular reactions ranged between 0.23 - 0.38. The products obtained in the ITAP reactions were in a size range of 124 - 3 246 bp (Table 1).

Our results are consistent with a high degree of ISSR polymorphism (82 %) reported in rye by Matos *et al.* (2001) and in barley (83 %) by Fernandez *et al.* (2002). Bento *et al.* (2008) have detected polymorphism of 65 % in octoploid triticale using the IRAP and REMAP methods, and of 68 % using the ISSR whereas Kalinka (2010) has analyzed triticale polymorphism of 74 % using the ISSR and REMAP, and of 89 % using the IRAP. These results confirm the usefulness of the selected marker systems in the analysis of the genomic

Table 1. Averaged coefficients from all test plants obtained by the use of ISSR, IRAP, REMAP, and ITAP marker systems.

Marker system	Primer	Total number of bands	Polymorphic band number	[%]	PIC	Product size [bp]
ISSR	IS1	22	21	95	0.41	277 - 2213
	IS2	32	28	87	0.35	255 - 1291
	IS3	21	17	81	0.34	241 - 1443
	IS4	25	24	96	0.40	203 - 1846
	IS5	23	23	100	0.41	349 - 2044
	IS6	18	15	83	0.33	98 - 1075
	IS7	20	19	95	0.42	205 - 1115
	IS8	22	21	95	0.39	491 - 1480
	IS9	17	16	94	0.35	319 - 1188
	IS10	25	23	92	0.38	230 - 1431
	IS11	20	18	90	0.35	391 - 1596
	IS12	16	14	87	0.36	185 - 1480
	IS13	15	15	100	0.36	361 - 1627
	IS14	25	20	80	0.37	196 - 991
	IS15	17	16	94	0.40	282 - 1737
	IS16	23	21	91	0.37	261 - 1371
	total	341	311	-	-	-
	mean	21.31	19.33	91.25	0.37	-
IRAP	IR1	21	15	71	0.28	142 - 571
	IR2	21	20	95	0.38	120 - 1167
	IR3	25	23	92	0.38	91 - 1342
	IR4	15	12	80	0.34	169 - 1046
	IR5	21	19	90	0.39	169 - 1046
	IR6	25	21	84	0.34	110 - 1416
	total	128	110	-	-	-
	mean	21.33	18.33	85.33	0.35	-
REMAP	IR3/IS14	11	9	82	0.30	137 - 792
	IR5/IS5	7	5	71	0.25	129 - 1373
	IR5/IS12	11	10	91	0.39	151 - 1286
	IR6/IS12	17	14	82	0.33	144 - 2007
	IR6/IS14	11	7	64	0.26	194 - 1046
	IR6/IS11	16	12	75	0.29	95 - 1427
	IR1/IS12	8	5	62	0.26	95 - 662
	IR1/IS11	17	16	94	0.38	95 - 724
	IR4/IS12	9	7	78	0.32	245 - 1318
	IR4/IS11	10	7	70	0.29	224 - 677
	IR3/IS12	10	6	60	0.22	84 - 663
	IR5/IS4	12	11	92	0.36	278 - 1653
	IR5/IS3	4	2	50	0.19	143 - 452
	IR6/IS3	8	4	50	0.19	198 - 778
	IR3/IS11	13	8	61	0.22	176 - 1231
	total	164	123	-	-	-
	mean	10.93	8.20	72.13	0.28	-
ITAP	IT1	14	13	93	0.38	389 - 1364
	IT2	12	7	58	0.23	124 - 1131
	IT3	11	10	91	0.39	460 - 3246
	IT4	10	7	70	0.28	387 - 1222
	IT5	19	15	79	0.35	460 - 1716
	total	66	52	-	-	-
	mean	13.2	10.4	78.2	0.33	-

structure of the hybrid plants studied.

The most important step in the analysis of the nucleotide sequence rearrangements was an assessment of lack of bands in the hybrids that were present in the parental species, and the emergence of new types of hybrid bands absent in the parental species. Hypothetically, on the principle of addition in triticale, all bands that had been present in both parental species

should occurred. Nevertheless, this general rule did not apply to the hybrid plant genomes. Bands that were detected in triticale can be divided into bands unique to wheat (PCR products amplified using a DNA template derived from the wheat genome), bands specific for rye (PCR products obtained from a DNA template derived from the genome of rye), as well as bands common for both wheat and rye. In contrast, elimination of bands in triticale was related to lack of a band that was present in the parental species.

The percentage of eliminated bands specific to rye was higher compared to wheat-derived bands and ranged from 73.64 % (Ostka Strzelecka × Dańkowskie złote) to 80.60 % (STHN 1002/1003 × Dańkowskie złote). Nevertheless, the percentage of band deletions characteristic for wheat was also fairly high, and ranged from 57.61 % (a combination of STHN 1002/1003 and Dańkowskie złote) to 76.48 % (a combination of Tonacja and Dańkowskie złote). Least frequent was the elimination (the subject to rearrangement) of bands common to wheat and rye – from 38.61 % (a cross of Zyta and Dańkowskie złote) to 63.83 % (a cross of Tonacja and Dańkowskie złote). The results indicate that the genome of the hybrid resulting from crossing Dańkowskie złote and Tonacja was subjected to the largest changes/rearrangements. The occurrence of new types of bands in the hybrids, absent in the parental forms, was the least frequent phenomenon as it ranged from 14.53 % (combinations of Ostka Strzelecka and Dańkowskie złote) to 17.94 % (combinations of STHN 5067 and Dańkowskie złote) (Table 2).

Table 2. Averaged summarized rates of the number of eliminated bands in the type of wheat (PCR products amplified using a DNA template derived from the wheat genome), type of rye (PCR products obtained from a DNA template derived from the genome of rye) and common to both wheat and rye, as well as new types of bands present in hybrids in generations F_2 and F_3.

Hybrid combination (wheat × rye)	New bands form [%]	Elimination of wheat and rye bands [%]	Elimination of rye bands [%]	Elimination of wheat bands [%]
Ostka Strzelecka × Dańkowskie złote	14.53	40.12	73.64	57.95
STHN 1002/1003 × Dańkowskie złote	14.62	41.97	80.60	57.61
STHN 5067 × Dańkowskie złote	17.94	50.49	78.95	68.75
Tonacja × Dańkowskie złote	16.90	63.83	80.57	76.48
Zyta × Dańkowskie złote	17.91	38.61	78.60	55.56

There was a variation observed in the scale of changes depending on the cross combination of newly synthesized forms of triticale. Most of the changes detected using the selected marker systems were related to the loss of bands observed in the parental hybrid species (an average of 48.50 to 58.25 % depending on the cross combination). The appearance of new types of hybrid bands were detected much less frequently (an average of 14.53 to 17.94 % depending on the cross combination) (Table 3 Suppl.). The present study has found a higher degree of nucleotide sequence rearrangements in various triticales compared to experiments conducted by other authors (Bento *et al.* 2008, Kalinka 2010). This may be due to a higher number of primers applied, evaluation of bands/products with a greater than an average size range (99 to 2 220 bp), as well as the incorporation of an additional marker system (ITAP) which analyzed changes in transposon-rich regions. Additionally, the current study examined the early generation of triticale that could have a significantly lower stability, which is highly probable, as early generations of allopolyploids (F_2 and F_3) were tested. Ma and Gustafson (2008) analyzed hexaploid and octoploid triticale and their parental species as well as wheat-rye hybrids of F_1 generation and showed a different degree of sequence diversity in hexaploid and octoploid triticale, as the latter one has fewer changes than the former. On average, in octoploid triticale, the loss of 30 % of bands was observed whereas 40 % in hexaploid (Ma and Gustafson 2008). Bento *et al.* (2008) and Kalinka (2010) have used a combination of three methods (the ISSR, IRAP, and REMAP) when studying octoploid triticale and its parental forms. These authors have demonstrated a higher percentage of band deletions compared to the emergence of new types of bands not observed in any of the parental species, which is consistent with the results of this work. The study of Kalinka (2010) has shown that about 40 % of bands are subjected to change whereas in our study, this number was considerably higher and amounted to 69.06 %. Such differences in the percentage of rearrangements may result from a fact that Kalinka (2010) analyzed later generations of triticale (F_5 and F_8) and most of the changes in the genomes of triticale are believed to occur immediately after the incident of polyploidization. In contrast, the research of Ma and Gustafson (2006) has shown that most of the changes occurr in F_1 generation before doubling the number of chromosomes. In addition, their results indicate that the response of the rye genome occurs primarily in intergeneric crossbreeding and not during polyploidization. Changes in subsequent generations of triticale after doubling the number of chromosomes are relatively small, and their level become fixed in successive generations (Ma and Gustafson 2008), which is related to mechanisms leading to stabilization of the triticale genome. In the present study, we did not observe stabilization of the triticale genome, expressed in

decreasing levels of rearrangements in successive generations, as the two early generations (F_2 and F_3) of triticale analyzed showed similar changes.

In conclusion, these examinations allow the observation of the genetic instability of analyzed triticale generations. The level of observed changes in triticale, which came from five different crossings, was similar, which indicates that it is hard to predict whether these plants will be suitable for breeding in the future. However, analyses of such early generations of triticale can enable finding a certain pattern of transformations which occur in early generations of allopolyploid and allow detecting the beginning of stabilization of these forms. It is worthwhile noticing that due to DNA elimination (up to 14 % of a total genome), differences between diploid genomes included in the same nucleus increase in triticale allopolyploids (Shaked *et al.* 2001). This result may increase the fertility of polyploids and can lead to disomic inheritance of descendant plants. Thus, the influence on the stability of progeny plants may be reflected in the use of forms which we tested for future crop breeding.

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