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CHARACTERIZATION OF GENOMIC POLYPLOIDS PLASTICITY IN THE WHEAT-RYE SYSTEM

TESE APRESENTADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM BIOLOGIA

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Aos meus pais;
à minha irmã;
às minhas sobrinhas.

Abstract

Polyploidization is a remarkable driving force in plant evolution, where hybridization and chromosome doubling result in huge genomic stress. Deeper knowledge about parental genomes behaviour in a hybrid nucleus and on processes underlying genetic and epigenetic modifications induced by polyploidization is essential to understand such evolutive process.

Using triticale as model species we evaluated the impact of polyploidization through molecular and cytological approaches. Genomic rearrangements involving retrotransposons and microsatellites related sequences include both repetitive and coding sequences, and revealed a preferential loss of rye-origin bands. Chromosome distribution of such marker sequences demonstrated moreover enrichment in heterochromatic domains. Intensification of rye genome rearrangements was also disclosed in wheat lines with disomic additions of rye chromosomes, probably due to unbalanced genomic constitution.

An integrative review of genomic modulation evaluated in Triticeae hybrid/polyploidy species unravelled furthermore higher restructuring of larger parental genomes, emphasizing the importance of genome size homogenization.

Epigenetic analysis of nucleolar dominance in wheat addition line with rye nucleolar chromosomes revealed an unexpected up-regulation of ribosomal genes from wheat-origin, suggesting that mutual and opposite expression patterns modifications are induced by genome interactions.

Altogether we demonstrate that heterochromatic domains are highly involved in parental genomes adjustments required to polyploids stabilization.

Key-Words: Polyploidization, Triticeae, Triticale, Wheat, Rye, Wheat-rye addition-lines, Retrotransposons, Microsatellites, Genome Restructuring, rDNA expression.

Título: Caracterização da plasticidade genómica de poliplóides no sistema trigo-centeio.

Resumo

A poliploidização é uma das principais forças evolutivas em plantas, sendo essencial um conhecimento profundo dos processos subjacentes às alterações genómicas e epigenéticas associadas a este processo evolutivo.

O triticale foi utilizado como espécie modelo para avaliar o impacto da poliploidização utilizando técnicas moleculares e citológicas. Identificaram-se rearranjos genómicos envolvendo sequências repetitivas e codificantes associadas a retrotransposões e microssatélites, afectando preferencialmente o genoma de centeio. A distribuição cromossómica dessas sequências demonstrou a sua predominância em domínios heterocromáticos. Paralelamente, em linhas de trigo com a adição dos cromossomas de centeio, observou-se uma intensificação de rearranjos nesse genoma, provavelmente resultante do desequilíbrio genómico parental.

A integração dos estudos realizados em híbridos/poliplóides pertencentes à tribo Triticeae demonstrou a reestruturação preferencial do genoma parental maior, realçando a importância da homogeneização genómica.

A análise epigenética dos genes ribossomais na linha de trigo com introgressão de cromossomas nucleolares de centeio, indica que o processo de dominância nucleolar induz modificações mútuas dos padrões de expressão genica dos progenitores.

Este trabalho enfatiza assim a importância da modulação dos domínios heterocromáticos parentais na estabilização dos organismos poliplóides.

Palavras-chave: Poliploidização, Triticeae, Triticale, Trigo, Centeio, Linhas de Adição trigo-centeio, Retrotransposões, Microssatélites, Reestruturação Genómica, expressão de rDNA.

Preamble

Polyploidization occurred throughout the evolutionary history of all eukaryotes, especially in flowering plants including many important crops. Such process is an important speciation mechanism with profound impacts on biodiversity and ecosystems. Hybridization and chromosome doubling induce significant genetic and epigenetic modifications but underlying mechanisms need yet to be disclosed (Chen 2007; Ainouche and Jenczewski 2010).

The research project we developed intend to contribute for a deeper understanding of polyploidization consequences at the genomic and epigenetic levels, studying some hybrid genotypes of the wheat-rye system, as the synthetic polyploid triticale and the entire set of wheat lines with the disomic addition of rye chromosomes.

The major goal of this project was to disclose modifications induced by polyploidization at the genomic level through comparative analysis of triticale with the respective wheat and rye parental lines. With such purpose PCR-based techniques were adopted, namely Inter Retrotransposons Amplified Polymorphism (IRAP), Retrotransposons Microsatellite Amplified Polymorphism (REMAP), and Inter Simple Sequence Repeat (ISSR) which target repetitive elements with high copy number in plants genomes as retrotransposons and microsatellites. In this study we pretend to evaluate the rate of genomic rearrangements induced in triticale and characterize the sequences involved. The genomic effects of the introgression of smaller rye chromatin fractions in wheat background was also pursued through the analysis of the entire set of wheat-rye addition lines produced by triticale backcross to wheat parental line followed by selfing generations.

Polyploidization is known to affect also repetitive coding sequences in hybrid genotypes through epigenetic remodeling. One of the best known phenomenon of gene expression modulation in polyploid organisms is nucleolar dominance, where only one parental set of ribosomal rRNA genes are transcribed (dominant NORs), whereas rRNA genes inherited from the other parent are silent (under dominant

NORs). While the behaviour of the under dominant NORs was already extensively analyzed, that of dominant NORs was never assessed. The transcriptional activity of wheat dominant NORs in the wheat-rye system was investigated using quantitative real-time PCR in the wheat line with the addition of rye nucleolar chromosome pair 1R.

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Chapter I

Introduction

1. Introduction

1.1. Genomes organization

In plants, genome size may vary as much as 1000-fold, ranging from 125 Megabases (Mbp) in *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000), to 12 000 Mbp in *Triticum durum* (Boyko et al., 1984) or 123 000 Mbp in *Fritillaria assyriaca* (Bennett and Smith, 1976). Most of the large plant genomes are mainly composed by repeated sequences and a small fraction of single or low copy sequences (Li et al., 2004b). Recently, an integrative view of plant genomes as systems whose architecture and organization is structured by repetitive elements was developed allowing a deeper understanding of theirs importance and roles (Morgante, 2006).

Triticeae genomes, on average, contain about 8% genes and gene related sequences and as much as 90% or even slightly more of repetitive sequences (reviewed in Dvořák, 2009). Repetitive DNA sequences were extensively studied in some large cereal genomes from three different Triticeae clades, namely *Hordeum vulgare*, *Triticum monococcum*, and *Secale cereale*, representing 76%, 80%, and 92%, respectively (Flavell et al., 1974).

Repetitive sequences comprehend two basic categories: tandem repeated sequences and interspersed sequences. The first category includes satellite DNA (repeated sequences present as long uninterrupted arrays); microsatellite or simple sequence repeats (short repeats with one to six base pairs); and ribosomal DNA (45S and 5S rRNA genes). The second class consists mainly of transposable elements (TE) (Li et al., 2004a).

1.1.1. Tandem repetitive sequences

Tandem repeats may have high copy numbers but usually only account for a relatively small portion of the genome (Li et al., 2004a). Tandem repeats are mainly located in centromeric and telomeric domains of Triticeae chromosomes, but can also be present in interstitial heterocromatic domains (Dvořák, 2009).

Although it was not yet conclusively proved that tandem repeats are sufficient or necessary for centromere function, a prominent role in their correct chromatin conformation was suggested (Lamb et al., 2007). Tandem repeats presence in centromeric regions was also confirmed in several other species, such as rice and *Arabidopsis*, but primary sequence and organization is highly variable even among closely related species (Dvořák, 2009). Sequence motifs of centromeric repeats are usually distinct from those present in subtelomeric regions, although some repeat families may be present in subtelomeric regions in one species and in centromeric regions of others (Dvořák, 2009).

Telomeric regions of barley (Roder et al., 1993), rye (Schwarzacher and Heslop-Harrison, 1991) and wheat (Werner et al., 1992) chromosomes were shown to be capped with TTTAGGG sequence repeats, the most common telomeric repeat in plants (Lamb et al., 2007) first characterized in *A. thaliana* (Richards and Ausubel, 1988). The number of those repeats ranges between 2-5 Kbp in *Arabidopsis* (Shakirov et al., 2005), is higher than 25 Kbp in barley (Roder et al., 1993), varies between 8-50 Kbp in rye, and between 15-175 Kbp in wheat (Vershinin and Heslop-Harrison, 1998).

Juxtaposed to the arrays of telomeric repeats are telomere-associated sequences (TAS). The presence of TAS was detected in all Triticeae species investigated to date. Different numbers of TAS families are present in distinct Triticeae species, probably due to divergence, amplification, and deletion processes which occurred in distinct Triticeae lineages (Dvořák, 2009). TAS sequences were firstly isolated in rye and classified accordingly to repetition periodicities into the following four major families: 120 bp, 480 bp (renamed 350-family since this one was more common among Triticeae), 610 bp and 630 bp (reviewed in Dvořák, 2009). In rye, TAS are present in high copy number, representing 8 to 12% of the genome (Bedbrook et al., 1980). Arrays of the 120 bp family are often located in interstitial regions but the remaining rye TAS families are almost exclusively on sub-telomeric domains (Jones and Flavell, 1982). Later on such TAS families were also mapped in other genomes from the Triticeae tribe. For instance, the 120 bp family present in $1.5x10^6$ copies in *S. cereale* genome, was also detected in reduced amounts in subtelomeric domains of *Triticum*, *Aegilops*, *Hordeum*, and *Critesion* chromosomes. The 350-family present at least in $2x10^5$ copies in *S. cereale*, was observed in other Triticeae genera, such as *Agropyron*, *Critesion*, *Australopyrum*,

Pseudoroegneria, *Psathyrostachys*, and *Leymus*. New TAS families were further identified in other Triticeae, such as in *Ae. tauschii* (pAS1/pTA1/Afa family with 340 bp), *Leymus racemosus* (*pLrTai-I* family with 570 bp), and *Hordeum* spp. (HvT01 family with 118 bp) (reviewed in Dvořák, 2009).

Microsatellites or simple-sequence repeats (SSRs) are widespread in eukaryotic genomes. Microsatellites consist of short motifs of one to six base pairs, repeated in tandem arrays up to 100 bp with identical, composite or degenerate motifs, and distributed through the whole genome (Chambers and MacAvoy, 2000). SSRs are highly abundant within genomes, and present high variation in the number of repeats in different *loci* and between individuals (Cuadrado and Schwarzacher, 1998). It has been estimated that on average a dinucleotide repeat longer than 20 bp in length occurs every 33 Kbp in plant genomes (Wang et al., 1994). Although microsatellites are present in both non-coding and coding regions, their frequency is higher in transcribed regions, especially in untranslated ones (UTRs) (Rakoczy-Trojanowska and Bolibok, 2004). In cereals 1.5%–7.5% of expressed sequences tags (ESTs) consist of SSRs (Li et al., 2004b). Trinucleotide repeats are the most common motifs in SSRs, followed by either dinucleotide or tetranucleotide repeats (Varshney et al., 2005). In the majority of plant species, AAG is the most frequent triplet motif, although CCG is the commonest one in cereal species (reviewed in Li et al., 2004b).

Ribosomal genes are also organized in tandem repeated sequences. 45S rRNA genes are organized in tandem arrays of rDNA units clustered at the nucleolus organizer regions (NORs). In plant genomes, each unit with approximately 10 Kb comprehends 18S, 5.8S, and 25-28S coding sequences, as well as transcribed and nontranscribed spacers (Heslop-Harrison, 2000). Hundreds or thousands of copies of 45S rRNA genes can be present in plant genomes, representing up to ~10% in some species (~8% in *Arabidopsis*) (Rogers and Bendich, 1987). 5S rDNA units usually consist of a highly conserved 120 bp coding region and a nontranscribed spacer of variable length. The number of 5S rDNA repeats *per* genome can be over 75 000 (Sastri et al., 1992) organized in tandem at one or more *loci* (Dvořák, 2009; Heslop-Harrison, 2000).

Transcription and processing of 45S and 5S ribosomal rRNA genes and assembly of ribosomal sub-units occurs in the nucleolus, the largest and most visible nuclear domain. The nucleolus contains rRNA transcripts at different maturation stages and a multitude of proteins required for ribosomal subunits assemble (reviewed in Brown and Shaw, 2008). The nucleolus also has functions in processing and assembly a variety of ribonucleoprotein particles associated with cell cycle control and senescence as well as stress sensors. Furthermore, nucleoli might also be involved in transcriptional gene silencing, mRNA surveillance and nonsense-mediated decay, and mRNA export (reviewed in Brown and Shaw, 2008).
In *S. cereale* a single 45S *Nor locus* is present on chromosome 1R (Appels et al., 1980), while *T. aestivum* has six *Nor loci* present on chromosomes 1A, 5A, 1B, 6B, 5D and 7D (Mukai et al., 1991; Jiang and Gill, 1994). NORs located on chromosomes 1B and 6B comprehend 90% of total wheat rDNA sequences and are responsible for 90% of rRNA transcripts, and hence are called *major* NORs. By opposition, the remaining are designated as *minor* NORs (Appels et al., 1980; Martini and Flavell, 1985). Nucleolar volume is poorly correlated with the number of ribosomal RNA genes allocated in each NOR domain, being mainly associated with the number of active rRNA genes present (Flavell and Odell, 1979; Martini and Flavell, 1985).

1.1.2. Transposable elements

Interspersed repeated sequences correspond mainly to transposable elements (TEs), which are ubiquitous in all organisms and drive the structure, function, and evolution of genes and genomes (Bennetzen, 2000). TEs were discovered by Barbara McClintock in 1944 while studying the effect of X-rays in maize chromosomes. She observed high levels of maize seedlings with bizarre phenotypes associated with distinct chlorophyll intensities and distribution patterns in defined leaf sectors. Latter on McClintock correlated such phenotypes with gene expression regulation mediated by mitotic events that lead one daughter cell to gain something that the other cell has lost. She called such elements “controlling elements” and proved that they could transpose or change position on chromosomes (McClintock, 1984).

In general terms, TEs are stretches of DNA that move throughout the genome and typically fall into two basic classes, based on their transposition intermediate: RNA (retrotransposons or class I) and DNA (DNA transposons or class II).

Retrotransposons move via RNA intermediates by a 'copy-and-paste' mechanism and are subdivided into long-terminal repeat (LTR) and non-LTR type. LTR-Retrotransposons are further divided into *Copia* and *Gypsy* accordingly with the architecture of the coding sequence (composed of GAG-Capsid protein, Aspartic proteinase, Integrase, Reverse transcriptase and RNase H genes). Terminal-repeat retrotransposons in miniature (TRIMs) also belong to LTR-Retrotransposons but lack coding sequences and thus are non-autonomous. Non-LTR retrotransposons are divided into long (LINE) and short (SINE) interspersed nuclear elements.

DNA transposons move via DNA intermediates, by either a 'cut-and-paste' mechanism or DNA replication. Most DNA transposons are bordered by terminal inverted repeats (TIRs) and are either autonomous (with the transposase gene) or non-autonomous (lacking the transposase gene). Miniature inverted-repeat transposable elements (MITEs) represent a heterogeneous group of very short TEs and finally Helitrons are TEs that replicate via a rolling-circle mechanism (reviewed in Wicker et al., 2007).

TEs represent a considerable fraction of plant genomes, especially in plants with big genomes. Maize is composed of approximately 50% of TEs, in a proportion of 98% retrotransposons and 2% DNA transposons (Meyers et al., 2001) and in *Aegilops tauschii* genome TEs represent ~68%, corresponding 78% to retrotransposons and 22% to DNA transposons (Li et al., 2004a). On the other hand, *Arabidopsis thaliana* comprehends 5.6% of retrotransposons in its genome (Peterson-Burch et al., 2004) and 16% TEs are present in the rice genome, represented by 88% of retrotransposons and 12% of DNA transposons (Mao et al., 2000).

TEs can be a source of mutations, deletions, insertions, frameshifts, inversions, translocations and duplications. Because of their properties TEs greatly contribute to structural repatterning of genomes and can also affect gene expression and function by inserting into genic regions. Furthermore, TEs expression and mobility seem to respond to specific stimuli, such as environmental stress conditions (Bennetzen, 2000).

In *Arabidopsis* both *Copia*-like and *Gypsy*-like retrotransposons tend to cluster within pericentromeric heterochromatin. This association is significantly pronounced for all three *Gypsy*-like retrotransposons identified (Metavirus, Tat and Athila), and Tat and Athila are strictly associated with pericentromeric heterochromatin (Peterson-Burch et al., 2004). In Triticeae species many TEs are non-randomly distributed along chromosomes as shown in maize (SanMiguel et al., 1996) and wheat, where several intergenic sequences are enriched in LTR retroelements, showing a complex nested insertion pattern (Wicker et al., 2001). Complete and truncated copies of *Gypsy*-like retrotransposons were shown to exist in primary constrictions of barley, wheat, *Aegilops*, and rye chromosomes (Dvořák, 2009). In *A. tauschii*, each TE family either has specific chromosomal territories or shares them with other families. In such regions where distinct TE families coexist, nested insertions and illegitimate recombination can occur extensively between families, probably leading to internal deletions, usually detected in a vast majority of TEs (Li et al., 2004a). In Triticeae species CACTA DNA transposons tend to be associated with genes (Wicker et al., 2003), and MITEs are usually inserted into genes or their immediate surrounding sequences (Sabot et al., 2005) and the same is observed in other cereal grasses (Bureau and Wessler, 1994). A bias insertion of TEs is responsible for nonrandom distribution observed among TE families within chromosomes. Such is the case of centromere proximal regions of *Ae. tauschii* chromosomes enriched in LTR retrotransposons Laura and Erica while centromere distal regions are enriched in CACTA family Caspar (Li et al., 2004a).

1.2. Polyploidization as an evolutive process

In polyploids more than two basic sets of chromosomes (genomes) share the same nucleus. Polyploidization is a natural process that occurs spontaneously and can also be artificially recreated. Accordingly with genomes origin polyploids can be classified into autopolyploids or allopolyploids and can comprehend different ploidy levels. Autopolyploids result from genome doubling within the same

species, and allopolyploids are formed by the combination of two or more distinct but usually related genomes (Ma and Gustafson, 2008).

Two prevailing models can be considered for the natural emergence of allopolyploids, namely: the “two-step” model involving interspecific hybridization followed by chromosome doubling of the F1 hybrid; and the “one-step” model based in fertilization of unreduced gametes from different diploid species or in direct interspecific hybridization between distinct autotetraploid species (Chen, 2010). As a small amount of unreduced gametes usually occur via restitution in the first or second meiotic division, the “one-step” model is considered the predominant one in natural conditions. Although spontaneous chromosome doubling is a rare event in nature (Chen and Ni, 2006), such procedure is the most common process used to produce synthetic polyploids, due to the discovery of colchicine chromosome-doubling properties (Blakeslee and Avery, 1937).

Polyploids are very common among plants and rare among animals, however some polyploid organisms exist among invertebrates (insects) and vertebrates (fish, amphibians and reptiles). The South American rodent *Tympanoctomy barrerae* is the unique polyploid mammal reported until now, although its tetraploid nature is still controversial (reviewed in Chen, 2007).

Polyploidy provides an obvious route to the rapid emergence of new species. Increased gene and genome dosages in autopolyploids and allopolyploids induce however genome instabilities and disrupting effects, such as nucleus and cell enlargement, regulatory incompatibilities, propensity for production of aneuploid cells through mitosis and meiosis, and reproductive failures (Chen, 2007; Comai, 2005). Polyploid organisms able to overcome such instabilities can become efficient competitors to the diploid relatives. Well documented polyploidy advantages are heterosis, gene redundancy, loss of self-incompatibility and gain of asexual reproduction (Comai, 2005).

Heterosis or hybrid vigor describes polyploids higher fitness of a polyploidy when compared with the parental species, encompassing higher biomass, stature, growth rate, and/or fertility. The level of heterosis in hybrids is directly dependent of the genetic distance between parents, leading higher genetic distances to greater heterosis. Heterosis major advantage in polyploids is the ability to diversify gene function by altering redundant copies of important or essential genes (Comai, 2005). *Arabidopsis suecica* (2n = 4x = 26) is an example of a natural allotetraploid,

formed between *A. thaliana* (2n = 4x = 20) and *A. arenosa* (2n = 4x = 32) ~12 000 to 300 000 years ago, that presents organs with higher dimensions in comparison with the parental species (Chen, 2010).

The study of circadian-mediated metabolic pathways revealed important clues regarding epigenetic regulation of growth vigor in hybrids and allopolyploids. Ni et al. (2009) studying natural *A. suecica* and the corresponding syntetic polyploid observed that the epigenetic repression of circadian clock genes, *Circadian Clock Associated 1* (*CCA1*) and *Late Elongated Hypocotyl* (*LHY*), during the day induces the expression of reciprocal regulators, *Timing of Cab Expression 1* (*TOC1*) and *Gigantea* (*GI*), and also of downstream genes involved in chlorophyll and starch metabolic pathways. The authors suggested that such alterations are correlated with the enhanced production of chlorophyll and starch detected in allotetraploids and F1 hybrids in comparison with parental lines, explaining the higher growth vigor and increased biomass.

1.2.1. Natural polyploids

Polyploidization is a frequent event throughout plant evolution. It has been estimated that 30 to 70% of plants species have a polyploid origin, assessment that reaches close to 100% if paleopolyploids are included (Wendel, 2000; Wolfe, 2001). Recent studies revealed that many species, such as maize (*Zea mays* L.), *Arabidopsis*, soybean (*Glycine max* L.), cotton (*Gossypium*), and sorghum (*Sorghum bicolor* L.), traditionally recognized as diploids, are ancient polyploids (paleopolyploids) (reviewed in Ma and Gustafson, 2005). The estimated proportion of polyploid flowering plants is higher than 70%, being the vast majority (75%) allopolyploids (Chen, 2010).

Many important crops such as *Brassica,* wheat, cotton, oats, canola (or oilseed rape), potato, peanuts, sugarcane, coffee, strawberry and banana are also polyploid species (Chen, 2010). The genus *Brassica* is the perfect example of reciprocal hybrids and allopolyploids formed between three diploid species, which is known as U-triangle. The three ancestral diploid species, *B. nigra* (BB, 2n=2x=16), *B. oleracea* (CC, 2n=2x=18), and *B. campestris or rapa* (AA, 2n = 2x = 20), combined and created the following contemporary allotetraploid species: *B.*

napus (AACC, 2n = 4x = 38), *B. juncea* (AABB, 2n = 4x = 34), and *B. carinata* (BBCC, 2n = 4x = 36) (U, 1935).

Wheat is another classical model species in the study of evolution mediated by poliploidization. The genera *Aegilops* and *Triticum* include several diploid species (2n = 14) which produced through allopolyploidization several tetraploid and hexaploid species, most of which have been domesticated (Salse et al., 2008). Durum wheat or pasta wheat (*T. turgidum*, AABB, 2n = 4x = 28) is an allotetraploid formed approximately half a million years ago from crosses between two diploid wild grasses, namely *T. urartu* (AA, 2n = 14) and the donor of the B genome (BB, 2n = 14). The exact donor of the B genome is still unknown, although wild goatgrass *Ae. speltoides* is believed to be the closest known relative (Chen, 2010; Feldman and Levy, 2005; Salse et al., 2008). Approximately 8000–10 000 years ago, hexaploid wheat or bread wheat (*T. aestivum*, 2n = 6x = 42, AABBDD) was probably formed in farmers fields through hybridization between a domesticated tetraploid wheat and the wild diploid grass *Triticum tauschii* (DD, 2n = 14) (Chen, 2010).

Initially it was considered a unique origin for each polyploid species, although later evidences clearly indicated recurrent origins in several polyploids, represented by multiple lineages independently formed (Soltis and Soltis, 1999), as described in some plant *taxa* such as *Brassica*, *Gossypium*, *Senecio*, *Spartina*, *Tragopogon* and *Triticum*. *Tragopogon miscellus* and *T. mirus*, for instance, may have formed as many as 20 and 12 times, respectively, only in the past 60–70 years in eastern Washington and adjacent Idaho (USA), and *Draba norvegica* (Brassicaceae) has formed at least 13 times in a small area of Scandinavia (Soltis and Soltis, 1999). *T. aestivum* has also multiple origins since at least two genetically distinct progenitors have been proved to contribute with D genome (Caldwell et al.; 2004). Nevertheless, some polyploid species such as *Arachis hypogaea*, *Spartina anglica*, *Gossypium hirsutum*, and *Arabidopsis thaliana* seem to result from a single or few hybridization events (Chen, 2010; Soltis and Soltis, 1999; Soltis and Soltis, 2000).

Multiple origins may represent an enhanced source of genetic diversity and when distinct polyploid lineages come into contact and hybridize, subsequent segregation and recombination amplifies even more such variability (Soltis and Soltis, 2000). New allopolyploids may moreover cross-hybridize to diploid or

autopolyploid progenitors, leading to zones with high genetic diversity, where polyploids, progenitors and intermediate forms coexist (Chen and Ni, 2006).
Hybridization can also increase the potential of progeny invasiveness when compared with progenitors. Plants with a history of hybridization may have fitness advantage, presenting enhanced adaptiveness to new ecological niches (Ellstrand and Schierenbeck, 2000). Allopolyploid *Spartina townsendii*, an hybrid between *S. alternifolia* and *S. stricta* genomes, for instance is so vigorous that replaced the parental forms and spread all over southern England and France (Baumel et al., 2001). Increased invasiveness potential was also observed in *Senecio* polyploids. The diploid *S. squalidus* native in France, after its introduction in Britain, hybridized with native *S. vulgaris* originating the allopolyploid *S. cambrensis* that spread throughout Wales and England (Abbott and Lowe, 2004).

1.2.2. Synthetic polyploids

Many polyploids are ancient, and their exact progenitors are often unknown. Thus, newly synthesized polyploids produced with known progenitors are the best models to study processes underlying the emergence of polyploid species. This approach has been widely applied in many species, such as wheat (*Triticum* ssp.), triticale (X-*Triticosecale* Wittmack), *Arabidopsis*, *Brassica*, and cotton (*Gossypium* ssp.) (Dong et al., 2005; Liu et al., 2001; Ma et al., 2004; Madlung et al., 2005; Ozkan et al., 2001; Salmon et al., 2005).
Synthetic *Brassica* polyploids derived from reciprocal interspecific hybridizations of single plants of diploid species *B. rapa* (A genome), *B. nigra* (B genome), and *B. oleracea* (C genome), designated as AB (A x B), BA (B x A), AC (A x C), and CA (C x A), were used to identify the phase of genomic changes emergence and to evaluate the influence of cytoplasmic-nuclear interactions (Song 1995). Many other synthetic *Brassica* polyploids were produced to study genetic and epigenetic modifications induced by polyploidization (Albertin et al., 2007; Gaeta et al., 2007; Lukens et al., 2006). To evaluate chromosome evolution in recently formed polyploids, the synthetic allotetraploid *Tragopon mirus* (*T. dubius* x *T. porrifolius*) was produced and compared with natural populations (<150 years old) (Lim et al., 2008). Several other studies in *Tragopon* polyploids were oriented to compare

mating systems (Cook and Soltis, 2000) or expression of homoeologous *loci* between natural and synthetic polyploids (Tate et al., 2006).

New synthetic allotetraploids of *Arabidopsis suecica* (2n = 4x = 26) were produced by crossing the spontaneous autotetraploid *A. thaliana* line (2n = 4x = 20), isolated from tissue culture (Valvekens et al., 1988), and *A. arenosa* (2n = 4x = 32) (Comai, 2000). These synthetc lines were used to study genomic changes (Madlung et al., 2005), epigenetic modulation (Wang et al., 2004) and the role of small RNA in allopolyploid plants (Chen et al., 2008).

Species from the genera *Aegilops* and *Triticum* are also important models in polyploidy studies, comprising many allotetraploid and allohexaploid species with marked economic importance. Synthetic polyploid wheat species are easy to produce and already allowed relevant comparisons on nuclear DNA amount determined by flow cytometry (Eilam et al., 2008) and amplified fragment length polymorphism (AFLP) profiles (Shaked et al., 2001) between polyploids and parental species.

Due to its polyploid nature bread wheat genome is very plastic and tolerates structural and numerical chromosome changes magnificently explored by E.R. Sears (1954) to produce and select several series of aneuploid lines, such as: monosomic lines in which a pair of chromosomes is represented by only one homologue (2n=6x=41); nullisomic-tetrasomic lines in which a pair of homologous chromosomes is missing and is compensated by four copies of a homoeologous chromosome (2n=6x=42); ditelosomic lines where a chromosome pair is represented by two telocentric chromosomes, either from the short or long arms (2n=6x=40+2t); and double-ditelosomic lines in which one chromosome pair is represented by a pair of telocentrics from each arm (2n=6x=40+4t) (Faris et al., 2002).

The crossability of bread wheat with related species allows the addition of whole genomes or the introgression of individual alien chromosomes to the wheat complement. Dissecting the genome of a related species in the form of wheat-alien addition, substitution and translocation lines allows therefore the functional analysis of individual alien chromosomes or chromosome segments in wheat background. The use of gametocidal (*Gc*) factors allowed moreover the construction of wheat lines with deleted chromosomes. Some chromosomes that were introduced into *Triticum* by interspecific hybridization with related *Aegilops*

species have *Gc* genes, which guarantee their transmission maintaining chromosome stability in gametes. Plants monosomic for *Gc* chromosome produce normal gametes when Gc chromosome is present, or non-functional gametes with structural chromosome aberrations in its absence. However, when the damage caused by chromosome breakage is insufficient to derail the gamete, chromosome deletions can be transmitted to the offspring (Endo, 2007). The use of the described gametocidal factors (*Gc*) was also used to produce wheat lines carrying deleted chromosomes from related species, like rye or barley, as well as wheat-alien translocations lines (Endo, 2007).

Triticale, the first synthesized amphidiploid cereal, is a chromosome-doubled intergeneric hybrid obtained through the cross of distinct wheat species (*Triticum* spp., AA, AABB, and AABBDD) and rye (*Secale cereale* L., RR), producing various genome combinations and ploidy levels, such as tetraploid AARR, hexaploid AABBRR and octoploid AABBDDRR. When compared with other allopolyploids, triticale is a very complex genome because of its high ploidy level, large genome size and parental genomes filogenetic distance (Ma et al., 2004). Triticale is a however very useful model species to study evolutive processes mediated by polyploidization due to accumulated pedigree knowledge. Synthetic triticale has furthermore been used in the production of wheat–rye addition lines. By backcrossing triticale to parental wheat and selfing for several generations, individuals with a single chromosome pair from rye (wheat-rye addition lines) can be produced. During meiosis of plants produced by the backcross, the two sets from wheat chromosomes undergo normal pairing and segregation, whereas rye chromosomes remain as univalents and are randomly segregated. Gametes from such plants contain therefore a full set of chromosomes from wheat, and from none to a full set of rye chromosomes and further repeatedly selfing induces the reduction of rye chromosomes (O'Mara, 1940).

Polyploid formation places the hybrid under considerable genomic stress, leading to genetic and epigenetic modifications. It has been suggested that allopolyploid genomes underwent two different phases: a Revolutionary phase, occurring immediately after hybridization, responsible for rapid genetic and epigenetic changes; and an Evolutionary phase that corresponds to long term events, such as slow changes in DNA sequences and functional alterations (Feldman and Levy,

2005; Levy and Feldman, 2002). The term “diploidization” has been used to describe revolutionary alterations that occur during the formation of a newly formed polyploid, including both genetic and epigenetic events, and which facilitate the establishment of the newly formed allopolyploid (Feldman and Levy, 2009).

1.3. Genomes remodeling in polyploids

Polyploid formation generates several adjustments inducing an irreversible burst of paternal genomes reorganisation and modification (Jones and Hegarty, 2009). Cytogenetic studies provided the earliest evidences of genomic shock consequences and meiotic instabilities inherent to newly formed hybrids and polyploids (McClintock, 1929; Navashin, 1934).

Meiotic instabilities can origin aneuploid gametes, more characteristic in autopolyploids and F1 hybrids than in allopolyploids. Aneuploid frequencies vary between species and accordingly to polyploid type (Comai, 2005). Studies performed in *Arabidopsis* and *Trapogonon* polyploids support the idea that aneuploidy is a direct result of meiotic dysfunction since a variety of karyotypic changes is observed in gametes (Lim et al., 2008; Madlung et al., 2005). Analysis of reproduction in newly synthesised *Tragopogon* allopolyploids revealed an initial redution or absence of fertility, followed by gradual increases in the following generations (Lim et al., 2008).

The occurrence of homoeologous recombination depends on the history of allopolyploids, since it can be frequent in newly formed allopolyploids, contributing to genome restructuring, and is rare in established allopolyploids (Comai, 2000). Also in *Tragopogon* polyploids the study of natural populations of allotetraploids *T. mirus* and *T. miscellus* suggest that chromosomal rearrangements are common and emerged soon after polyploidization (Lim et al., 2008). Although homoeologous exchanges occur more frequently during early stages of allopolyploid evolution, the process may continue over millions of years like in *Nicotiana* allopolyplois, where chromosomes are mosaics of homoeologous segments (Lim et al., 2007). Homoeologous chromosomes exchanges underlie the

creation of novel allele combinations, enhancing phenotypic variation in newly formed *B. napus* polyploids (Gaeta et al., 2007; Udall et al., 2005).
Chromosome homologous pairing in allopolyploid meiosis may however be enforced by genetic mechanisms. In *Triticum turgidum* and *T. aestivum*, *Ph1* (*homologous pairing*) gene is required to avoid homoeologous pairing and certainly represents an important adaptation to polyploidy. In the presence of *Ph1* gene chromosome pairing is restricted to homologous chromosomes leading to the diploid-like behaviour characteristic of wheat allopolyploid genome (Gaeta and Pires, 2010). A similar system was suggested to exist in allotetraploid *Brassica* species (Comai, 2005).

Chromosome alterations were visualized in rye-origin chromosomes of triticale through C-banding, represented by reduction of telomeric bands or absence of interstitial C-bands (Jouve et al., 1989). Subtelomeric heterochromatin deletions were also observed through fluorescent in situ hybridization (FISH) in triticale and wheat-rye addition and substitution lines, accompanied by a reduction of rye subtelomeric pSc200 sequence FISH signals (Alkhimova et al., 1999; Cuadrado and Jouve, 1994).
The study of F8 generation of tetraploid *Lolium perenne x Festuca pratensis* (2n=4x=28) using genomic in situ hybridization (GISH) revealed extensive recombination between homoeologous chromosomes of both genomes. Substitution of *Festuca*-origin chromosomes by the *Lolium*-origin ones resulted in a mean chromosome number of 17.9 from *Lolium* and 9.7 from *Festuca* instead of the normal 14 chromosomes for each parental genome. Concordantly, imbalanced chromatin content was also detected (62,1% from *Lolium* and 37,9% from *Festuca*) revealing genome conflict situation throughout 8 cycles of sexual reproduction, in favour to the dominant *Lolium* genome (Canter et al., 1999). *Lolium* genome-dominant behaviour was confirmed more recently in a tetraploid between *F. pratensis* and *L. multiflorum* (Jones and Hegarty, 2009), and in a tetraploid between *F. pratensis* and *L. perenne* (Zwierzykowski et al., 2006*)*, although reasons underlying such behaviour are not yet understood (Jones and Hegarty, 2009). Similar results were also reported in *Triticum* (2n = 4x = 28) x *Leymus* (2n = 4x = 28) hybrids, as allopolyploids stabilised with six chromosome pairs of *L. mollis* origin and 15 chromosome pairs from wheat origin (Anamthawat-

Jonsson, 1999). Inter-genomic invasion of chromatin segments was also observed in allotetraploid wheat *Triticum dicoccoides* (2n = 4x = 28, genome AABB), where part of A-genome heterochromatic clusters is replaced by satellite DNA from B genome, resulting in B-genome sequences enrichment (Belyayev et al., 2000).

Polyploidization has generally been assumed to induce a burst of transposition events, but results gathered so far revealed that it is not clear. TE-related genome restructuring events were studied through comparisons of hybrids and allopoplyploids with respective parental genomes using sequence-specific amplified polymorphism (SSAP). Polyploid-specific SSAP fragments detected in distinct polyploids and absent in parental diploids are often considered as indicative of transposition. However, since those new fragments may simply result from molecular changes of TE sequences, TEs transposition must be additionalty validated (Parisod et al., 2010). In natural allopolyploid *Spartina anglica* scarce evidence of transposition in natural populations was identified. Even though, few possible new TEs insertions were suggested to occur in the allopolyploid, but a transposition burst did not occur in the TE families investigated (Parisod et al., 2009). The same was concluded for the natural allopolyploid *A. suecica* (Hazzouri et al., 2008). In various synthetic allopolyploids of *A. suecica*, although TEs transcriptional activity was detected, only a few transposition events involving *Sunfish* DNA transposons could probably occurred (Madlung et al., 2005).
On the other hand, evidences of amplification of Tnt1 retrotransposon (a young and active TE element) was detected in synthetic allopolyploids of *N. tabacum*, although interestingly TEs proliferation was only detected in the fourth generation (Petit et al., 2010). In synthetic *B. napus* up to 20% non-additive SSAP fragments involving MITEs were detected, being transposition events only rarely recognised (Parisod et al., 2010).
Structural rearrangements after polyploidization targeting TEs genome fraction seem to preferentially affect one parental genome. For instance, in synthetic *N. tabacum*, structural changes are revealed predominantly by losses of SSAP fragments of paternal origin (Petit et al., 2010). Contrastingly, in natural *S. anglica* losses of SSAP fragments from maternal origin seem to predominate, occurring mostly after hybridization (Parisod et al., 2010) rather than after genome doubling as observed in synthetic *N. tabacum* (Petit et al., 2010).

In the synthetic allopolyploid between *Ae. sharonensis* and *T. monococcum* the study of Wis 2-1A retrotransposon revealed no evidence of polyploidy induced transposition (Kashkush et al., 2003). The same conclusion was achieved in the first three generations of synthetic *T. aestivum*, in relation to seven retrotransposon and one CACTA DNA transposon (Parisod et al., 2010). Globally, the described results indicate that although TE-related restructuring events were detected in some polyploid species, a burst of transposition immediately after polyploidization is not a common phenomenon (Parisod et al., 2010).

1.4. Epigenetic changes in polyploids

Epigenetics refers to heritable changes in gene expression that occur without DNA sequence changes, but which may result in significant morphological and genetic consequences. Many processes are associated with epigenetic phenomena, such as DNA methylation, histone modification and small RNA.

The best-studied epigenetic marker is cytosine methylation, the most important post-replication modification of DNA in eukaryotes, that regulates gene expression and is implicated in chromatin organization and genomic imprinting (Finnegan et al., 1998). In plants, the most commonly methylated sites are dinucleotide CpG or trinucleotides CpNpG (Gruenbaum et al., 1981). Several evidences showed that genome methylation re-patterning is commonly induced by polyploidization (reviewed in Ma and Gustafson, 2005), although results obtained are extremely variable between different polyploid species (Doyle et al., 2008). Changes in methylation patterns are not only associated with altered gene expression but may also involve repetitive, non-coding and unknown sequences (Ma and Gustafson, 2005). Furthermore, altered patterns of genome methylation status induced through polyploidization can ultimately contribute to facilitate the process of diploidization (Ma and Gustafson, 2005).

Song et al. (1995) reported apparently random changes in polyploid genome methylation patterns in *Brassica* allotetraploids when compared with diploid parents, including hyper and hypomethylation at CpG or CpNpG sites. Those

results were later confirmed in 49 synthetic lines of *B. napus*, unraveling CpG methylation changes on 2% to 7% of the sequences analyzed by methylation-sensitive AFLP technique (Lukens et al., 2006). Reexamination of 47 of those lines after four generations showed ~3% of additional changes (Gaeta et al., 2007).

The study of synthetic *Arabidopsis suecica* lines suggest that methylation patterns are subject to widespread modifications corresponding predominantly to DNA de-methylation events. Madlung et al. (2002) using also methylation-sensitive AFLP analysis, detected ~8% differences between parentals and the F3 progeny, resulting 63% of them from DNA de-methylation. In the initial F1 hybrid between *Spartina alterniflora* and *S. maritime* ~30% of parental methylation profiles were non-additive. Most of DNA methylation modifications detected in the F1 hybrid (~71%) were also present in the equivalent natural polyploid *S. anglica*, suggesting that methylation changes are mainly due to hybridization rather than polyploidy (Salmon et al., 2005).

In newly synthesized allopolyploids of *Aegilops-Triticum* DNA methylation analysis revealed changes in low-copy sequences corresponding preferentially to hypermethylation rather than hypomethylation (Liu et al., 1998). Subsequent studies demonstrated that cytosine methylation alterations occurred in ~13% of the *loci* analyzed by methylation-sensitive AFLP analysis. Although such alterations were observed both in F1 hybrids and allopolyploids, higher frequencies were detected in the F1 hybrid (Shaked et al., 2001). Furthermore, Dong et al. (2005) using an identical methodology found ~20% differences in banding profiles between both synthetic and natural allohexaploid wheats and parental lines. Random and nonrandom modifications in DNA methylation patterns were identified in synthetic and natural polyploids, and revealed that both types were stably inherited. A recent study in the same model system, predominantly revealed hypermethylation changes in 54% of the CCGG sites flanking distinct transposable elements families in the first four generations of newly formed allohexaploid wheats (Yaakov and Kashkush, 2011a).

Synthetic *Gossypium* allohexaploids and allotetraploids are the exception to the previous described changes in methylation patterns, since the first five polyploid generations studied did not displayed any alterations in banding patterns obtained with methylation-sensitive AFLP, indicating that epigenetic changes following polyploid synthesis were absent or minimal in these situations (Liu et al., 2001).

Nucleolar Dominance is an epigenetic regulated gene silencing phenomena, extensively described in hybrids or allopolyploids in which only one parental set of ribosomal RNA (rRNA) genes is transcribed (dominant species), being silenced hundreds or thousands of rRNA genes inherited from the other parent (underdominant species). Such designation is related with the fact that only transcriptionally active rRNA genes originate nucleoli, the sites of ribosomal subunits assembly (Pikaard, 2000).

Navashin (1934) was the first scientist describing the emergence of nucleolar chromosome morphological modifications in hybrids between different species from the genus *Crepis.* Morphological changes affecting all chromosomes from one parent, such as chromosomes thickening or shortening, were termed “amphiplasty” or “neutral amphiplasty”, whereas changes affecting only particular chromosomes were designated as “differential amphiplasty”, latter referred as Nucleolar Dominance. Navashin used the term “differential amphiplasty” to describe alterations observed in a particular chromosome group - D chromosomes - presenting at metaphase a distal small segment (the satellite), connected to the remainder of the chromosome by a thin strand of chromatin (the secondary constriction). In interspecific hybrids, only D-chromosomes from one parental species show this characteristic morphology, while those from the other progenitor failed to display the secondary constriction. Additionally, Navashin also realized that this morphological alteration always affected chromosomes from the same parental species in reciprocal crosses. Erasure of differential amphiplasty occurs after backcrossing F1 *Crepis* hybrids to the underdominant parental species, evidencing the reversibility of the process (Navashin, 1934). Navashin’s observations therefore demonstrated that the information for the under-dominant species needed to form nucleoli in the hybrids was suppressed rather than eliminated (Jones and Hegarty, 2009).

Barbara McClintock (1934) associated such affected chromosome domains to the *loci* responsible for nucleoli formation and used for the first time the term “nucleolar organizer” (or Nucleolus Organizer Region, NOR). McClintock also suggested that NORs might bear redundant genetic information, feature that was later confirmed when studies on NOR organization revealed a cluster of multiple copies of rRNA genes (Phillips et al., 1971; Wallace and Birnstiel, 1966).

Nucleolar Dominance was further observed in tens of others allopolyploids such as *Brassica*, wheat, triticale and *Arabidospis* (reviewed in Viegas et al., 2002). Recent experiments have demonstrated that Nucleolar Dominance involves epigenetic control of rRNA genes expression (Earley et al., 2006; Lawrence et al., 2004; Pikaard, 1999). This phenomenon affects hundreds to thousands of rRNA gene copies inherited from the under dominant genome and represents gene silencing on a multi-megabase level (Pikaard, 2000). Cytosine methylation and histone modifications play an important role in Nucleolar Dominance observed in *Brassica* (Chen and Pikaard, 1997), triticale (Neves et al., 1995) and Arabidopsis (Chen et al., 1998; Lawrence et al., 2004). Preuss et al. (2008) demonstrated that the activity of *de novo* cytosine methyltransferase DRM2, and methylcytosine binding domain proteins MBD6 and MBD10 are required for Nucleolar Dominance establishment in *A. suecica*. Furthermore this phenomenon involves siRNAs (small interference RNAs) that specify the *de novo* cytosine methylation patterns recognized by MBD6 and MBD10 in the large-scale silencing of rRNA gene *loci*. The involvement of histone deacetylase HDA6 in silencing of rRNA gene *loc*i from *A. thaliana* origin was also proved (Earley et al., 2006). Such observations support a model whereby cytosine methylation and repressive histone modifications specify one another in a self-reinforcing cycle that maintains in hybrids/polyploids the preferential silencing of rRNA genes from one parental species (Lawrence et al., 2004). Although epigenetic regulation of rRNA genes silencing in hybrid genotypes is well characterized, the ‘selection’ of the parental genome to be silenced is still unclear (Jones and Hegarty, 2009), even though there are evidences suggesting the preferential silencing of rRNA genes from the larger parental genome (Viegas et al., 2002). These authors propose a chromatin imprinting model where heterochromatin is a key player in Nucleolar Dominance scenario. This model suggests that differences in genome size between parental species, which mainly result from distinct repetitive sequences contents, are responsible for the establishment of the new epigenetic patterns associated with the silencing of heterochromatin-rich underdominant NORs.

In addition to Nucleolar Dominance, several other gene expression changes were detected in synthetic polyploids, mostly driven by hybridization rather than genome doubling. Comai’s group (Comai, 2000) research in synthetic lines of *A. suecica*

revealed variable phenotypes and demonstrated that gene silencing observed in the F2 generation after hybridization affects euchromatic regions, genes and repeated sequences related to transposons. Furthermore, those authors estimate that ~0.4% of the genes in allotetraploids are silenced. Subsequently, it was proved that changes in allotetraploids expression patterns are accompanied by nonrandom modifications of parental methylation states (Madlung et al., 2002).

McClintock in 1984 suggested that widespread changes in the activity of transposable elements can result from the “genomic shock” induced by the fusion of two different genomes into the same nucleus (McClintock, 1984). Indeed, some transposons including DNA transposons and retrotransposons were proved to be reactivated in allotpolyploids. In *Arabidopsis*, transcriptional activity associated with remodeling of CG methylation upon allopolyploidization was detected in several transposons, although transposition of those elements needs to be further confirmed (Madlung et al., 2005). Activation of Wis 2-1A retrotransposons leads to the synthesis of new transcripts from adjacent sequences, including genes antisense or sense strands (Kashkush et al., 2003). Despite changes reported in retrotransposons transcription, the available data in wheat synthetic allopolyploids do not prove the occurrence of transposable elements movement (Kashkush et al., 2003). Additionally, in the synthetic allohexaploid wheat it was also reported in the first four generations a massive alteration of methylation patterns in sequences adjacent to transposons (Yaakov and Kashkush, 2011a). Although several evidences show that TEs are highly affected upon polyploidization, leading mainly to de-methylation and transcriptional activation, the origin and the biological significance of those modifications are still unclear (Yaakov and Kashkush, 2011b).

Chapter II

Polyploidization as a retraction force in plant genome evolution: sequence rearrangements in Triticale

Bento M, Pereira HS, Rocheta M, Gustafson J P, Viegas W and Silva M (2008) Polyploidization as a Retraction Force in Plant Genome Evolution: Sequence Rearrangements in Triticale. PLoS ONE 3(1): e1402. doi:10.1371/journal.pone.0001402.

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Polyploidization as a Retraction Force in Plant Genome Evolution: Sequence Rearrangements in Triticale

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Background. **Polyploidization is a major evolutionary process in plants where hybridization and chromosome doubling induce enormous genomic stress and can generate genetic and epigenetic modifications. However, proper evaluation of DNA sequence restructuring events and the precise characterization of sequences involved are still sparse.** ***Methodology/Principal Findings.*** **Inter Retrotransposons Amplified Polymorphism (IRAP), Retrotransposons Microsatellite Amplified Polymorphism (REMAP) and Inter Simple Sequence Repeat (ISSR) largely confirmed the absence of any intraspecific variation in wheat, rye and triticale. The comparative analysis of banding profiles between wheat and rye inbred lines revealed 34% of monomorphic (common to both parental species) bands for the ten different primer combinations used. The analysis of triticale plants uncovered nearly 51% of rearranged bands in the polyploid, being the majority of these modifications, due to the loss of rye bands (83%). Sequence analysis of rye fragments absent in triticale revealed for instance homology with hydroxyproline-rich glycoproteins (HRGP), a protein that belongs to a major family of inducible defence response proteins. Conversely, a wheat-specific band absent in triticale comprises a nested structure of *copia*-like retrotransposons elements, namely *Claudia* and *Barbara*. Sequencing of a polyploid-specific band (absent in both parents) revealed a microsatellite related sequence. Cytological studies using Fluorescent *In Situ* Hybridization (FISH) with REMAP products revealed a widespread distribution of retrotransposon and/or microsatellite flanking sequences on rye chromosomes, with a preferential accumulation in heterochromatic sub-telomeric domains.** ***Conclusions/Significance..*** **Here, we used PCR-based molecular marker techniques involving retrotransposons and microsatellites to uncover polyploidization induced genetic restructuring in triticale. Sequence analysis of rearranged genomic fragments either from rye or wheat origin showed these to be retrotransposon-related as well as coding sequences. Further FISH analysis revealed possible chromosome hotspots for sequence rearrangements. The role of chromatin condensation on the origin of genomic rearrangements mediated by polyploidization in triticale is also discussed.**

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INTRODUCTION

Polyploidization is an evolutionary process where two or more genomes are joined into the same nucleus. There are numerous examples of polyploidization in the plant kingdom, where chromosome fusions/fissions and rearrangements occurred following speciation. In fact, many modern day diploids are believed to have arisen from ancient polyploidization events (reviewed in [1]). Polyploidization is responsible for the emergence of genotypic plasticity, providing polyploids with the ability to tolerate genomic variations better than their diploid progenitors. This dynamic process has been explored in cereal species, which share a monophyletic origin. Although phylogenetic and molecular studies demonstrated large differences in genome size between cereal species, gene number and synteny are conserved (reviewed in[2]). Comparisons at the genetic map level show high genome colinearity conservation between cereal species, occasionally disrupted by gross chromosomal translocations [3].

Polyploid formation places the hybrid under a considerable amount of stress and/or genetic shock, which in turn can leads to a number of genetic and epigenetic modifications. Solely genetic changes include translocations and transpositions as well as sequence deletions and insertions, while epigenetic changes take into account non-additive gene regulation, transposon transcription, silencing or sub-funtionalization of homoeologous genes, and chromatin condensation [4–6]. Previous studies on wild wheat polyploid relatives, such as *Triticum* and *Aegilops* spp., suggested that the genetic and epigenetic changes that occurred were not random, but rather directed and reproducible [7–11]. Directed and stable modifications have also been reported in *Brassica* [12] as well as in *Arabidopsis* [13]. In the allopolyploid *A. suecica*, a product of hybridization of *A. thaliana* and *A. arenosa*, genetic changes involving the loss of one parental-specific rDNA *locus* were observed in both naturally occurring as well as synthetic polyploids [14,15]. Genome rearrangements have also been extensively studied in triticale, a man-made wheat (*Triticum* ssp.)/rye (*Secale cereale* L.) allopolyploid (X *Triticosecale* Wittmack) [16,17]. Amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) analyses have established the occurrence of genetic and epigenetic modifications in triticale, mostly attributable to the rye parental genome [17]. Moreover, most of the modifications revealed by AFLP occurred to a

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2. Polyploidization as a Retraction Force in Plant Genome Evolution: Sequence Rearrangements in Triticale

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Key words: Polyploidization, genome restructuring, retrotransposons and microsatellites, IRAP, REMAP and ISSR, triticale.

2.1. Abstract

Background

Polyploidization is a major evolutionary process in plants where hybridization and chromosome doubling induce enormous genomic stress and can generate genetic and epigenetic modifications. However, proper evaluation of DNA sequence restructuring events and the precise characterization of sequences involved are still sparse.

Methodology/Principal Findings

Inter Retrotransposons Amplified Polymorphism (IRAP), Retrotransposons Microsatellite Amplified Polymorphism (REMAP) and Inter Simple Sequence Repeat (ISSR) largely confirmed the absence of any intraspecific variation in wheat, rye and triticale. The comparative analysis of banding profiles between wheat and rye inbred lines revealed 34% of monomorphic (common to both parental species) bands for the ten different primer combinations used. The analysis of triticale plants uncovered nearly 51% of rearranged bands in the polyploid, being the majority of these modifications due to the loss of rye bands (83%). Sequence analysis of rye fragments absent in triticale revealed for instance homology with hydroxyproline-rich glycoproteins (HRGP), a protein that belongs to a major family of inducible defense response proteins. Conversely, a wheat-specific band absent in triticale comprises a nested structure of *copia*-like retrotransposons elements, namely *Claudia* and *Barbara*. Sequencing of a polyploid-specific band (absent in both parents) revealed a microsatellite related sequence. Cytological studies using Fluorescent *In Situ* Hybridization (FISH) with REMAP products revealed a widespread distribution of retrotransposon and/or microsatellite flanking sequences on rye chromosomes, with a preferential accumulation in heterochromatic sub-telomeric domains.

Conclusions/Significance

Here, we used PCR-based molecular marker techniques involving retrotransposons and microsatellites to uncover polyploidization induced genetic restructuring in triticale. Sequence analysis of rearranged genomic fragments

either from rye or wheat origin showed these to be Retrotransposon-related as well as coding sequences. Further FISH analysis revealed possible chromosome hotspots for sequence rearrangements. The role of chromatin condensation on the origin of genomic rearrangements mediated by polyploidization in triticale is also discussed.

2.2. Introduction

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A. thaliana and *A. arenosa*, genetic changes involving the loss of one parental-specific rDNA *locus* were observed in both naturally occurring as well as synthetic polyploids [14,15]. Genome rearrangements have also been extensively studied in triticale, a man-made wheat (*Triticum* ssp.)/rye (*Secale cereale* L.) allopolyploid (X *Triticosecale* Wittmack) [16,17]. Amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) analyses have established the occurrence of genetic and epigenetic modifications in triticale, mostly attributable to the rye parental genome [17]. Moreover, most of the modifications revealed by AFLP occurred to a greater degree immediately after hybridization, especially concerning sequences from rye origin, compared to the continuous variation events that occurred at a very small rate following chromosome duplication [18]. Using the same techniques on newly formed polyploids of *Aegilops* and *Triticum*, rapid alterations of chromosome- and genome-specific sequences were demonstrated, with the preferential loss of parental bands [8,9]. It must be emphasized here that both AFLP and RFLP analyses were performed utilizing non-methylation and methylation sensitive restriction enzymes, thereby uncovering differences which cannot be attributable solely to sequence modifications.

The exact level of DNA sequence restructuring events involved in this important evolutionary process remains to be determined. In addition, precise information regarding the actual sequences involved is scarce. In order to better understand the genomic processes underlying polyploidization, microsatellite and retrotransposon PCR-based molecular marker techniques were utilized to evaluate exclusively genetic rearrangements in triticale. Microsatellites, or Simple Sequence Repeats (SSRs), are polymorphic *loci* present in nuclear DNA that consist of repeating units of 1-6 base pairs in length. They are typically neutral, co-dominant and widely spread throughout the genome. Retrotransposons are ubiquitous in the plant kingdom, being the main constituent of large plant genomes [19]. These transposable genetic elements require the action of reverse transcriptase on an RNA intermediate to integrate in the host genome by a ´copy and paste´ method of transposition [20]. They are conventionally divided into groups, depending whether or not they possess long terminal repeats (LTRs). The LTR retrotransposons are further classified into the *Ty1- copia* and *Ty3- gypsy* families (Figure 1). Due to their dynamics and mobility, it is widely accepted that

these elements generate molecular modifications and increase genome size, and therefore have an important role in genome evolution and speciation. Actually, an increase in Retrotransposon-related transcripts has been detected in both wheat and Arabidopsis synthetic polyploids [7,13,21,22], although actual transposition of these elements has never been proved in newly synthesized polyploids.

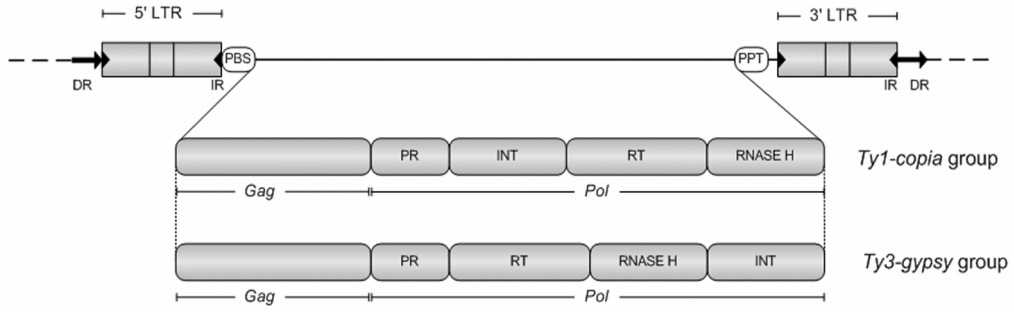

Figure 1. Structural features of *copia* and *gypsy* retrotransposons. LTR, long terminal repeat; Gag, core structural protein genes; PR, protease; RT, reverse transcriptase; INT, integrase; PBS, primer binding site; PPT, polypurine tract; DR, direct repeat; IR, inverted repeat (adapted from [20]).

In this study, we demonstrate that Inter Retrotransposons Amplified Polymorphism (IRAP), Retrotransposons Microsatellite Amplified Polymorphism (REMAP), and Inter Simple Sequence Repeat (ISSR) are powerful tools for polyploid analysis. A high rate of polyploidization induced DNA sequence rearrangements in retrotransposon and/or microsatellite associated sequences were uncovered in triticale. These techniques also permitted the isolation of restructured fragments for sequence analysis, unambiguously demonstrating that repetitive and coding sequences are involved in the evolutionary process mediated by polyploidization. FISH for chromosome mapping of sequences associated to the retrotransposon *Nikita* and/or to the microsatellite (CT)9G showed these to be widely distributed throughout all rye chromosomes, with preferential localization in major heterochromatic domains, suggesting that chromatin condensation plays an important role in polyploid evolution.

2.3. Results

IRAP, REMAP and ISSR profiles enhance wheat and rye phylogenetic relationships

To test if the IRAP, REMAP and ISSR techniques constitute efficient tools to evaluate genomic rearrangements within allopolyploid triticale, we initially analyzed the banding profiles obtained with different primer combinations in wheat and rye parental plants. Seven primer combinations were tested with IRAP and REMAP using the primer for the LTR sequence of the barley retrotransposon Nikita by itself as well as combined with three LTR primers from additional barley retrotransposons (Sabrina, Sukkula, and Stowaway), and with three anchored microsatellite primers (GA)9C, (CT)9G, and (CA)9G (Table 1). ISSR banding profiles were obtained for all three di-nucleotide repeats. In order to reduce the potential number of fragments amplified and obtain consistent, easily analyzable and reproducible banding patterns, a single LTR primer from each retrotransposon was utilized on all IRAP and REMAP combinations (Figure 2).

Table 1. Primers used for PCR analysis.

Primer		Sequence
LTR	**Barley Retrotransposon**	
C0699	Nikita	5′-CGCTCCAGCGGTACTGCC
C0945	Sabrina	5′-GCAAGCTTCCGTTTCCGC
9900	Sukkula	5′-GATAGGGTCGCATCTTGGGCGTGAC
Stowaway	Stowaway	5′-CTTATATTTAGGAACGGAGGGAGT
SSR		
(GA)9C		5′-GAGAGAGAGAGAGAGAGAC
(CT)9G		5′-CTCTCTCTCTCTCTCTCTG
(CA)9G		5′-CACACACACACACACACAG

The banding profiles yielded a considerable number of distinct and reproducible bands in all the lines and for all primer combinations analyzed. Only bands between 100 and 1650 bp were scored, as this gel region produced the highest quality profiles, allowing for discrimination of major bands against a low

background. Qualitative differences between profiles and minor non-reproducible IRAP, REMAP and ISSR bands were not considered. Differences in intensities between bands were obvious within the same species for each primer combination but did not show any direct relation with the size of the amplified fragment.

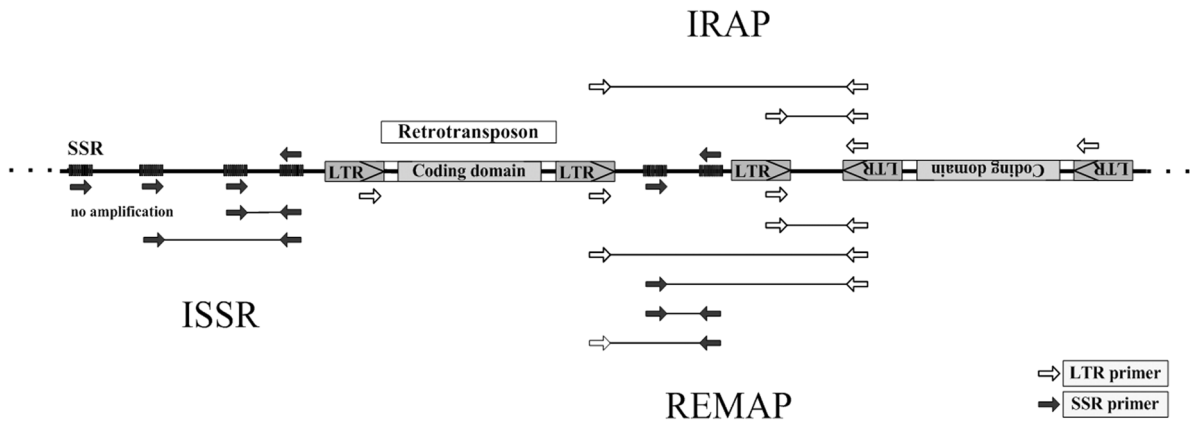

Figure 2. Principles of the IRAP, REMAP and ISSR procedures. IRAP: LTR primers (white arrows) facing outward from the ends of LTRs will amplify intervening DNA from retrotransposons or loose LTRs in opposite orientations. Retrotransposons or loose LTRs in the same orientation will not result in amplification products. **REMAP**: LTR primers are used together with SSR primers (black arrows), the products of amplification will be the combination of all retrotransposons or loose LTRs and SSRs in opposite orientations, sites with the same orientation will not result in amplification products. **ISSR**: SSR primers (black arrows) will amplify intervening DNA from SSRs in opposite orientations, sites with the same orientation will not result in amplification products.

Banding profiles from wheat and rye genomes obtained with five of the ten primer combinations are shown in Figure 3a. The IRAP and REMAP results are summarized in Table 2 and the ISSR results in Table 3. The total number of bands per species as well as the number of monomorphic (similar in both parental species) and polymorphic (observed in only one parental species) bands observed for all ten primer combinations are shown. Although our results confirm the phylogenetic proximity between wheat and rye genomes, the number of polymorphic bands obtained with IRAP and REMAP was nevertheless 65% (74 of 114 total number of bands observed in wheat and rye) and 68% (39 of 57 total number of bands observed in wheat and rye) with ISSR, proving these markers are excellent tools to discriminate between closely related species. Intraspecific comparisons were also performed through the analysis of three distinct wheat and rye plants using primer Nikita (Supporting information Figure S1). The banding profiles were identical for all plants of the same species, confirming their high inbred nature.

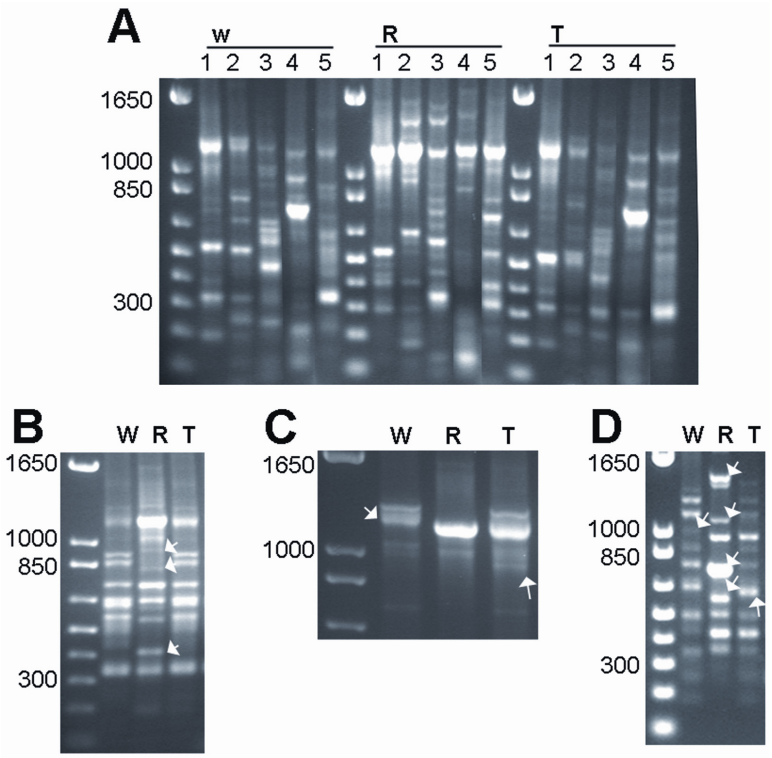

Figure 3. IRAP, REMAP and ISSR banding profiles of wheat (W), rye (R), triticale (T). (a) grouped by species, obtained with IRAP and REMAP utilizing primer combination Nikita/Nikita (1), Nikita/Sabrina (2), Nikita/Sukkula (3), Nikita/(GA)9C (4), Nikita/(CA)9G (5). **(b)** Banding profiles obtained with REMAP utilizing primer combination Nikita/(CA)9G,arrows indicate the three polyploid rearranged bands of rye genome origin absent in triticale. **(c)** Detailed section of banding profiles obtained with IRAP utilizing primer Nikita, arrows indicate a rearranged band of wheat genome origin absent in triticale and a novel band in triticale. **(d)** Banding profiles obtained with ISSR utilizing primer combination (CT)9G/(CT)9G, arrows indicate five rearranged bands in the polyploid, one of wheat genome origin absent in triticale, four of rye genome origin absent in triticale and one novel band in triticale.

Comparison of banding profiles between primer combinations revealed that they were reproducible and unique to each primer combination. However, profiles resulting from combinations of primers did not necessarily result in additive profiles of reactions in which each primer is used alone. For instance, the entire banding pattern obtained with primer Nikita was not observed in the other IRAP and REMAP reactions where this primer was utilized in combination with other microsatellite or retrotransposon primer. Since the results obtained from three technical replicates for each PCR experiment were identical the amplification products obtained in each reaction seem therefore to represent a reproducible competition between the primer and its binding sites for each specific reaction mixture.

Table 2. IRAP and REMAP analysis in triticale allopolyploid and its diploid progenitors**.**

	Number of bands with different primer combinations							
	IRAP				REMAP			
Primer combined with Nikita	**Nikita**	**Sabrina**	**Sukkula**	**Stowaway**	**(GA)9C**	**(CT)9G**	**(CA)9G**	**Total**
Wheat	7	7	11	4	6	9	12	56
Rye	5	9	13	8	5	8	10	58
Monomorphic [i]	1	1	7	2	2	-	7	**40** (35%)
Polymorphic [ii]	10	14	10	8	7	17	8	**74** (65%)
Polyploid **expected** [iii]	11	15	17	10	9	17	15	**94**
Polyploid **conserved** [iv]	9	10	14	6	7	11	12	**69** (73%)
Polyploid **observed**	10	10	14	6	8	13	12	**73** [ii]
Polyploid **rearranged** [v]	3	5	3	4	3	8	3	**29**
Eliminated from wheat	1	1	1	1	-	1	-	**5** (17%)
Eliminated from rye	1	4	2	3	2	5	3	**20** (69%)
Novel in triticale	1	-	-	-	1	2	-	**4** (14%)

[i] Monomorphic bands: common to both triticale progenitors [40 (20 wheat + 20 rye) / 114 (56 wheat + 58 rye) = 35.1%];
[ii] Polymorphic bands: observed in only one triticale progenitor;
[iii] Polyploid expected bands: the ones expected as an addition pattern of parental bands observed;
[iv] Polyploid conserved bands: parental bands observed in the polyploid;
[v] Polyploid rearranged bands: parental bands absent in the polyploid and novel bands only observed in the polyploid.

Analyzing banding profiles of all the reactions tested resulted in the following maximum and minimum number of bands: (i) IRAP and REMAP bands observed in wheat and rye ranged between a minimum of four in wheat (primer combination Nikita/Stowaway) and a maximum of thirteen in rye (primer combination Nikita/Sukkula); (ii) the number of monomorphic bands ranged between zero (primer combination Nikita/(CT)9G) and seven (primer combinations Nikita/Sukkula and Nikita/(CA)9G); (iii) with ISSR, the number of bands observed in wheat and rye ranged between a minimum of four (with primer (CA)9G) and a maximum of fourteen (with primer (GA)9C) in rye with the number of monomorphic bands ranging between zero (with primer (CA)9G) and seven (with primer (GA)9C).

Parental genomic rearrangements in triticale

Since the parental materials were strictly inbred over many years, any genomic changes revealed in the triticale should be directly related to genome responses to polyploidization.

Table 3. ISSR analysis in triticale allopolyploid and its diploid progenitors**.**

	Number of bands with different di-nucleotide primers			
	ISSR			
	(GA)9C	**(CT)9G**	**(CA)9G**	Total
Wheat	13	12	5	30
Rye	14	9	4	27
Monomorphic [i]	7	2	-	**18** (32%)
Polymorphic [ii]	13	17	9	**39** (68%)
Polyploid **expected** [iii]	20	19	9	**48**
Polyploid **conserved** [iv]	14	14	9	**37** (77%)
Polyploid **observed**	15	15	9	**39**
Polyploid **rearranged** [v]	7	6	-	**13**
Eliminated from wheat	-	1	-	**1** (8%)
Eliminated from rye	6	4	-	**10** (77%)
Novel in triticale	1	1	-	**2** (15%)

[i] Monomorphic bands: common to both triticale progenitors [18 (9 wheat + 9 rye) / 57 (30 wheat + 27 rye) = 32%];
[ii] Polymorphic bands: observed in only one triticale progenitor;
[iii] Polyploid expected bands: the ones expected as an addition pattern of parental bands observed;
[iv] Polyploid conserved bands: parental bands observed in the polyploid;
[v] Polyploid rearranged bands: parental bands absent in the polyploid and novel bands only observed in the polyploid.

In fact, genomic rearrangements were observed as non-additive IRAP, REMAP and ISSR banding profiles in triticale compared to those of the wheat and rye parental species. All seven IRAP and REMAP and two of the three ISSR primer combinations showed the occurrence of rearrangements in triticale (Figure 3a, Table 2 and 3) which resulted from the loss of parental bands in triticale or, conversely, novel bands appearing in the allopolyploid that were not seen in the parental genomes. Moreover, the presence in the banding profile obtained for the parental genome mixture, with Nikita and Nikita/(CT)9G primer combinations (Supporting information Figure S2) of the triticale rearranged bands (lost parental bands), confirmed that the genome modifications revealed resulted from polyploidization. On the other hand, to evaluate if the restructuring events detected in the polyploid genotype were present in all plants analyzed, IRAP utilizing primer Nikita was performed with DNA extracted from three triticale plants (Supporting information Figure S1). The individual banding profiles clearly demonstrated no differences between individuals, indicating that triticale plants are stable for the molecular markers selected.

Table 4. Summary of rearrangements identified in triticale by IRAP, REMAP, and ISSR.

	Bands observed	Rearranged bands (% of observed)		
		Eliminated	Novel	TOTAL
IRAP	29	14 (48%)	1 (3%)	**15 (52%)**
REMAP	24	11 (46%)	3 (13%)	**14 (58%)**
ISSR	30	11 (37%)	2 (7%)	**13 (43%)**
Total	83	36 (43%)	6 (7%)	**42 (51%)**

To evaluate the frequency of genome rearrangements in triticale, the number of polymorphic wheat and rye bands was added to the number of monomorphic bands (a band present in both parental profiles was counted only once), and the result was compared with the number of bands observed in triticale profiles. These calculations demonstrated that only 73% (69 bands observed / 94 expected) of the parental bands were conserved in triticale with IRAP and REMAP (Table 2), and 77% (37 bands observed / 48 expected) with ISSR (Table 3). Out of the seven IRAP and REMAP primer combinations analyzed, a total of twenty-nine rearrangements were detected in triticale, representing the loss of five wheat origin bands (17%) and twenty rye origin bands (69%), as well as the appearance of four novel bands (14%) not seen in either parental species. The rearranged bands observed with the seven IRAP and REMAP experiments were as follows: (i) IRAP with primer Nikita resulted in the loss of one band from rye, the loss of one band from wheat and the appearance of one novel band (Figure 3c); (ii) primer combination Nikita/Sabrina resulted in the loss of four bands from rye and the loss of one band from wheat; (iii) with IRAP primer combination Nikita/Sukkula the loss of two bands from rye and the loss of one band from wheat was observed; (iv) IRAP utilizing primer combination Nikita/Stowaway resulted in the loss of three bands from rye and the loss of one band from wheat; (v) REMAP utilizing primer combination Nikita/(GA)9C resulted in the loss of two bands from rye and the appearance of one novel band; (vi) primer combination Nikita/(CT)9G revealed the loss of five bands from rye, the loss of one band from wheat and the appearance of two novel bands in triticale; and (vii) primer combination Nikita/(CA)9G indicated

the loss of three rye origin bands in triticale (Figure 3b). Thirteen rearranged bands were identified in triticale with the three ISSR primers tested, corresponding to loss of one wheat origin band (8%) and from the loss of ten rye origin bands (77%) and the occurrence of two novel bands (15%) not present in either of the parental species (Table 3). The rearranged bands observed with the three ISSR experiments were as follows: (i) ISSR with primer (GA)9C resulted in the loss of six bands from rye and the appearance of one novel band; and (ii) primer (CT)9G indicated the loss of four bands from rye, one band from wheat and the appearance of one novel band in the triticale (Figure 3d).

Analyzing the total number of IRAP, REMAP and ISSR rearrangements clearly indicated that the loss of parental bands in triticale occurred much more frequently (43%) than the appearance of novel bands (7%), (Table 4). Amongst triticale lost bands, most involved rye specific bands (10 from IRAP, 10 from REMAP and 10 from ISSR reactions), comparing to the loss of six wheat specific bands (4 from IRAP; 1 from REMAP and 1 from ISSR reactions). The loss of a monomorphic band (common to wheat and rye progenitors) was never observed in triticale. Conversely, a total of six novel bands were detected in the allopolyploid with the molecular markers used (1 with IRAP, 3 with REMAP and 2 with ISSR, respectively).

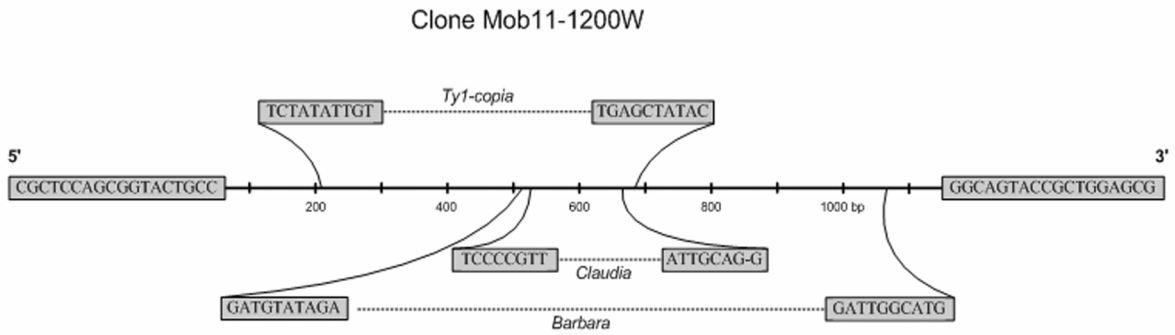

Figure 4. Structure of putative partial retrotransposon-like sequence Mob-11-1200 from *T. aestivum* with 1200 bp. The terminal boxes at each end represent the primer sequence used in the amplification (C0699). Shown in the expansions are three *copia*-like retroelements that seem to have partial inserts inside clone Mob11-1200. Boxes indicate the beginnings and the ends of these retrotransposons. Accession numbers: *Barbara* retrotransposon (*T. monococcum*, AF326781); *Claudia* retrotransposon (gi:18496650); *Ty*-1 *copia* retrotransposon (*O. sativa* gi:.57114405).

Sequences analysis of polyploid rearranged bands

Three rearranged polymorphic fragments uncovered in triticale arising from IRAP and REMAP with three different primer combinations were gel-isolated, purified and cloned for sequence analysis. These included one band from the wheat

parental genome absent in triticale, one band from the rye parental genome missing in the polyploidy, as well as a novel triticale-specific band. Sequence analyses of these three rearranged bands is described below.

A putative copia-like retrotransposon

IRAP using only the Nikita primer resulted in a 1219 bp rearranged fragment of wheat origin absent in triticale, which was named MoB-11-1200W (accession number EF486520). Utilizing NCBI, TIGR, and PlantSat (repetitive plant sequences) databases, nucleotide alignments show 71, 79, 80 and 84% homology with *copia*-like retrotransposons *Claudia*, *Ty*-1 from *O. sativa*, *Barbara* and an unnamed retrotransposon from *T. monococcum* 7Am, respectively (Figure 4). Immediately before the 3' LTR, MoB-11-1200W presented a short polypurine tract (PPT) with 13 purines (AAAAAGGGGGAGA) (Figure 5a) and downstream of the PPT an inverted repeat with four nucleotides (TTGT) indicative of the beginning of 3' LTR. Neither in the clone or in the aligned retroelements was found between the polypurine tract and the right LTR, a dinucleotide characteristic of the LTR end-sequence of retroviruses and retrotransposons [23]. This absence seems to be a characteristic of this retroelement group. Based on the nucleotide alignment, a phylogenetic consensus tree (Figure 5b) was constructed using the Neighbor-Joining method [24] on the basis of a distance matrix calculated with the Bionumerics software (version 3.5). There was one major clade supported by a bootstrap value of 100% that included all the elements except *Claudia*, which appears as an out-group. Inside the major clade two clades supported by bootstrap values of 61 and 96% were selected, the first one including the retroelement *Barbara* from *T. monococcum* and the second with *T. monococcum 7 Am* and Mob-11-1200W clone.

An hydroxiproline-rich glycoprotein sequence

The 963 bp rearranged band of rye parental origin absent in triticale was isolated from the REMAP Nikita/(CA)9G) and named MoB-111-1000R (accession number EF486521). The aminoacid residues were tested against the NCBI, TIGR, and PlantSat (repetitive plant sequences) databases (Figure 6a). The alignment shows 50 and 73% homology with several sequences that code for a hydroxyproline-rich glycoprotein from *A. thaliana* and *O. sativa*, respectively.

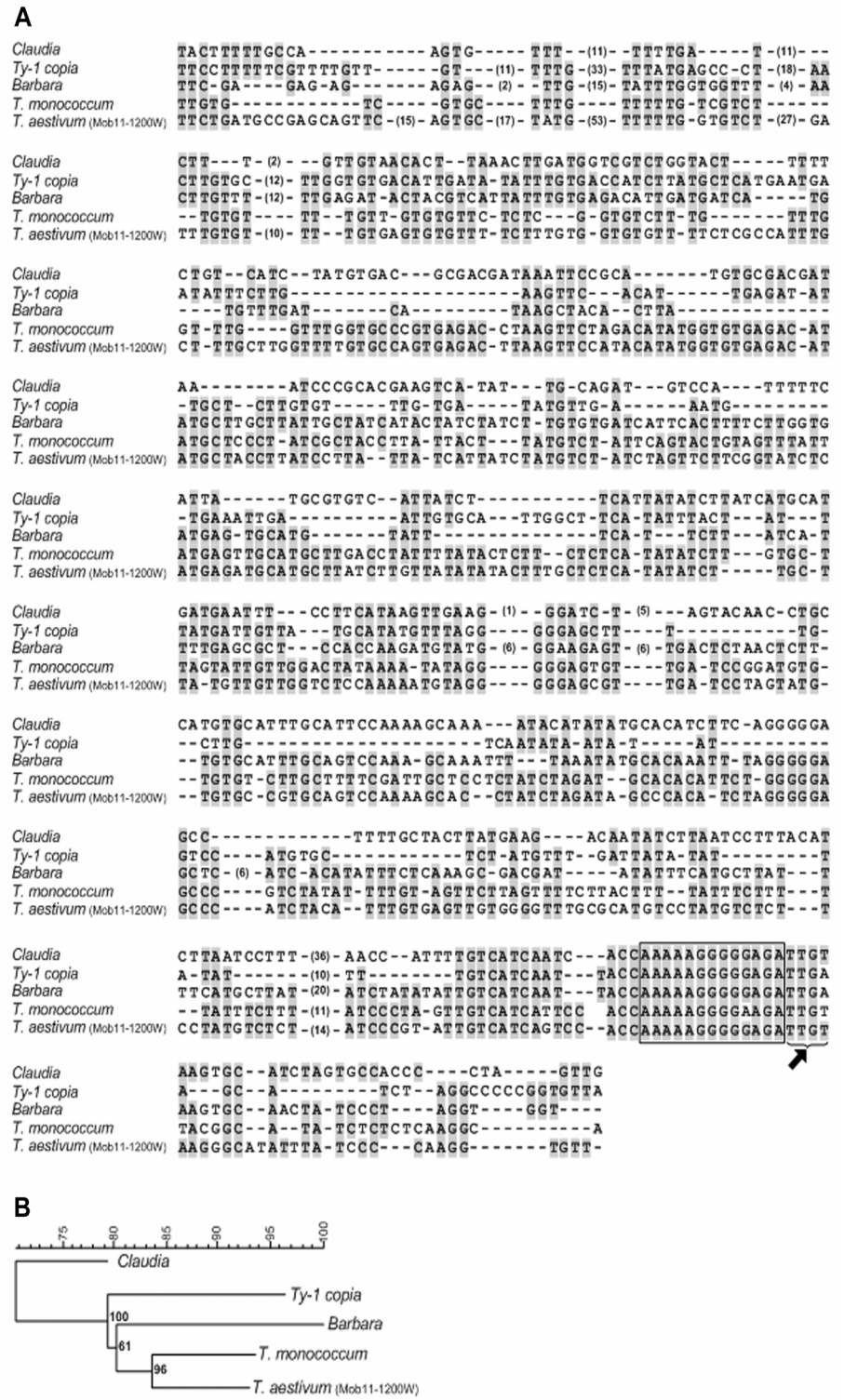

Figure 5. Sequence alignment of the clone Mob-11-1200 from *T. aestivum* with several *copia* retrotransposons. (a) Only partial sequences, corresponding to the conserved parts of *pol* region are shown. The polypurine tract (PPT) are boxed and arrow indicate the inverted repeat (IR) signature just before the beginning of the 3' LTR. Numbers in brackets indicate the number of nucleotides separating the sequences. **(b)** Based on previous nucleotide alignment, a tree was constructed utilizing the Neighour-Joining method [24]. The numbers on the branches represent bootstrap support for 1,000 replicates. Numbers in bold represent cophenetic correlations, which confirm the stability of the tree nodes. Sequences were aligned using Bionumerics software (version 3.5). Accession numbers: *Barbara* retrotransposon (*T. monococcum*, AF326781); *Claudia* retrotransposon (gi:18496650); *Ty*-1 *copia* retrotransposon (*O. sativa* gi: 57114405); *T. monococcum* 7Am (AF488415).

Based on the aminoacid alignment (Figure 6b), a phylogenetic consensus tree was constructed using the Neighbor-Joining method [24] on the basis of a distance matrix calculated with the Bionumerics software (version 3.5). The tree showed *A. thaliana* as an out-group and one major clade supported by a bootstrap value of 100% that included clone Mob-111-1000R and two independent sequences from *O. sativa*.

A characteristic triticale microsatellite

REMAP with primer combination Nikita/(CT)9G resulted in a novel 100bp triticale specific fragment that was not seen in either of the parental genomes, named Mob-110-100T which revealed to be mainly composed of microsatellites, and did not produce any significant alignment against the databases.

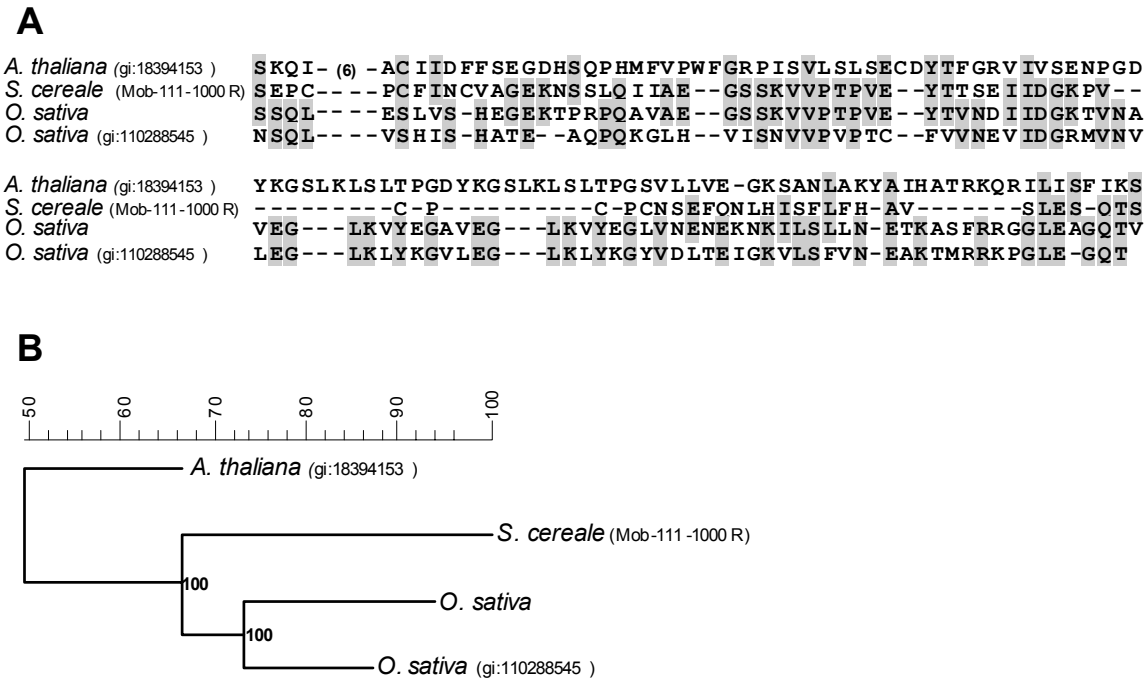

Figure 6. Alignments of conceptual translation of clone Mob-111-1000R. (a) The alignment shows homology with sequences coding for a hydroxiproline-rich glycoprotein. **(b)** Based on the alignment of amino acid residues, the tree was constructed utilizing the Neighour-Joining method [24]. The numbers on the branches represent bootstrap support for 1,000 replicates. Sequences were aligned using Bionumerics software (version 3.5). Accession numbers: *A. thaliana* (gi: 18394153); *O. sativa* (gi:20270098); *O. sativa* (gi: 110288545).

Chromosome mapping of Nikita LTR and microsatellite flanking sequences in rye

In order to characterize the genomic distribution of the amplified products, the complete REMAP amplification reaction with primer combination Nikita/(CT)9G as well as the rDNA unit from wheat (pTa71) were utilized as probes to perform

fluorescent *in situ* hybridization (FISH) on rye. The REMAP probe was labeled with digoxigenin and detected with anti-digoxigenin FITC conjugated (green), the rDNA probe was labeled with biotin and detected with streptavidin Cy3 conjugated (red) and DAPI was used as a DNA counterstain (Figure 7). Spreads of meristematic root-tip cells allowed for the visualization of specific REMAP sequences throughout the cell cycle, namely on interphase nuclei (Figure 7a), prometaphase (Figure 7b) and metaphase chromosomes (Figure 7c).

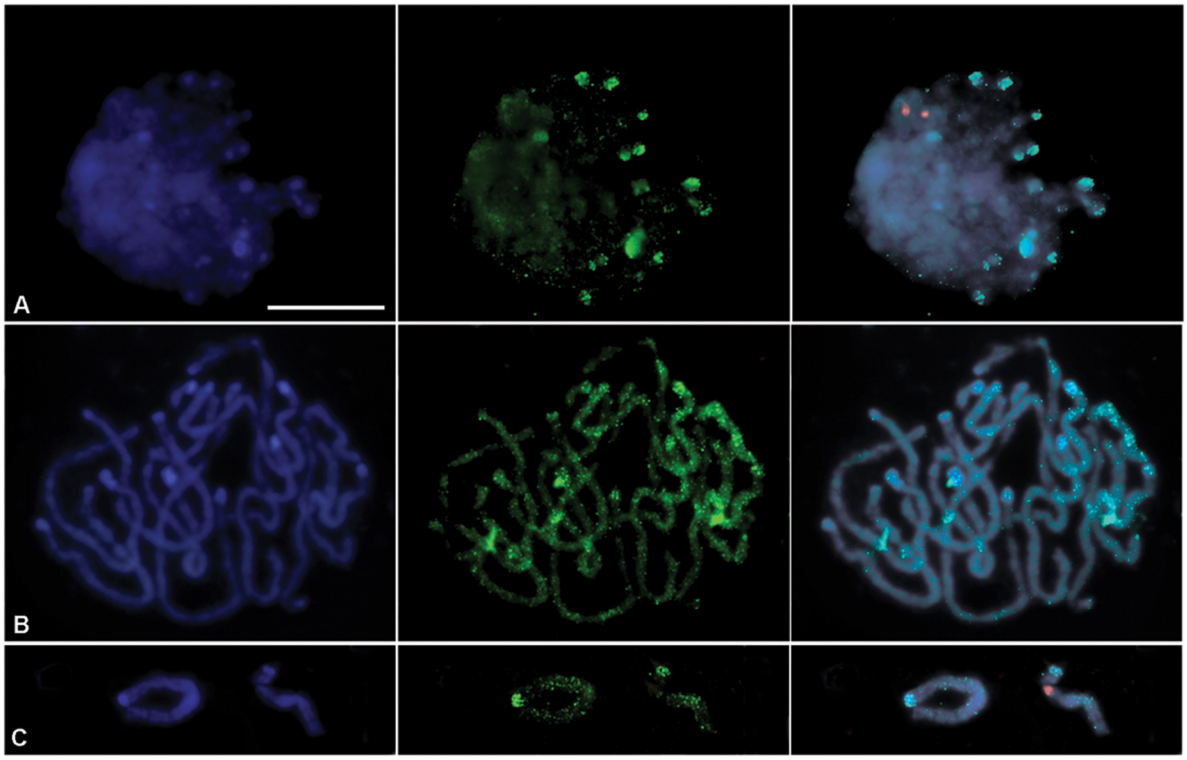

Figure 7. FISH on rye meristematic root-tip cells with REMAP product and pTa71. The following probes were used: total amplification product of REMAP reaction with primer combination Nikita/(CT)9G (green signal) and rDNA unit pTa71 (red signal). DNA was counterstained with DAPI (blue). **(a)** Interphase**)**; **(b)** Prometaphase; and **(c)** Metaphase chromosomes. Bar 10 µm.

In rye interphase nuclei, the nuclear Rabl organization of chromosomes was evident as low intensity DAPI staining in one nuclear pole clearly contrasted to large DAPI-positive signals characteristic of heterochromatic sub-telomeric domains observed in the opposite pole (Figure 7a). Detailed analysis of interphase nuclei shows ten heterochromatic DAPI positive domains co-localizing with the most intense aggregation of FISH REMAP signals and numerous dot-like signals dispersed throughout the entire nucleus. Due to chromosome condensation a widespread distribution of FISH signals was observed throughout rye

prometaphase chromosomes (Figure 7b). It was possible to distinguish two signals on both chromatids in some interstitial domains, as well as a marked sub-telomeric accumulation on most chromosomes. Rye metaphase chromosomes clearly demonstrated absence of signal in centromeric and rDNA domains and on sub-telomeric regions that are not DAPI positive (Figure 7c). Taken together, FISH analysis of sequences amplified by REMAP with Nikita/(CT)9G showed a wide distribution throughout the rye genome although preferentially accumulated at heterochromatic domains.

2.4. Discussion

Retrotransposon rich genomic domains are volatile and susceptible to a variety of responses to diverse stresses, such as those induced by polyploidization. In this study, large scale screening of retrotransposon and microsatellite rich genomic regions using IRAP, REMAP, and ISSR procedures uncovered reproducible polyploidization induced DNA rearrangements, measured as the lack of additive parental banding profiles in triticale. Nine of the ten IRAP, REMAP and ISSR primer combinations tested disclosed rearranged fragments. Only ISSR with primer (CA)9G produced a completely additive banding profile. Taken together, IRAP and REMAP with seven primer combinations and ISSR with three di-nucleotide sequences resulted in a total of 83 bands in the polyploid genotype, and 42 DNA sequence rearrangement events (51%). Of these, 36 corresponded to the loss of parental bands and 6 to the emergence of novel ones (Table 4). Interestingly, the intensities of monomorphic bands did not significantly differ between wheat and rye, suggesting that either the number of amplified *loci* or tandem repeats per *locus* are conserved between the two species. Using those markers we identified a higher proportion of rearrangements in triticale than the one observed in previous studies which is moreover exclusively associated with sequence modifications. Previous studies of polyploids using RFLP [9,17] and AFLP [17] identified both genetic and epigenetic genome restructuring events, since cytosine methylation sensitive as well as non-sensitive restriction enzymes were used.

To our knowledge, IRAP and REMAP [25] and ISSR [26] have only been used once for polyploid analysis, when it was shown that parental genomes were highly conserved in the natural allopolyploid *Spartina anglica*. However, these molecular marker techniques designed by Kalendar and co-workers [27] to characterize cultivars and varieties in several species [27-29], not only provide an original approach to detect genetic rearrangements induced by polyploidization, but also contribute to a greater resolution of the specific sequences affected by polyploidy evolutionary process. The control experiment we designed with the wheat+rye test tube indeed reinforces that this technique is accurate in evaluating genomic restructuring events and detecting rearranged bands induced by polyploidization. Three rearranged fragments, representing the loss of a wheat parental band, loss of a rye parental band, and the appearance of a novel band in triticale were excised, purified and cloned for sequence analysis. The wheat-specific band (Mob-11-1200W) absent in triticale and obtained with IRAP showed significant homologies with sequences of *T. monococcum* (chromosome 7), *T. aestivum* cv Renan, *copia*-like retrotransposons previously identified in *Triticum*, namely *Claudia*, *Barbara*, *T. monococcum* 7Am (unnamed retrotransposon) and *Ty- copia* from *O. sativa*. Alignment data further revealed this clone to be fragmented by the insertion of three different *copia*-like retrotransposons, and showed a 13 nucleotide polypurine-tract (PPT), which serves as a putative priming site for plus strand DNA synthesis [30]. A four nucleotide inverted repeat (TTGT) different to that of *Ty*1-*copia* and *Barbara* retrotransposons (TTGA) was identified immediately after the PPT region, indicating the presence of two classes of *copia* elements [31]. This clone could therefore represent a partial sequence of a retrotransposon with similarity to other elements, suggesting the occurrence of recombination events between partial sequences of single or multiple retroelements and/or families of retrotransposons. The rearranged rye fragment (Mob-111-1000R) absent in triticale and obtained by REMAP revealed homology with hydroxyproline-rich glycoproteins (HRGP) from *A. thaliana* and *O. sativa*. HRGP belong to a major family of inducible defense response proteins involved in the natural resistance of plants to injury, disease and various stress conditions [32,33]. Perhaps the absence of this sequence in triticale is somehow related to its lower level of hardiness when compared to rye. The third sequence, isolated by REMAP, representing a novel band in triticale was found to be mostly microsatellite related,

suggesting that polyploidization affected the number of these types of repetitive sequences.

The sequencing data provided important insights into which genomic sequences are involved in polyploidization induced modifications and cereal speciation. Retrotransposon-related genome rearrangements disclosed in this work were not restricted to non-coding regions, supporting the model proposed by Vitte and Panaud [19], where the organization of large plant genomes includes extensive heterochromatin blocks mainly composed of retrotransposons interspersed with gene-rich regions. During polyploidization, there may be a directed and controlled loss of coding sequences, a process believed to be necessary to overcome gene redundancy and achieve genetic diploidization. Accordingly, Southern blotting with probes to different genes indicated gene loss in synthetic allopolyploids of *Triticum* and *Aegilops* [7]. Our banding profiles and sequencing results with LTR- and/or microsatellite-associated sequences suggest a direct role of retrotransposons in gene loss events. Although transcriptional activation of retrotransposons has been previously reported in wheat [7,22] and *Arabidopsis* [13,21], transposition of these elements has never been shown in newly synthesized polyploids.

The vast majority of rearrangements uncovered in the polyploid genotype corresponded to the loss of parental bands (86%, 36 out of 42, Table 4). This loss seems to have originated from sequence modification/elimination rather than a mutation in PCR primer binding sequences, since extensive sequencing of the LTR termini has shown that mutations are rare in these regions [34]. Moreover, studies on C values of various polyploids have indicated that genome downsizing might be a widespread phenomenon following polyploidy formation [35], and recently a reduction in DNA content was observed in six newly synthesized wheat allopolyploids [10]. Our results suggest that the decrease of DNA content previously reported in the course of triticale breeding [36] can be attributed to significant loss of retrotransposon and/or microsatellite flanking sequences. Our data showing that the majority of the missing bands in triticale corresponded to rye-specific bands (30 out of 36 lost bands), confirms previous data about the preferential occurrence of restructuring events in this parental genome [17]. This brings to mind the observed deletion of rye telomeric heterochromatin regions [37] responsible for the meiotic pairing failure and cytological instability of triticale [38]. Also reminiscent, albeit on another scale, is the preferential loss of 2R rye

chromosome (the rye chromosome with greater DNA content) which usually results in more stable triticale plants with better agronomic characteristics [16].

Chromosome mapping with FISH on rye meristematic cells showed. REMAP products to be dispersed throughout the rye genome, but completely absent from centromeric domains and nucleolar organizing regions. Dot-like FISH signals clearly demonstrated that the labeled sequences were tandemly arranged and clustered, with marked accumulation in condensed sub-telomeric domains, suggesting their putative role in the establishment of terminal heterochromatin. The preferential disposition of FISH signals on telomeric domains is further enhanced by the Rabl organization of rye interphase nuclei, where major heterochromatic telomeric domains localize to one hemisphere. This disposition is similar to that observed for conserved domains of the Ty1-*copia*, Ty3-*gypsy* and *LINE* groups of retroelements on *Ae. speltoides*, and less obvious in *H. spontaneum*, probably due to the absence of telomeric heterochromatic domains in this species [39].

Differences in genome size, genetic redundancy and chromatin organization patterns between wheat and rye certainly resulted in genomic conflicts in the newly formed polyploid. In the triticale nucleus, rye has a greater haploid DNA content when compared to that of each genome complement from the hexaploid wheat [40]. Most likely, the preferential elimination of LTR and microsatellite-related sequences from rye played a role in homogenizing parental genomic DNA content. As previously mentioned, cytogenetic mapping of triticale showed dense heterochromatic domains in rye, with a high density of heterochromatin in sub-telomeric regions, that is not characteristic in wheat [41]. On the other hand, structural alterations in chromosome condensation often activate mechanisms such as DNA recombination and/or damage repair, leading to sequence excision/modification, as recently described in Drosophila mutant lines for genes coding chromatin remodeling enzymes [42]. Our molecular and cytogenetic results not only show the power of REMAP, IRAP and ISSR in uncovering polyploidization induced genetic restructuring events, but also allow for interesting interpretations regarding the genomic processes involved. It is tempting to speculate for instance that Retrotransposons -rich rye heterochromatic domains when present in triticale loose their capacity to remain condensed, allowing for the occurrence of changes in retrotransposon and microsatellite flanking sequences, essential for polyploid

stability. The involvement of chromatin organization brings us back to the role of epigenetics on genetic adaptation and speciation. Future work will undoubtedly shed light into the complex interactions that occur between genetic and epigenetic events, and their role in molecular evolution.

2.5. Material and Methods

Plant material and DNA isolation. The following plant materials was used: synthetic octoploid primary triticale, (*Triticum aestivum* 'Chinese Spring' × *Secale cereale* 'Imperial'; 2n=8x, AABBDDRR) at least 35 generations old, and their exact progenitors, hexaploid wheat *T. aestivum* 'Chinese Spring' (2n=6x, AABBDD), and diploid rye *S. cereale* 'Imperial' (2n=2x,RR). Although rye is naturally a highly outbreeding species and highly polymorphic genotype, the rye cultivar 'Imperial' used is highly inbred and only inbred seeds has been maintained since 1944. Thus, parental species are very homozygous as a result of many years of selfing. Seeds stocks from all the genotypes were obtained from the USDA–Sears collection, Columbia, Mo. The 'Chinese Spring' wheat and 'Imperial' rye parents used in the present study were taken from the original seed envelopes used by E.R. Sears when he created the Chinese Spring/Imperial triticale. All seed stocks were germinated and grown in controlled conditions at a 16 hours light (20 ^{o}C) / 8 hours dark (20 ^{o}C) cycle. Genomic DNA was isolated from fresh young leaves of eight week old plants using modified cetyltrimethylammonium bromide (CTAB) method [43]. For fluorescent *in situ* hybridization (FISH), three rye plants were analyzed separately. Root tips were collected from one week old plants, washed, fixed in ethanol/acetic acid (3:1 vol/vol) for 24h at room temperature, and stored at -20ºC until use.

IRAP and REMAP procedures. IRAP and REMAP PCR were performed in a 20 µl reaction mixture as previously detailed [27]. Primers for the LTR regions of four barley (*Hordeum vulgare* L.) retrotransposons [25] and three anchored microsatellite primers were utilized. A total of seven primer combinations were tested (Table 1). To assess if the rearranged bands detected in triticale are

faithfully a result of polyploidization and did not result from competition between priming sites of one parent or the other, a mix of the parental wheat and rye DNA was used in the wheat+rye test tube.

ISSR procedure. To determine the specific contribution of SSRs to the observed sequence rearrangements, ISSR amplifications were performed on the same plant material used for IRAP and REMAP. All three di-nucleotide repeats that were used for REMAP and IRAP were tested, and SSR primers are shown in Table 1. The amplification conditions were the same as for IRAP and REMAP and for all PCR experiments at least three technical replicates were completed.

Electrophoresis and data analysis. PCR products were run on 1% agarose gels for 2-3h at 110 volts, detected by ethidium bromide staining, and photographed using BioRad GEL DOC 2000. IRAP, REMAP and ISSR data were analyzed, and DNA bands were identified using the following criteria:
[i] Monomorphic bands: common to both triticale progenitors (wheat and rye);
[ii] Polymorphic bands: observed in only one triticale progenitor (wheat and rye);
[iii] Polyploid expected bands: expected from an additive profile of the characteristic wheat and rye parental bands;
[iv] Polyploid conserved bands: parental bands detected in the polyploid;
[v] Polyploid rearranged bands: parental (wheat and rye) bands absent in the polyploid as well as novel bands observed exclusively in the polyploid.
Once rearranged sequences were identified and verified for their reproducibility, three bands were gel-isolated, purified, cloned, and finally sequenced following the procedures described in Rocheta *et al.* [44]. The sequences obtained were used for BLAST on NCBI, TIGR, and PlantSat (repetitive plant sequences) databases as described in the results section. Cluster analysis were performed utilizing the Neighbor-Joining method [24] on the basis of a distance matrix calculated with the Bionumerics software (version 3.5).

FISH. REMAP PCR reaction with primer CO699/(CT)9G was used as probe for *in situ* hybridization. The reaction conditions and amplification program were similar to those of normal REMAP, except that 1µl (1ηmol/µl) of digoxigenin-dUTP or biotin-dUTP (Roche, Gipf-Oberfrick, Switzerland) was added to the reaction

mixture in order to label all PCR products. Ribosomal specific probe pTa71 [45] was used as control, labeled by nick translation using biotin-dUTP or digoxigenin-dUTP. Root tips were prepared as previously described for cytological analysis [46], with the following modifications. Fixed root tips were digested with pectinase/cellulase in 1×EB for 2h15min at 37ºC, and squashes were performed in 60% glacial acetic acid. Nuclei and chromosomes were counterstained with 4',6-diamidino-2-phenylindole hydrochloride (DAPI) in Citifluor antifade mounting medium (AF1; Agar Scientific). Samples were examined using a Zeiss Axioskop 2 epifluorescence microscope and images were obtained using a Zeiss AxioCam digital camera. Digital images were processed using PHOTOSHOP (Adobe Systems).

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2.7. Supporting Information

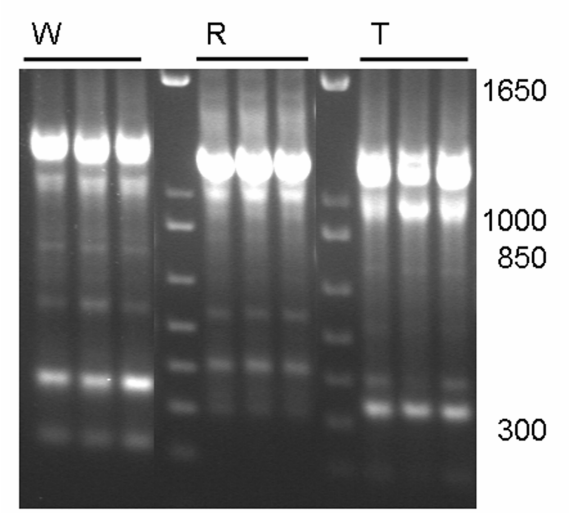

Figure S1. IRAP banding profiles - individual plants of each genotype. IRAP banding profiles obtained with primer Nikita from three individuals of each genotype: (W) Wheat, (R) Rye, and (T) Triticale.

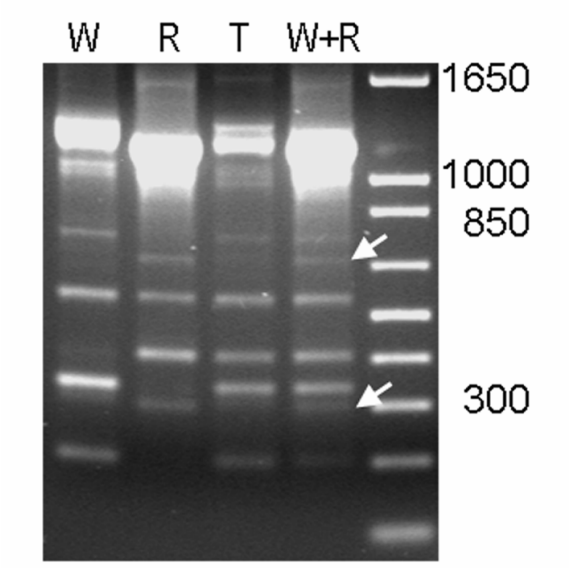

Figure S2. IRAP banding profiles - wheat, rye, triticale and wheat+rye test tube. IRAP banding profile obtained with primer Nikita of wheat (W), rye (R), triticale (T), and wheat+rye test tube (W+R) showing triticale rearranged bands. Arrows indicate two rearranged band of rye genome origin absent in triticale but present in the wheat+rye test tube.

Chapter III

Genome merger: from sequence rearrangements in triticale to their elimination in wheat–rye addition lines

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ORIGINAL PAPER

Genome merger: from sequence rearrangements in triticale to their elimination in wheat–rye addition lines

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Abstract Genetic and epigenetic modifications resulting from different genomes adjusting to a common nuclear environment have been observed in polyploids. Sequence restructuring within genomes involving retrotransposon/microsatellite-rich regions has been reported in triticale. The present study uses inter-retrotransposon amplified polymorphisms (IRAP) and retrotransposon microsatellite amplified polymorphisms (REMAP) to assess genome rearrangements in wheat–rye addition lines obtained by the controlled backcrossing of octoploid triticale to hexaploid wheat followed by self-fertilization. The comparative analysis of IRAP and REMAP banding profiles, involving a complete set of wheat–rye addition lines, and their parental species revealed in those lines the presence of wheat-origin bands absent in triticale, and the absence of rye-origin and triticale-specific bands. The presence in triticale × wheat backcrosses (BC) of rye-origin bands that were absent in the addition lines demonstrated that genomic rearrangement events were not a direct consequence of backcrossing, but resulted from further genome structural rearrangements in the BC plant progeny. PCR experiments using primers designed from different rye-origin sequences showed that the absence of a rye-origin band in wheat–rye addition lines results from sequence elimination rather than restrict changes on primer annealing sites, as noted in triticale. The level of genome restructuring events evaluated in all seven wheat–rye addition lines, compared to triticale, indicated that the unbalanced genome merger situation observed in the addition lines induced a new round of genome rearrangement, suggesting that the lesser the amount of rye chromatin introgressed into wheat the larger the outcome of genome reshuffling.

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Introduction

Merging plant genomes is a major evolutionary process, resulting mainly through polyploidization, and has been estimated to have occurred in 30–70% of all plant species. The adjustment of different genomes to a shared nuclear environment can induce genomic and epigenomic variation. Genetic changes include translocations and transpositions as well as sequence deletions and insertions, while epigenetic changes include non-additive gene regulation, transposon transcription, silencing or sub-functionalization of homoeologous genes, and chromatin remodeling (Ma and Gustafson 2008). Several studies in wheat (*Triticum* ssp.), *Arabidopsis*, and *Spartina* polyploids have suggested that the genomic changes that occur during polyploid formation are not random, but rather directed and highly reproducible (Ainouche et al. 2008; Chen et al. 2008; Kashkush et al. 2002; Liu et al. 1998; Madlung et al. 2005; Ozkan et al. 2003; Shaked et al. 2001). The preferential loss of parental-specific bands has been revealed in newly formed polyploids involving *Aegilops* and *Triticum* species using amplified fragment length polymorphism (AFLP) and

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3. Genome merger: from sequence rearrangements in triticale to their elimination in wheat-rye addition lines

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Key words: genome restructuring, retrotransposons and microsatellites, IRAP and REMAP, wheat-rye addition lines.

3.1. Abstract

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Merging plant genomes is a major evolutionary process, resulting mainly through polyploidization, and has been estimated to have occurred in 30-70% of all plant species. The adjustment of different genomes to a shared nuclear environment can induce genomic and epigenomic variation. Genetic changes include

translocations and transpositions as well as sequence deletions and insertions, while epigenetic changes include non-additive gene regulation, transposon transcription, silencing or sub-functionalization of homoeologous genes, and chromatin remodeling (Ma and Gustafson 2008). Several studies in wheat (*Triticum* ssp.), *Arabidopsis*, and *Spartina* polyploids have suggested that the genomic changes that occur during polyploid formation are not random, but rather directed and highly reproducible (Ainouche et al. 2008; Chen et al. 2008; Kashkush et al. 2002; Liu et al. 1998; Madlung et al. 2005; Ozkan et al. 2003; Shaked et al. 2001). The preferential loss of parental-specific bands has been revealed in newly formed polyploids involving *Aegilops* and *Triticum* species using amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) (Liu et al. 1998; Ozkan et al. 2003). Similar techniques also disclosed genetic and epigenetic modifications in the synthetic allopolyploid triticale (X *Triticosecale* Wittmack) preferentially affecting rye-origin repeated and low-copy sequences (Ma et al. 2004).

Transposable elements constitute a decisive driving force in genome evolution and are crucial in plant genome adjustment and speciation through sequence modifications and changes in genome size (Charles et al. 2008). Although polyploidy induced retrotransposon transposition was never demonstrated, an increase in retrotransposon-related transcripts has been detected in wheat synthetic neopolyploids (Kashkush et al. 2002, 2003) as well as in *Arabidopsis* polyploid genotypes (Adams and Wendel 2005; Josefsson et al. 2006; Madlung et al. 2005). The analysis of *Spartina* polyploids through sequence specific amplified polymorphism (SSAP), revealed band elimination involving retroelements (Ainouche et al. 2008). Recently, the analysis of triticale through Inter Retrotransposon Amplified Polymorphism (IRAP), and Retrotransposon Microsatellite Amplified Polymorphism (REMAP) revealed the disappearance of wheat- and rye-origin bands and the appearance of triticale-novel bands in the polyploid genotype. A significant rate of polyploidization-induced rearrangements was thus uncovered in retrotransposon and/or microsatellite associated sequences, preferentially allocated in major heterochromatic domains, and affecting mainly rye-origin bands (Bento et al. 2008).

Polyploidization is the beginning of alien chromatin introgression in important crops through chromosome engineering. The synthetic intergeneric allopolyploid

triticale resulting from the hybridization of wheat (*Triticum* ssp.) and rye (*Secale cereale* L.) has been used in the production of wheat-rye addition, substitution, and translocation lines through initial backcrosses to the wheat parent leading to rye chromosome, miss-segregation in meiosis and their concomitant modification and/or loss. Auto-fertilization of those BC plants therefore leads to plants disomic for wheat chromosomes and with reduced numbers of rye chromosomes, which represent the starting point for the stable introgression of rye chromatin in wheat background thus increasing wheat genome diversity (O'Mara 1940).

The present work analyses DNA sequence restructuring assessed through microsatellite and retrotransposon PCR-based molecular marker techniques of an entire set of wheat lines containing the addition of each individual rye chromosome pair. In all wheat-rye addition lines, IRAP and REMAP analyses revealed the absence of rye-origin and triticale-novel sequences and the presence of wheat-origin bands absent in the triticale parent. Moreover, we demonstrated that rye-origin band loss occurring in triticale corresponded to sequence rearrangements only involving primer annealing sites, whereas rye-specific band losses observed in the addition lines result from massive sequence elimination events.

3.3. Material and Methods

Plant material and DNA isolation. The following plant materials were used: hexaploid wheat *T. aestivum* L. 'Chinese Spring' (2n=6x=42, AABBDD), diploid rye *S. cereale* L. 'Imperial' (2n=2x=14, RR), the correspondent synthetic octoploid triticale, (*T. aestivum* 'Chinese Spring' × *S. cereale* 'Imperial'; 2n=8x=56, AABBDDRR), and the set of seven wheat-rye addition lines, each composed of the entire hexaploid wheat genome plus a single pair of rye homoeologous chromosomes. The rye cultivar 'Imperial' used is highly inbred and the octoploid triticale and their corresponding wheat-rye addition lines are at least 35 generations old (Ma et al. 2004). All seeds used in the present study were from the original E.R. Sears seed stocks and were obtained from the USDA–Sears collection, Columbia, Missouri. We have also analyzed the BC triticale x wheat F_1 (AABBDDR) produced using 'Chinese Spring' wheat.

Seeds from all genotypes were germinated and grown in controlled conditions at a 16h light (20^{o}C) / 8h dark (20^{o}C) cycle. Genomic DNA was isolated from fresh young leaves of eight week old plants using modified cetyltrimethylammonium bromide (CTAB) method (Saghaimaroof et al. 1984).

For all cytogenetic analyses, three plants of each accession were analyzed separately. Root tips were collected from one week old plants, cold treated for 24 hours, fixed in ethanol/acetic acid (3:1 vol/vol) for 24h at room temperature, and stored at -20ºC until use.

PCR amplification, electrophoresis and data analysis. PCR was performed for IRAP and REMAP analyses and to amplify rye sequences, using the primers presented in Table 1. IRAP and REMAP PCR reactions were performed as previously detailed (Kalendar et al. 1999). A total of five combinations of primers for the LTR regions of three barley (*Hordeum vulgare* L.) retrotransposons and for two-anchored microsatellite were used (Bento et al. 2008). The selected retrotransposons (Nikita, Sabrina, and Sukkula) have proven to be useful in DNA fingerprinting and evolutionary studies, not only in barley but also in related taxa (Leigh et al. 2003).

To test the presence of characteristic rye sub-telomeric sequences primers were designed for pSc200, a 521 bp clone of an *S. cereale* DNA sub-telomeric tandem repeat (accession number Z50039) (Vershinin et al. 1996). To characterize rearranged DNA sequences previously observed in (Bento et al. 2008), primers were designed to amplify several internal segments of the rye-origin sequence MoB-111-1000R (accession number EF486521) obtained with REMAP Nikita/(CA)9G.

PCR amplification of all sequences was performed in 10µl reactions containing: 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM MgCl2, 0.25mM dNTP's, 1mM each primer, 0.5 U *Taq* polymerase and 25ng DNA template; using the following program: 4 min at 94ºC; 30 cycles of 30s at 94ºC, 30s at 54ºC, 30s at 72ºC; termination by 4 min of final extension at 72ºC. PCR products were run on 1% agarose gels for 2-3h at 110 volts, detected by ethidium bromide staining, and photographed using Bio-Rad GEL DOC 2000. Selected bands were gel isolated, purified, cloned, and finally sequenced following the procedures described in (Rocheta et al. 2006). Homology between internal fragments and original rye-

origin bands were verified using BioEdit version 7.0.5.3 sequence alignment editor.

Table 1. Primers used for PCR analysis.

Primer	Sequence
pSc200	
For	5'- TCTTTGATCACCGTTTCTTCG
Rev	5'- CCCCACCCATGTATGGATAA
LTR	
C0699 (Nikita)	5'-CGCTCCAGCGGTACTGCC
C0945 (Sabrina)	5'-GCAAGCTTCCGTTTCCGC
9900 (Sukkula)	5'-GATAGGGTCGCATCTTGGGCGTGAC
SSR	
(CT)9G	5'-CTCTCTCTCTCTCTCTCTG
(CA)9G	5'-CACACACACACACACACAG
MoB-111-1000R [1-3]	
For	5'- ATTAGTATGCTGCCGTCGTG
Rev	5'- ATTACCTTCAAACCCTCC
MoB-111-1000R [1]	
For	5'- ATTAGTATGCTGCCGTCGTG
Rev	5'- ACCACTTTGCTGCTTCCTTC
MoB-111-1000R [2]	
For	5'- AGTGTCCTTAGGATGGACAACTG
Rev	5'- GTGGTTCCAACTCCTGTA
MoB-111-1000R [3]	
For	5'- GTGGTTCCAACTCCTGTA
Rev	5'- ATTACCTTCAAACCCTCC

GISH. Genomic *in situ* hybridization (GISH) was performed on root tip meristematic cells prepared as previously described (Silva et al. 2008). Fixed root tips were digested with pectinase/cellulase in 1×EB for 2h 15min at 37ºC, and squashes were performed in 45% glacial acetic acid. Rye total genomic DNA was used as a probe labeled by nick translation with biotin-dUTP (with wheat total genomic blocking DNA) and detected with streptavidin Cy3-conjugated. Nuclei and chromosomes were counterstained with 4',6-diamidino-2-phenylindole hydrochloride (DAPI) in Citifluor antifade mounting medium (AF1; Agar Scientific). Samples were examined using a Zeiss Axioskop 2 epifluorescence microscope and images were obtained using a Zeiss AxioCam digital camera. Digital images were processed using PHOTOSHOP (Adobe Systems).

3.4. Results

Cytological and molecular identification of rye chromosomes in wheat-rye addition lines

The wheat-rye addition lines were produced through controlled BC of the octoploid triticale to the wheat parent, followed by successive generations of self-fertilization and subsequent rye chromosome selection. The first backcross (BC) generation yielded plants with the hexaploid wheat genome plus the rye genome in a haploid condition. Selfed BC plants contained the complete wheat complement and variable numbers of rye chromosomes, from one to seven. Selected BC plants which were monosomic addition lines, contained the complete wheat complement and one rye chromosome, produced disomic addition lines (O'Mara 1940). To confirm their genomic constitution, both cytological and molecular methodologies were used.

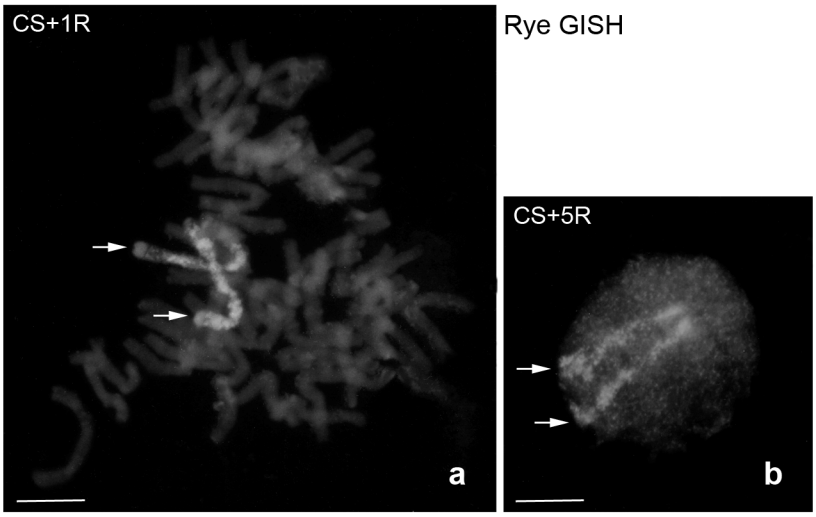

Fig. 1 GISH on meristematic root-tip cells using rye total genomic DNA as a probe for the confirmation of rye chromosomes in wheat-rye addition lines. **a**. Metaphase cell of CS+1R wheat-rye addition line; hybridization signal revealing the pair of 1R homologous chromosomes (arrows). **b**. Interphase nucleus of CS+5R wheat-rye addition line, hybridization signal revealing the pair of 5R homologous chromosomes as string domains (arrows). Bar = 5μm.

The presence of a pair of rye chromosomes in each wheat-rye addition line was confirmed using GISH with rye total genomic DNA in both metaphase and interphase cells (Figure 1a and 1b, respectively). In interphase nuclei GISH allowed for the detection of the characteristic nuclear Rabl organization since rye chromosomes are observed as thin linear "strings" throughout the nucleus, with

DAPI-positive sub-telomeric heterochromatic domains of both homologous localized in the same nuclear hemisphere.

The presence of rye chromosomes in the addition lines was further confirmed by PCR amplification of pSc200, a rye-origin sub-telomeric tandem repeat (accession number Z50039) that contains a 381 bp repeat unit. Primers amplified a 446 bp fragment, as a result of the amplification from the forward primer of one repeat to the reverse primer in the next repeat unit (Electronic supplementary material, Figure S1). The pSc200 banding profiles produced from wheat, rye, triticale and the seven wheat-rye addition lines are presented in Figure 2a. The band amplified from rye genomic DNA was ~450 bp, matching the referred fragment size and was also present in triticale. As no amplification product was detected from wheat DNA, pSc200 PCR experiments were used to confirm the presence of rye chromosomes in a wheat background. Higher exposure of the gel presented in Figure 2a, revealed seven wheat-rye addition lines bands similar to the ones observed in rye and triticale (Figure 2b). Thus, the pSc200 sequence identified all genotypes containing rye chromatin.

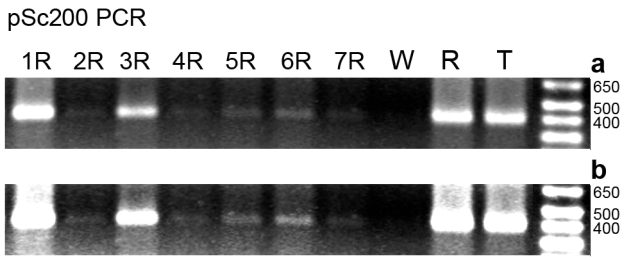

Fig. 2 pSc200 PCR banding profiles of wheat (W), rye (R), triticale (T) and seven wheat-rye addition lines (numbers correspond to rye homologous chromosomes pair). A rye-origin band with ~450 bp is detected in triticale and wheat-rye addition lines. No amplification in wheat was observed. **b.** is an overexposed copy of **a**, reveling pSc200 bands in all wheat-rye addition lines. Molecular weight marker: 1kb+.

Triticale- and rye-origin bands are absent in wheat-rye addition lines and present in triticale x wheat backcross

Five IRAP and REMAP primer combinations were used in the analysis of wheat-rye addition lines, namely the primer for the LTR sequence of the barley retrotransposon Nikita by itself and in combination with other LTR primers from the barley retrotransposons Sabrina and Sukkula, and with the (CT)9G and (CA)9G anchored microsatellite primers. The banding profiles yielded reproducible arrays of distinct bands for the three replicates performed for all primer combinations

analyzed. Only bands between 100 and 1650 bp were scored, as this gel region produced the highest quality profiles. Banding profiles from wheat, rye, triticale and the seven wheat-rye addition lines obtained with two of the five primer combinations studied are shown in Figures 3, 4, and 5 and the results obtained from all primer combinations are presented in Table 2.

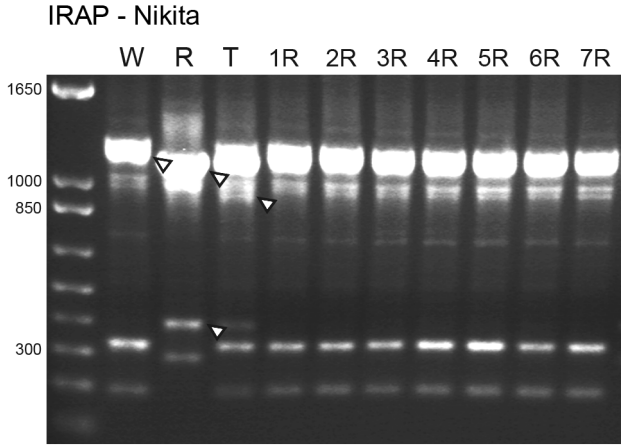

Fig. 3 IRAP banding profiles of wheat (W), rye (R), triticale (T) and the seven wheat-rye addition lines, obtained with primer Nikita (numbers correspond to rye homologous chromosomes pair). Arrowheads indicate a wheat band absent in triticale and present in all the addition lines and three bands absent in all the addition lines: two rye-origin bands and one triticale-origin band. Molecular weight marker: 1 kb+.

IRAP and REMAP banding profiles comparing wheat-rye addition lines profiles with wheat and triticale progenitors were analyzed. The results revealed that banding patterns of all wheat-rye addition lines were identical to wheat progenitor banding profiles for all the primer combinations evaluated. All wheat-origin bands were present in the addition lines, even those absent in the triticale. However, a contrasting situation was observed for rye-origin bands present in the triticale, and for triticale-specific bands. In wheat-rye addition lines we observed the absence of parental triticale bands with the following origins:

- Rye-origin DNA bands observed in rye and triticale;
- Triticale-origin DNA bands absent in wheat and rye.

The seven addition line IRAP banding profiles obtained using the Nikita primer (Figure 3) revealed one wheat-origin band absent in triticale, the loss of two rye-origin bands (present in triticale), and the loss of one triticale-origin band. The Nikita/Sabrina and Nikita/Sukkula IRAP primer combination analysis revealed the presence of all wheat-origin bands including one absent in triticale, and the absence of the four rye-origin bands present in the triticale. REMAP Nikita/(CT)9G

(Figure 5) detected the presence, in addition lines, of one wheat-origin band (absent in triticale) and the absence of the three rye- and two triticale-origin bands. Nikita/(CA)9G REMAP (Figure 4) was used as a control experiment since the banding profiles obtained for wheat and triticale and the wheat-rye addition lines banding profiles were identical.

Table 2. IRAP and REMAP analysis in wheat lines with the addition of rye chromosomes comparing with triticale and wheat progenitors**.**

	Number of bands with different primer combinations					
		IRAP		**REMAP**		**Total**
Primer combined with Nikita	**Nikita**	**Sabrina**	**Sukkula**	**(CT)9G**	**(CA)9G**	
Wheat [a]	7	7	11	9	12	46
Triticale [a]	10	10	14	13	12	59
Addition lines	7	7	11	9	12	46
Addition lines lost bands [b]	3	4	4	5	0	16
Rye-origin	2	4	4	3	-	13
Triticale-origin	1	-	-	2	-	3

[a] Results published in (Bento et al. 2008)
[b] Addition lines lost bands: rye-origin lost bands - bands observed in rye profiles as well as in triticale profiles, and absent in all the wheat–rye addition lines; triticale-origin bands - bands observed in triticale considered as novel, since they are not observed in wheat and rye progenitors profiles, and absent in all the wheat–rye addition lines

Since all rye-origin bands present in triticale and all triticale-origin bands were absent in all wheat-rye addition lines, detailed experiments, to evaluate the

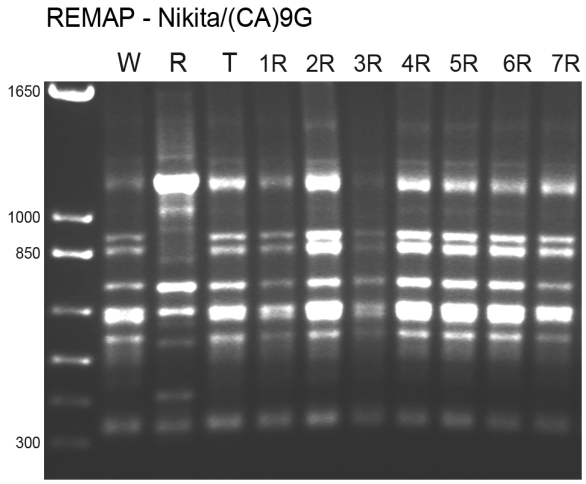

Fig. 4 REMAP banding profiles of wheat (W), rye (R), triticale (T) and the seven wheat-rye addition lines, obtained with primer combination Nikita/(CA)9G (numbers correspond to rye homologous chromosomes pair). Banding patterns observed in all the addition lines corresponds to the sum of banding profiles detected in the parental genotypes triticale and wheat.
Molecular weight marker: 1 kb^{+}.

accuracy of the techniques used, were performed to consider the possibility that the loss of rye/triticale origin bands in the addition lines resulted from rye sequence-reduction in comparison to wheat-origin sequences.

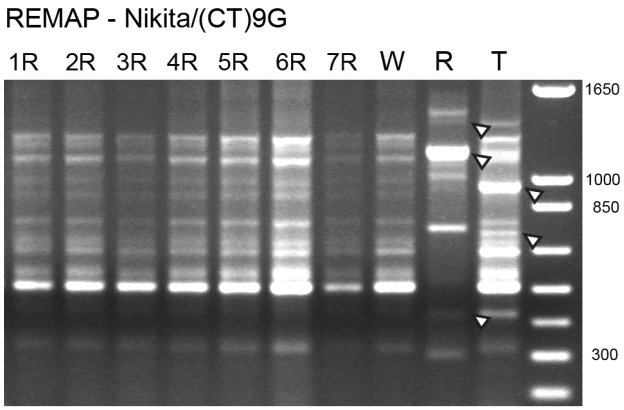

Fig. 5 REMAP banding profiles of wheat (W), rye (R), triticale (T) and the seven wheat-rye addition lines, obtained with primer combination Nikita/(CT)9G (numbers correspond to rye homologous chromosomes pair). Arrowheads indicate five bands absent in all the addition lines: three rye-origin bands and two triticale-origin bands. Molecular weight marker: 1 kb^{+}.

Several IRAP and REMAP experiments were performed using a DNA template mixture containing a fixed quantity of wheat DNA and decreasing quantities of rye DNA until 1:1/7 of wheat : rye ratio, which corresponds to the addition of one rye chromosome to the wheat genome in comparison with rye genomic DNA present in triticale. Figure S2 of Electronic supplementary material corresponds to the IRAP experiment obtained with primer Nikita using decreasing concentrations of rye DNA mixed with wheat DNA, showing the presence of two rye-origin bands in all rye dilutions. This experiment proved that the absence of rye bands in the wheat-rye addition lines was not the result of a technical artifact.

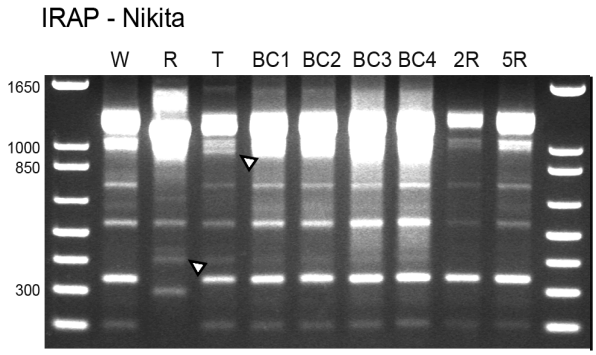

Fig. 6 IRAP banding profiles obtained with primer Nikita of wheat (W), rye (R), triticale (T), four different individual plants from triticale-wheat backcross (BC1 – BC4) and two wheat-rye addition lines (CS+2R and CS+5R). One rye-origin band (~400 bp) observed in triticale and one triticale-origin band (~900 bp) are both observed in the four BC individual plants and are absente in the two wheat-rye addition lines (arrowheads). Molecular weight marker: 1 kb^{+}.

To assess the timing course of genome rearrangements detected in wheat-rye addition lines, Nikita IRAP experiments were performed on four plants that resulted from the triticale x wheat BC. Banding profiles from backcross plants, wheat, rye, triticale and two wheat-rye addition lines (2R and 5R) (Figure 6) revealed the presence of all wheat-origin, one rye-origin (~400 bp) and one triticale-origin (~900 bp) bands in the four BC plants analyzed.

The overall characterization of wheat-rye addition lines using IRAP and REMAP demonstrated that in these genotypes rye- and triticale-origin bands were absent. Moreover, all bands from wheat-origin were present, including those absent in triticale. To confirm that wheat-origin bands absent in triticale and present in the addition lines corresponded to the same wheat sequence, we further analyzed the MoB-11-1200W wheat-origin sequence (accession number EF486520), which was gel-isolated from the Nikita IRAP banding profiles of wheat-rye addition lines CS+1R and CS+7R, purified and cloned for sequencing. Sequencing analyses of these bands (Electronic supplementary material, Figure S3) revealed that they share 84% and 83% homology, respectively, with the wheat sequence previously characterized.

Triticale rye-origin sequences are eliminated in wheat-rye addition lines

The absence of rye- and triticale-specific bands in the addition lines raises questions about the processes underlying such absences, such as sequence rearrangements or deletions. Primers to amplify four internal segments of the REMAP rye-origin 964 bp sequence MoB-111-1000R (accession number EF486521) were designed (Figure 7). The results obtained in rye confirmed the amplification of four bands with the expected sizes:

- one whole internal fragment: MoB-111-1000R [1-3] with 798 bp;
- three partial internal fragments: MoB-111-1000R [1] with 480 bp, MoB-111-1000R [2] with 483 bp and MoB-111-1000R [3] with 323 bp.

Unlike the original MoB-111-1000R rye band, the whole internal segment with the expected 798 bp was detected in rye and in triticale (Figure 8). The ~800 bp bands amplified from rye and triticale DNA were gel-isolated, purified and cloned for sequencing, revealing high homology between the original MoB-111-1000R sequence and MoB-111-1000R [1-3] internal segment amplified from both rye (100%) and triticale (98%).

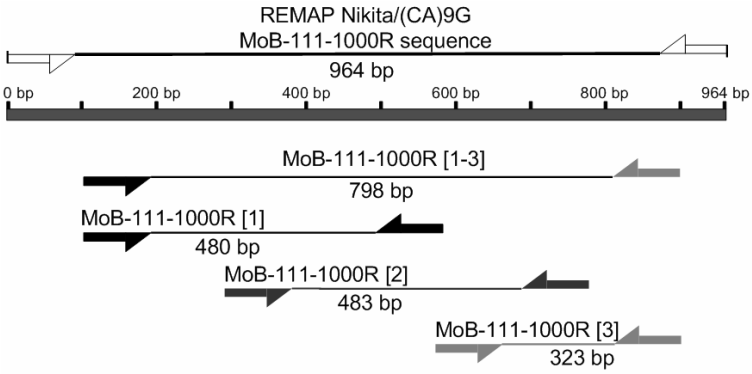

Fig. 7 Dimensions (bp) of fragments expected from the amplification of four internal segments of the rye-origin sequence MoB-111-1000R (accession number EF486521): MoB-111-1000R [1-3], MoB-111-1000R [1], MoB-111-1000R [2], and MoB-111-1000R [3].

The amplification with primers designed to MoB-111-1000R [1] revealed one ~450 bp band in wheat, distinct from the 480 bp band obtained in rye with both being present in triticale (Figure 9). Further sequencing of the 480 bp rye-origin sequence amplified from triticale showed that, as expected, it shared 97% homology with the sequence amplified from rye.

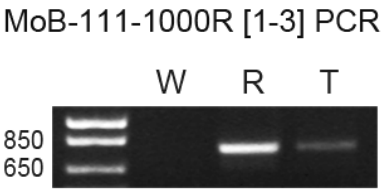

Fig. 8 PCR banding profiles of wheat (W), rye (R), triticale (T) obtained with primers designed to amplify the whole internal fragment of the sequence MoB-111-1000R (MoB-111-1000R [1-3]). As expected, only one band with ~800 bp was amplified from rye. A similar band was amplified from triticale and no amplification products were detected in wheat.

The amplification obtained using primers designed to MoB-111-1000R [2] revealed two different bands in wheat (approximately ~400 and ~450 bp) and one in rye (483 bp). The banding profile obtained in triticale showed the three bands resulting from the addition of the parental wheat and rye banding profiles (Figure 10). The sequencing of the MoB-111-1000R [2] sequences amplified from wheat, rye, and triticale revealed that they were all analogous sequences (Electronic supplementary material, Figure S4), and that both rye- and wheat-origin bands amplified from triticale shared extremely high levels of homology (more than 98%) with the ones amplified from the parental species. On the other hand, ~400 and ~450 bp wheat amplified sequences also shared a high level of homology (80% and 91%, respectively) with the sequence amplified from rye. The band

correspondent to MoB-111-1000R [3] was present in all three genotypes with a strong intensity in rye, a faint one in wheat and an intermediate one in triticale.

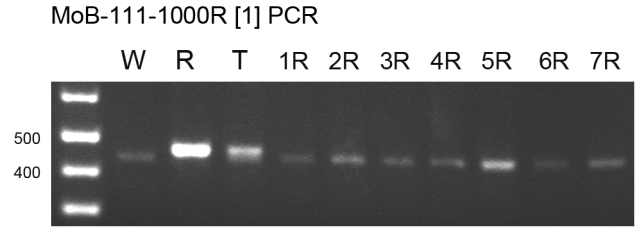

Fig. 9 PCR banding profiles of wheat (W), rye (R), triticale (T) and 7 wheat-rye addition lines (numbers correspond to rye homologous chromosomes pair), obtained with primers designed to amplify the first internal fragment of the sequence MoB-111-1000R (MoB-111-1000R [1]). In triticale a double band was observed, corresponding to the wheat and rye parental bands. In the addition lines, only the wheat-specific band is observed. Molecular weight marker: 1 kb^{+}.

The amplification of DNA from the wheat-rye addition lines with primers to MoB-111-1000R [1] and MoB-111-1000R [2] only revealed the wheat-origin bands, whereas the rye-origin bands were missing on all seven wheat-rye addition line banding profiles (Figures 9 and 10, respectively). It was therefore demonstrated that the rye MoB-111-1000R internal sequences maintained in triticale and in the triticale x wheat BC were absent in addition lines.

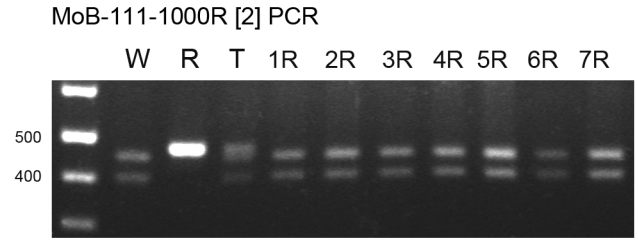

Fig. 10 PCR banding profiles of wheat (W), rye (R), triticale (T) and 7 wheat-rye addition lines (numbers correspond to rye homologous chromosomes pair), obtained with primers designed to amplify the second internal fragment of the sequence MoB-111-1000R (MoB-111-1000R [2]). In triticale, three bands were observed, corresponding to two wheat-origin bands plus one rye-origin band. In the addition lines, only the two wheat-specific bands were observed. Molecular weight marker: 1 kb^{+}.

3.5. Discussion

A high rate of induced DNA sequence rearrangements was recently demonstrated to occur due to polyploidization namely in triticale and those events are associated with retrotransposon and/or microsatellite-rich genomic regions (Bento et al. 2008)

as they were disclosed through the utilization of IRAP, REMAP and ISSR techniques (Kalendar et al. 1999). Detection of DNA sequence rearrangements in triticale were feasible due to parental genomic diversity unraveled by those important molecular tools, largely used also to characterize intraspecific variability in barley, *Vitis vinifera*, and *Pisum* ssp. (Kalendar et al. 1999; Pereira et al. 2005; Smykal 2006).

If no rearrangement events had resulted during the addition line creation, the presence of all bands observed in wheat plus those of triticale-origin would be expected in the global profiles of the seven wheat–rye addition lines. However, the genomic screening of wheat-rye addition lines compared to the triticale and wheat parents, accomplished through IRAP and REMAP using five different primer combinations, revealed the absence of all rye- and triticale-origin bands. Concerning the wheat genome, all wheat-origin bands absent in triticale were present in all wheat-rye addition lines, probably resulting from the BC of triticale to wheat. Moreover, the detailed analysis of one IRAP wheat-origin copia-like retrotransposon related sequence (MoB-11-1200W) amplified with Nikita IRAP (Bento et al 2008), confirmed that the wheat-origin sequence amplified in the addition lines share high homology with the original characterized wheat band.

Besides the substantial elimination of rye-origin sequences in wheat-rye addition lines, our results also disclosed another novel restructuring event where the triticale-origin bands that emerged from wheat-rye polyploidization were eliminated. The directed and controlled loss of non-coding and coding sequences has been suggested to overcome genome redundancy induced by different genomes merging (Ma and Gustafson 2005) and a preferential occurrence of restructuring events involving rye parental genome was observed in wheat-rye hybrids and triticale (Bento et al. 2008; Ma et al. 2004; Ma and Gustafson 2006; Tang et al. 2008). This tendency was also observed on AFLP banding profiles of wheat-rye addition lines (Ma et al. 2004) which showed that the rye-origin triticale bands were absent in the addition lines.

Differences in genome size, chromatin organization patterns and cell cycle duration are well known to cause genomic conflicts in newly formed hybrids. Genomic differences have been cytologically revealed in triticale as dense rye heterochromatic sub-telomeric domains not present in wheat (Bennett 1977; Neves et al. 1997), as we confirmed in wheat-rye addition lines through DAPI-

positive sub-telomeric heterochromatic bands on rye chromosomes and through PCR analysis of pSc200 rye-origin sequence. Wheat-rye addition line chromosome instability has been well documented (Alkhimova et al. 1999; Riley 1960) and seems to suggest a new round of genomic adjustment due to a greater genome composition unbalance. However, major genomic restructuring events are not an immediate result of triticale-wheat BCs, since bands absent in the addition lines are yet present in plants resulting from triticale x wheat BC. Thus, rye-origin sequence elimination must occur throughout self-fertilization of selected lines from the progeny of triticale x wheat BC. Accordingly, (Gustafson et al. 1983) found rye-chromosome alterations involving sub-telomeric heterochromatic bands, but only in a F3 generation of a triticale x wheat BC.

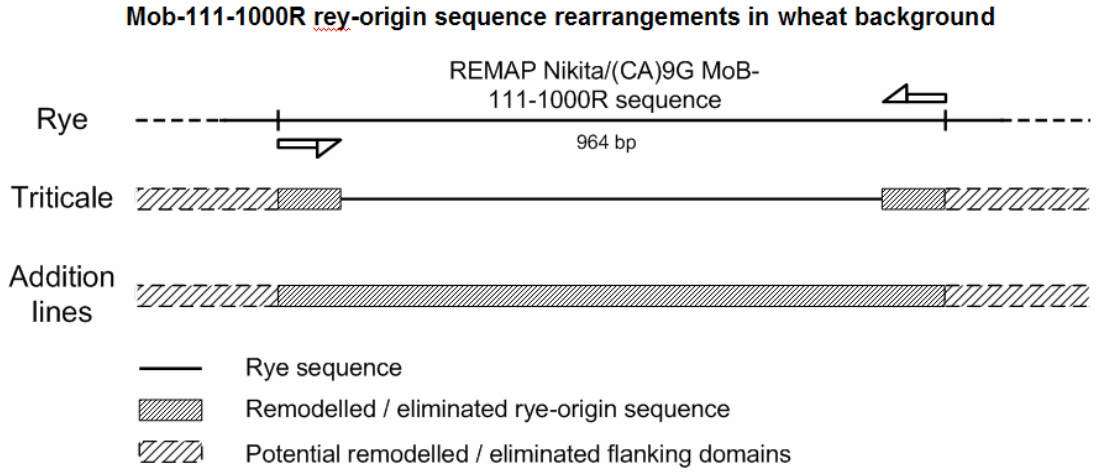

Fig. 11 Schematic representation of REMAP Nikita/(CA)9G rye-origin sequence (MoB-111-1000R) and its rearrangements when introgressed in wheat background. Although the original REMAP band is absent in triticale, internal fragments were confirmed by amplification and sequencing, revealing that polyploidization probably induced changes in flanking domains of primer annealing sites. In wheat-rye addition lines a major rearrangement of that sequence occurred as neither the original REMAP rye sequence, nor its internal segments are amplified, disclosing the occurrence of extensive alien chromatin elimination.

Transcriptional activation of retrotransposons has been previously reported in newly synthesized wheat amphiploids (Kashkush et al. 2002, 2003) and *Arabidopsis* (Adams and Wendel 2005; Josefsson et al. 2006; Madlung et al. 2005). On the other hand, the analysis of wheat-rye hybrids using wheat SSRs (Tang et al. 2008), and tobacco (*Nicotiana* ssp.) allopolyploids (Petit et al. 2007) and *Spartina* hybrids (Ainouche et al. 2008) through retrotransposon sequence-specific amplified polymorphism (SSAP), revealed only low frequent parental band disappearance events. A crucial question arises about the extension of the

restructuring events disclose by IRAP and REMAP as genomic diversity, assessed by those techniques, is based on banding profiles. Band loss can result either from minor changes in primer annealing sites or from gross elimination of DNA sequences, and both are indiscriminately described as genome rearrangements.

DNA sequence analysis of a rye-origin band absent in both triticale and wheat-rye addition lines largely confirmed the distinct levels of genome rearrangements resulting from merging genomes or chromosome introgression. Although the original rye REMAP hydroxyproline-rich glycoproteins (HRGP) coding related sequence (MoB-111-1000R) was absent in triticale, most of its internal sequence was present in triticale, demonstrating that polyploidization rearrangements could involve primer annealing sites. Conversely, in wheat-rye addition lines rearranged sequences present in triticale and its internal segments were eliminated. The disappearance of the MoB-111-1000R rye-origin band in triticale resulted from a small sequence rearrangement markedly contrasting with the deletion of an entire rye sequence demonstrated to occur in the wheat-rye addition lines (Figure 11). Genomic rearrangements are therefore much more drastic in wheat-rye addition lines than in triticale, indicating a massive elimination of rye sequences, when restricted amounts of rye chromatin were introgressed into a hexaploid wheat background.

The high level of rearrangements concerning microsatellite and/or retrotransposon-rich genome fractions identified in the wheat-rye addition lines demonstrated that genome reshuffling not only entailed polyploid induced genome adjustments, but enhanced more restricted chromatin introgression (i.e. chromosome pair additions, substitutions or translocations, involving the transfer of DNA from one species to another) frequently involved in plant breeding approaches.

Acknowledgments

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and PTDC/BIA-BEC/101964/2008). We would like to thank Augusta Barão for her excellent technical assistance and Leonor Morais-Cecílio for her help in image processing.

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3.7. Electronic Supplementary Material

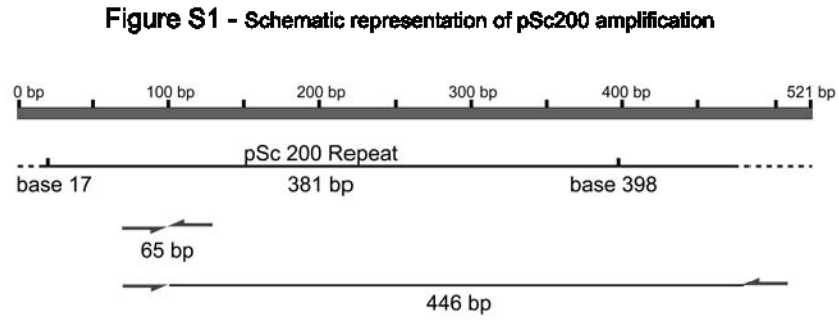


Figure S1 Dimension of amplified fragments expected from the sequence pSc200 of *S.cereale* DNA sub-telomeric tandem repeat (accession number Z50039) that comprehends a repeat unit with 381 bp.

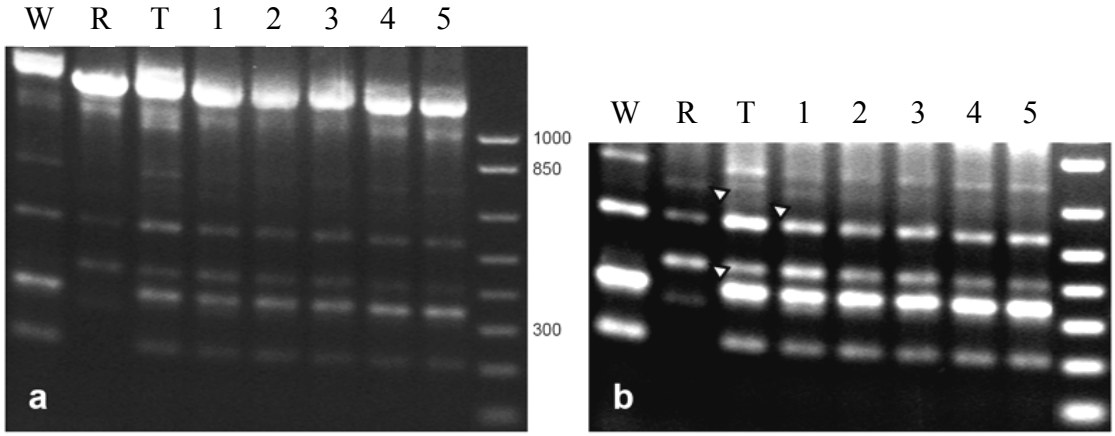
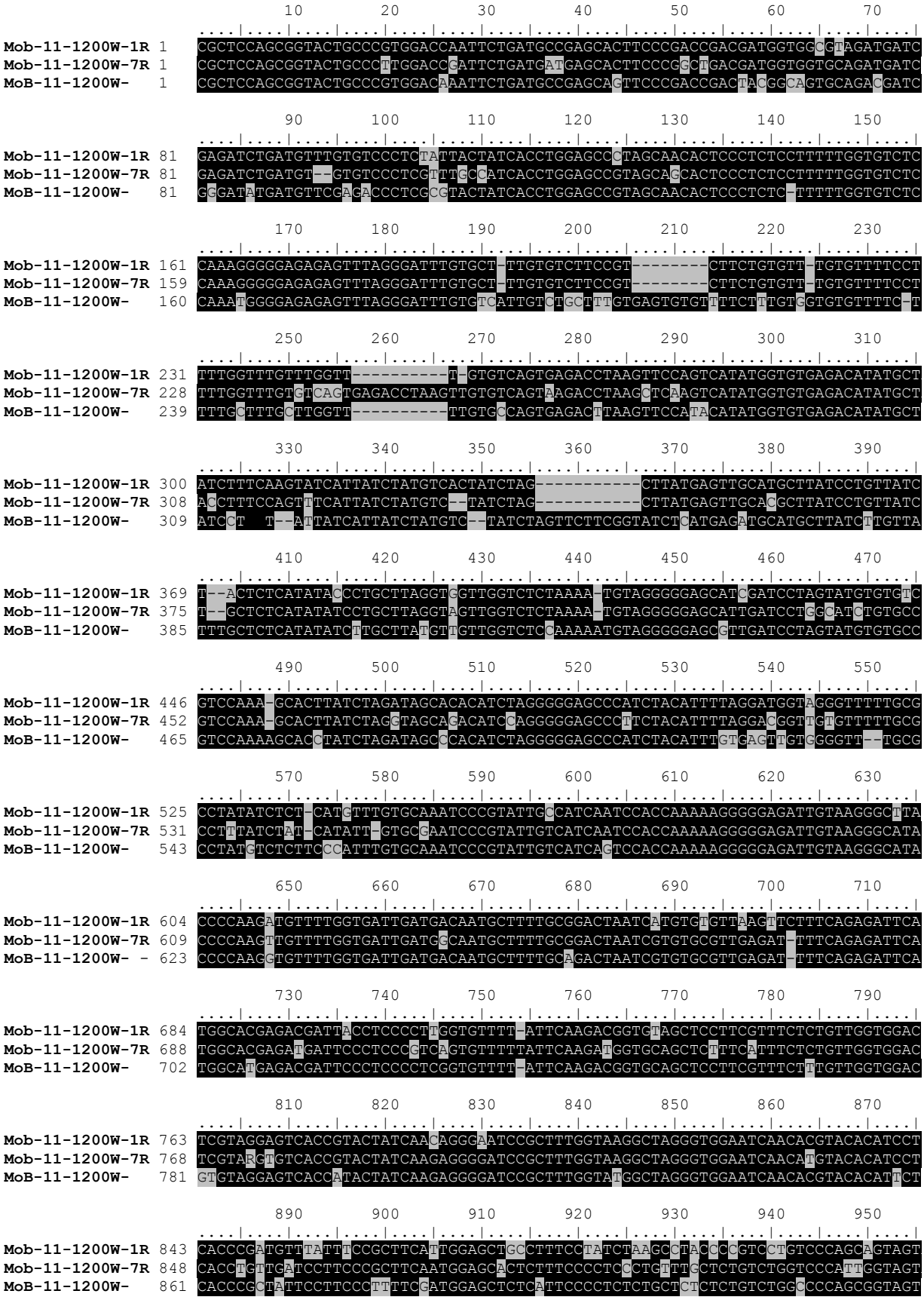


Figure S2 Nikita IRAP banding profiles of wheat (W), rye (R), triticale (T) and wheat DNA + rye DNA test tubes (1-5). Test tubes correspond to reactions that used as DNA template the result from the mixture of wheat DNA plus decreasing quantities of rye DNA (1/3,1/4, 1/5, 1/6, and 1/7). **b** is an overexposed copy of **a**, reveling two rye-origin polymorphic bands, absent in wheat–rye addition lines, present in all rye dilutions used (arrowheads). A triticale-origin band is absent in all rye dilutions (arrowhead). Molecular weight marker: 1kb+.


Mob-11-1200W-1R
Mob-11-1200W-7R
MoB-11-1200W-

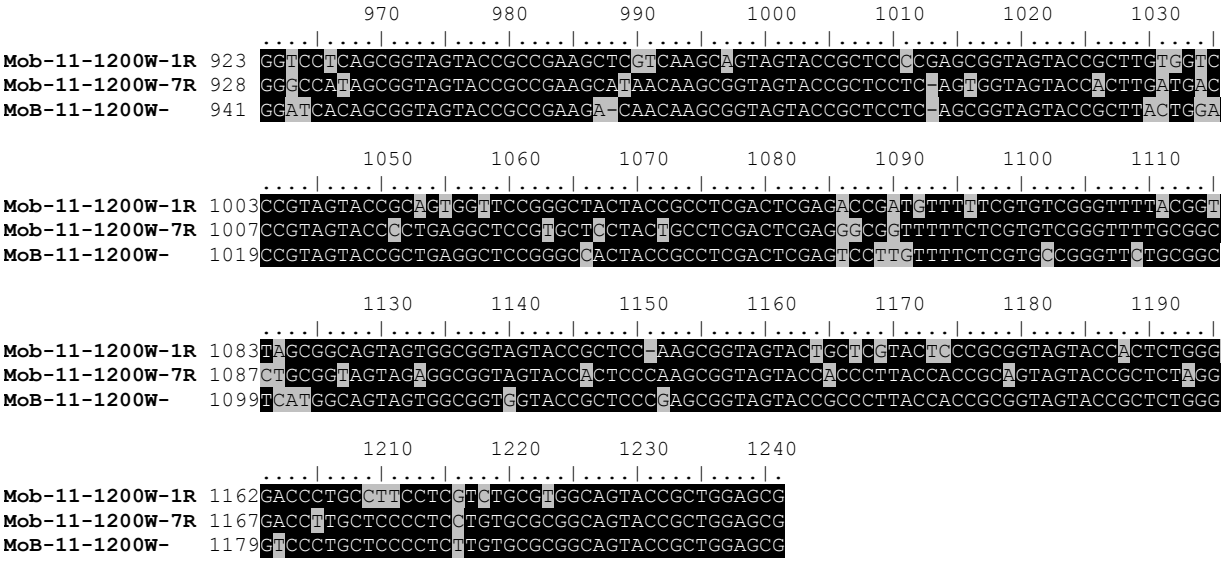


Figure S3 Alignments of the wheat-specific sequence MoB-11-1200W amplified from wheat and from CS+1R (MoB-11-1200W-1R) and CS+7R (MoB-11-1200W-7R)

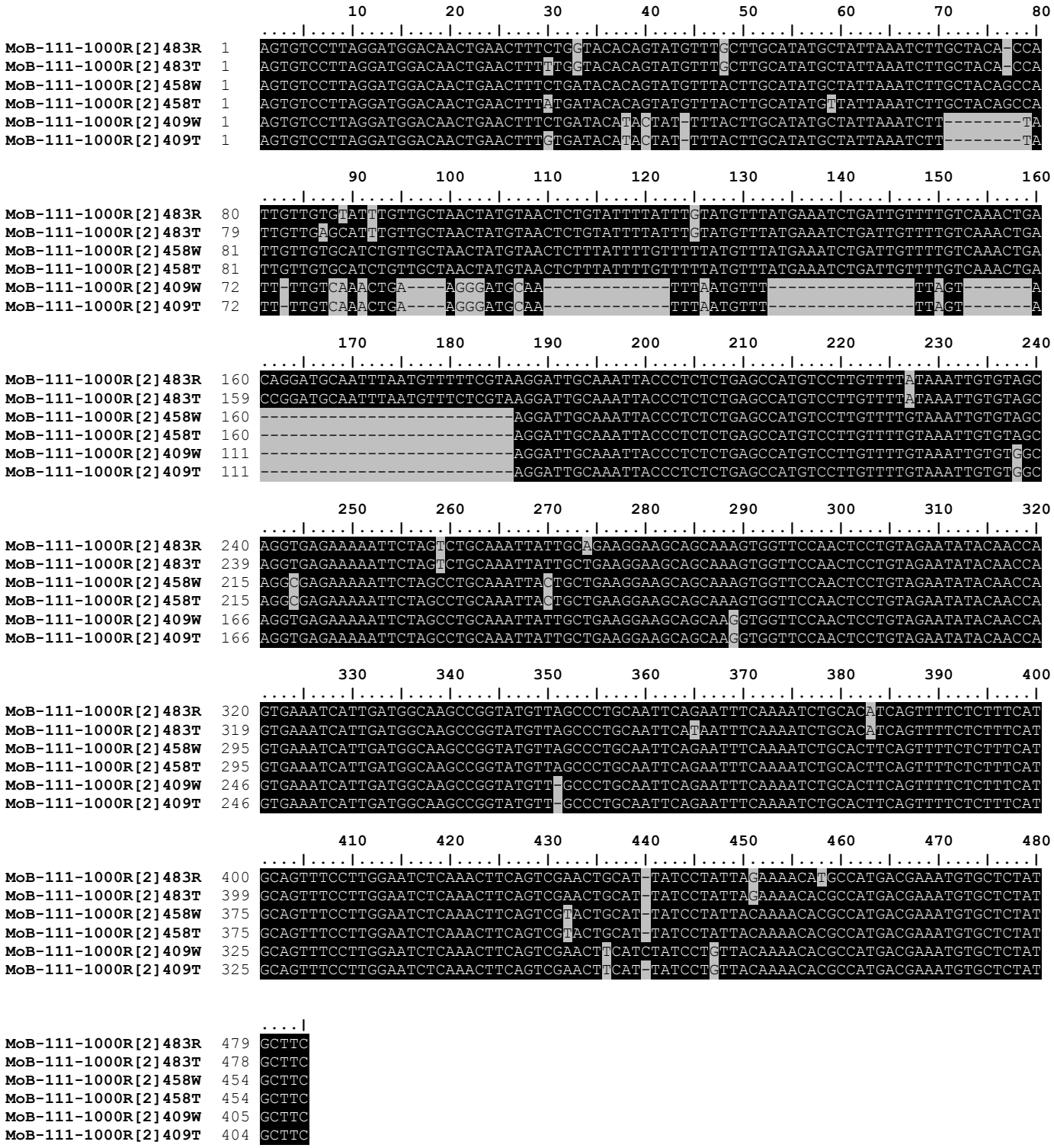


Figure S4 Alignment of the MoB-111-1000R [2] sequences amplified from wheat, rye, and triticale.

Chapter IV

Interplay of ribosomal DNA loci in nucleolar dominance: dominant NORs are up-regulated by chromatin dynamics in the Wheat-Rye system

Silva M, Pereira HS, **Bento M**, Ana-Paula Santos, Shaw P, Delgado M, Neves, N., Viegas W. (2008). Interplay of Ribosomal DNA Loci in Nucleolar Dominance: Dominant NORs Are Up-regulated by Chromatin Dynamics in the Wheat-Rye System. PLoS ONE 3(12): e3824. doi:10.1371/journal.pone.0003824.

Note: In this paper the author contributed to the molecular evaluation of rRNA gene expression.

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PLoS one

Interplay of Ribosomal DNA *Loci* in Nucleolar Dominance: Dominant NORs Are Up-Regulated by Chromatin Dynamics in the Wheat-Rye System

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Abstract

Background: Chromatin organizational and topological plasticity, and its functions in gene expression regulation, have been strongly revealed by the analysis of nucleolar dominance in hybrids and polyploids where one parental set of ribosomal RNA (rDNA) genes that are clustered in nucleolar organizing regions (NORs), is rendered silent by epigenetic pathways and heterochromatization. However, information on the behaviour of dominant NORs is very sparse and needed for an integrative knowledge of differential gene transcription levels and chromatin specific domain interactions.

Methodology/Principal Findings: Using molecular and cytological approaches in a wheat-rye addition line (wheat genome plus the rye nucleolar chromosome pair 1R), we investigated transcriptional activity and chromatin topology of the wheat dominant NORs in a nucleolar dominance situation. Herein we report dominant NORs up-regulation in the addition line through quantitative real-time PCR and silver-staining technique. Accompanying this modification in wheat rDNA trascription level, we also disclose that perinucleolar knobs of ribosomal chromatin are almost transcriptionally silent due to the residual detection of BrUTP incorporation in these domains, contrary to the marked labelling of intranucleolar condensed rDNA. Further, by comparative confocal analysis of nuclei probed to wheat and rye NORs, we found that in the wheat-rye addition line there is a significant decrease in the number of wheat-origin perinucleolar rDNA knobs, corresponding to a diminution of the rDNA heterochromatic fraction of the dominant (wheat) NORs.

Conclusions/Significance: We demonstrate that inter-specific interactions leading to wheat-origin NOR dominance results not only on the silencing of rye origin NOR *loci*, but dominant NORs are also modified in their transcriptional activity and interphase organization. The results show a cross-talk between wheat and rye NORs, mediated by ribosomal chromatin dynamics, revealing a conceptual shift from differential amphiplasty to 'mutual amphiplasty' in the nucleolar dominance process.

Citation: Silva M, Pereira HS, Bento M, Santos AP, Shaw P, et al. (2008) Interplay of Ribosomal DNA *Loci* in Nucleolar Dominance: Dominant NORs Are Up-Regulated by Chromatin Dynamics in the Wheat-Rye System. PLoS ONE 3(12): e3824. doi:10.1371/journal.pone.0003824

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Introduction

Chromatin, the living form of genetic information in eukaryotes, has particular organization and distribution patterns in the nucleus that are related to gene expression as observed in several biological systems, ranging from animals to plants. One of the most widespread features of this relationship concerns the association of decondensed chromatin (euchromatin) with transcriptional activity, based on a greater physical availability of DNA sequences to the transcription machinery. Heterochromatin, which is the cytological representation of chromosome domains that undergo dense packaging are mainly composed of repetitive DNA sequences [1,2] (review in [3]), and is involved in transcriptional silencing of genes located in cis or in trans co-arrangements by spreading of heterochromatinization [4]. Additional significance of heterochromatin also relies on its involvement in the RNA interference pathways that lead to transcriptional and post-transcriptional gene silencing [5]. Chromosome domains and their topology, in addition to other functionally relevant nuclear landscapes (e.g, transcription *foci*, RNA processing, DNA repair), point to the importance of functional compartmentalization of the nucleus [6]. One of the most representative features of intranuclear compartments ascribed to particular nuclear functions is the nucleolus, which largely results from transcription of 45S ribosomal RNA (rRNA) genes [7–9]. Besides nucleolar involvement in several cellular processes (review in [10]), the nucleolus has, nevertheless, a universal and fundamental function as the ribosome sub-unit production centre. The formation of the nucleolus is primarily dependent on the transcriptional activity of competent NORs (nucleolus organizer regions) that are composed of hundreds to thousands tandem copies of rRNA genes [9,11]. It has also been established that in virtually all eukaryotes there is an

4. Interplay of Ribosomal DNA *Loci* in Nucleolar Dominance: Dominant NORs Are Up-regulated by Chromatin Dynamics in the Wheat-Rye System

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4.2. Introduction

Chromatin, the living form of genetic information in eukaryotes, has particular organization and distribution patterns in the nucleus that are related to gene expression as observed in several biological systems, ranging from animals to plants. One of the most widespread features of this relationship concerns the association of decondensed chromatin (euchromatin) with transcriptional activity, based on a greater physical availability of DNA sequences to the transcription machinery. Heterochromatin, which is the cytological representation of chromosome domains that undergo dense packaging are mainly composed of repetitive DNA sequences [1,2] (review in [3]), and is involved in transcriptional silencing of genes located in cis or in trans co-arrangements by spreading of heterochromatinization [4]. Additional significance of heterochromatin also relies on its involvement in the RNA interference pathways that lead to transcriptional and post-transcriptional gene silencing [5]. Chromosome domains and their topology, in addition to other functionally relevant nuclear landscapes (e.g, transcription *foci*, RNA processing, DNA repair), point to the importance of functional compartmentalization of the nucleus [6]. One of the most representative features of intranuclear compartments ascribed to particular nuclear functions is the nucleolus, which largely results from transcription of 45S ribosomal RNA (rRNA) genes [7-9]. Besides nucleolar involvement in several cellular processes (review in [10]), the nucleolus has, nevertheless, a universal and fundamental function as the ribosome sub-unit production centre. The formation of the nucleolus is primarily dependent on the transcriptional activity of competent NORs (nucleolus organizer regions) that are composed of hundreds to thousands tandem copies of rRNA genes [9,11]. It has also been established that in virtually all eukaryotes there is an excess of cellular rRNA genes in relation to ribosome needs for protein synthesis [8,12]. Hence, most of the NORs have only part of their rRNA gene arrays being transcribed at any particular time, while the remaining

arrays adopt a heterochromatic configuration forming knobs at perinucleolar location [9,11]. In addition, rDNA physical organization seems to be correlated with the dynamic topology of rDNA *loci*. In fact, elegant studies in humans showed that NORs become associated in one large perinucleolar knob at early G1 phase [10,13], a tendency also detected in *Arabidopsis thaliana* through the observation of frequent association between NOR-bearing chromosomes [14].

In *A. thaliana* the regulation of rRNA gene array availability for transcription has shown to be mechanistically linked to epigenetic modulation in nucleolar dominance phenomena where whole-NOR epigenetic silencing is commonly observed in hybrids and polyploids (e.g. *A. suecica*, [11,15]). This process occurs in Drosophila [16], Brassica [17] and triticale [18] when only NORs from one progenitor are transcriptionally active and contribute to nucleolus formation dominating over the rRNA genes of the other species that are rendered silent. The cytological event was first described in *Crepis* hybrids, when Navashin pointed out morphological changes on a group of chromosomes of one progenitor, detecting the disappearance of their secondary constrictions where rRNA gene arrays are located [11,12,19,20]. At that time the process observed by Navashin was termed differential amphiplasty, since only one parental genome in several *Crepis* hybrids suffers consistently the modifications on chromatin morphology - the absence of secondary constrictions, i.e. NOR chromatin full compaction. Considering that studies on nucleolar dominance were focused in silenced NORs behavior [11,12], information on the transcriptional level and chromatin organization of dominant NORs is still sparse and needed for the growing understanding of the dynamic behaviour of these key genomic domains. To address this topic, the wheat-rye system was used in the current investigation. This biological system shows nucleolar dominance of wheat (*Triticum aestivum* L.)-origin NORs over rye (*Secale cereale* L.)-origin NOR *loci* [18], which is mediated by epigenetic cues such as DNA methylation [21,22]. Regarding the organization of ribosomal chromatin, rye NORs show a heterochromatic centromere proximal domain from which decondensed rDNA portions emerge toward the nucleolus [23,24]. In wheat the pair of major NORs on chromosome 1B present an organization similar to that of rye NORs, whereas the other pair of major NORs on chromosome 6B presents two heterochromatic domains (a centromere proximal and a distal one), with the decondensed rDNA domain positioned between them [25,26]. Using this system

we analyzed the potential changes in wheat-origin rDNA transcriptional activity, as well as in their organization and topology. We report that nucleolar dominance is a process where NORs of both parental species are modified in the wheat-rye combination (although in opposite functional directions), in contrary to the differential amphiplasty concept as described by Navashin in 1930s [19] affecting only under-dominant NORs. Our model points out that dominant NORs are up-regulated by rDNA chromatin co-dynamics with under-dominant rDNA *loci*, and presumably mediated by epigenetic modulation.

4.3. Results and Discussion

Expression of wheat rDNA *loci* is enhanced by rye nucleolar chromosomes.
Quantitative real-time PCR was used to evaluate wheat-specific rDNA transcription levels in wheat and wheat+1R1R addition line. The actin control amplified similar quantities of a single product with approximately 350 bp and a melting temperature (Tm) of 86 ^{o}C in all four genomes analyzed (Figure 1B). However, there were marked differences in the levels of wheat-specific rRNA expression between wheat, rye and the addition line wheat+1R1R (Figure 1A and B). Importantly, the wheat-specific primers amplified a single cDNA product with the expected size of approximately 300 base pairs (Supporting Information, Fig S1) and with a T_m=88.5 ^{o}C in the three genomes containing wheat genetic material, but no amplification product was observed in rye. Mean delta delta C_t ± standard deviation of wheat-specific rRNA was calculated for wheat and the wheat+1R1R addition line utilizing the addition line wheat+7R7R as standard. This resulted $\Delta\Delta C_t$ = 0.95 ± 0.46 for wheat, and $\Delta\Delta C_t$ = -1.03 ± 0.65 for the addition line wheat+1R1R (graphic representation in Figure 1C). These results indicate significantly different expression levels of wheat-specific rRNA between wheat and wheat+1R1R (Student's *t* test, p=0.0025). In comparison to the wheat+7R7R addition line, the fold variation in the expression of wheat-specific rRNA was calculated to be 0.52 (approximately 50% less) in wheat and 2.05 (approximately 200 % greater) in wheat+1R1R. In conclusion, the expression of wheat-specific

rRNA is approximately four fold higher in the wheat+1R1R addition line in comparison to wheat.

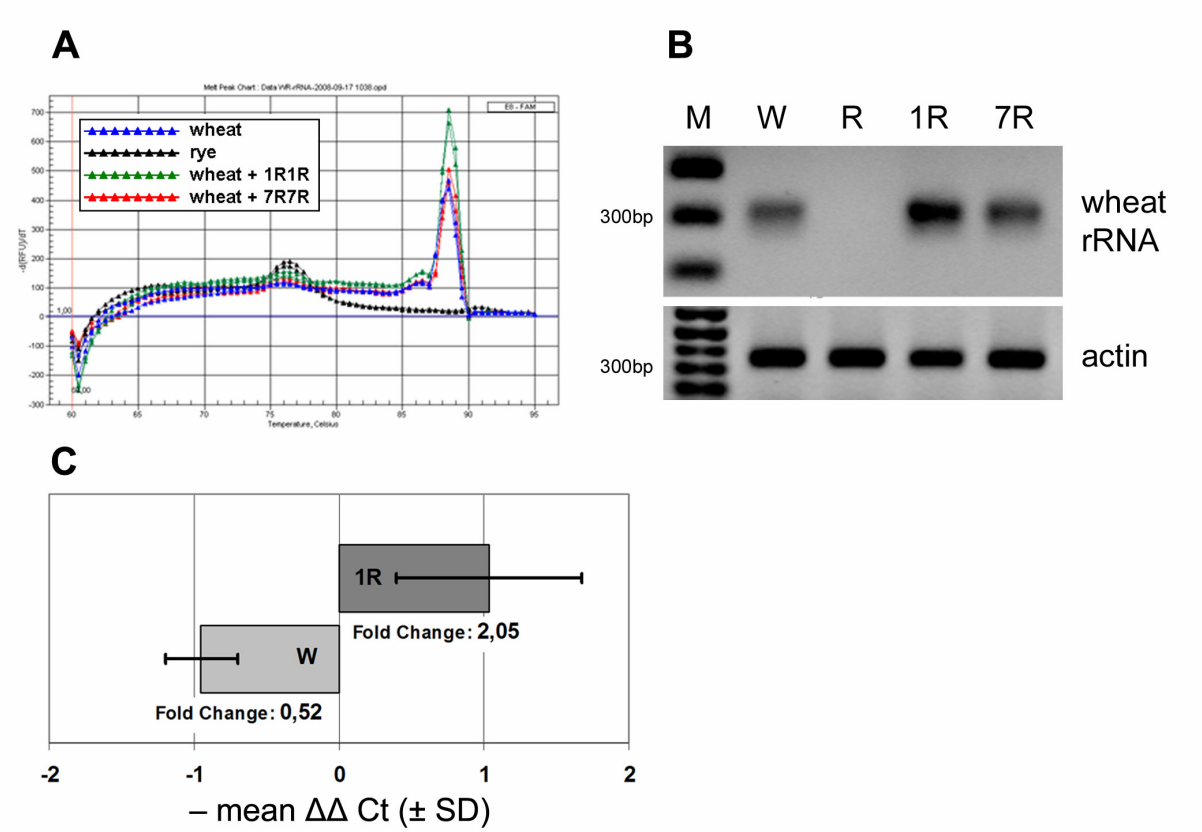

Figure 1 - Quantitative real-time PCR of wheat rRNA in wheat and wheat addition lines. A The melt curves for two replicates of cDNA isolated from wheat (blue), rye (black), wheat+1R1R (green), and wheat+7R7R (red) amplified with primers specific for wheat rRNA are shown. A single melt peak with Tm=88.5 in the three genomes containing wheat genetic material indicate a single amplification product. Due to the specificity of primers for wheat rRNA, there is no amplification of rye rRNA. **B** Quantitative real-time PCR products separated by gel electrophoresis. The results indicate greater expression of wheat-specific rRNA in the addition line wheat+1R1R (1R) in comparison to wheat (W) and addition line wheat+7R7R (7R). No amplification product was observed in rye (R). Actin controls are shown and M is the molecular weight marker (1 kb^+, 300 basepair band is shown on the left). **C** Transcription levels of wheat rRNAs in wheat and wheat+1R1R in respect to wheat+7R7R. Quantitative real-time PCR threshold cycles (Ct) were equilibrated with actin for wheat, wheat+1R1R, and wheat+7R7R (delta Ct). Wheat+7R7R mean delta Ct was utilized to calculate mean delta delta Ct (mean ΔΔ Ct) of two replicates of two wheat and wheat+1R1R cDNA dilutions. The graph illustrates – mean ΔΔ Ct ± standard deviation (SD) for wheat and wheat+1R1R, and the associated fold changes in transcription ($2^{-\text{mean }\Delta\Delta\text{ Ct}}$).

4.4. Concluding remarks

From our results, we propose that the presence of wheat and rye ribosomal chromatin in the same nucleus induces a mutual amphiplastic process instead of a unidirectional one, since the reduction of the activity of under-dominant NORs from

rye origin [21] was associated with an increase of the activity of wheat-origin dominant rRNA gene arrays (this work) in comparison with their original genomic backgrounds. In wheat and rye, DNA methylation has been previously associated with rRNA gene expression [22,27]. The silencing process of rye origin NORs in the wheat-rye system was also shown to be dependent of cytosine methylation status within rye rDNA intergenic spacer [21]. Therefore we expect that the bi-univocal interaction between NOR *loci* is associated with epigenetic modifications in *trans* that affect both the under-dominant (i.e. silent) NORs and the dominant rRNA gene arrays. This enlightens the significance of genome responses to challenges forwarded by Barbara McClintock in the mid 1980s [38]. This model is also in agreement with results that show that the epigenetic modifications that affect ribosomal *loci* expression are targeted to NORs rather than to particular rRNA genes [39]. Our model for rDNA transcription and organization seems also to associate with overall nuclear architecture of chromatin, namely Rabl versus non-Rabl interphase configurations. More studies on ribosomal chromatin topology and nucleolus biogenesis in other systems will contribute determinately for the understanding of the functional complexity of genome organization.

4.5. Materials and Methods

Plant material. Seeds from hexaploid wheat (*Triticum aestivum*, 2n=6x=42, genome designation AABBDD) cv. Chinese Spring, and from wheat-rye addition lines wheat+1R1R, wheat+6R6R and wheat+7R7R (wheat cv. Chinese Spring plus rye chromosome pairs 1, 6 and 7, 2n=44), were germinated for 3 days at 25ºC and further grown at climatic chambers with a photoperiod of 16 hours light (20 ^{o}C) / 8 hours dark (20 ^{o}C) cycle. Root tips from 3 days old seedlings were used for cytological analysis and leaves from three week old plants were used for RNA extraction. Seeds stocks from all the genotypes were originally obtained from the USDA–Sears collection, Columbia, Mo and were stored at the Secção de Genética, Instituto Superior de Agronomia, Lisbon.

Evaluation of rDNA expression levels through quantitative real-time PCR. Wheat-specific rDNA transcription levels were analyzed by Quantitative real-time-PCR with the BIO-RAD IQ 5 Multicolor Real-Time PCR detection System. Total RNA was extracted with the *mir*Vana™ miRNA Isolation Kit (Ambion, Cat# AM1560), following manufacturer's instructions. After verifying concentration and integrity, 1 µg of total RNA was utilized to perform a RNase free DNase digestion and for first strand cDNA synthesis with random primers (N_9) (Superscript II reverse transcriptase, Invitrogen Cat# 18064-014). PCR with primers specific for wheat rRNA (For 5'- TGGCACATTACGTGCCCG, Rev 5'- CTACCAGCACGGCCATCG) as well as the Actin2 gene product (For 5´- GCTGGATTCTGGTGATGGTGTGAG, Rev 5´- CAATGAGAGATGGCTGGAAGAGGAC) was performed with the BIO-RAD IQ SYBR Green Supermix (BIO-RAD Cat# 170-8880S). The Actin control gene was analyzed for three replicates of cDNA from each genome (dilution factor 1:10) and wheat-specific rDNA gene expression was quantified for two replicates of two cDNA dilutions (1:100 and 1:500). Each 20 µL PCR mix containing forward and reverse primers (0.1 pM each) was amplified for 35 cycles (95 ºC-5 min, 35 cycles of 95ºC 1 min, 60 ºC 1 min, and 72 ºC 1 min, and a final elongation step of 72ºC for 5 minutes). Melt curves were performed to ensure amplification of single products as well as to estimate their melting temperatures. Upon completion, PCR products were separated by 1.5 % agarose gel electrophoresis and detected by ethidium bromide staining.

To analyze the levels of wheat-specific rRNA between genomes, the mean actin cDNA threshold cycle (C_t) was used to calculate each delta C_t (ΔC_t = C_t wheat rRNA – mean C_t actin) associated with two replicates of both dilutions for each genome (wheat, wheat + 1R1R and wheat + 7R7R). In order to compare these between wheat and wheat + 1R1R, delta delta C_t ($\Delta\Delta C_t$ = ΔC_t of interest – mean ΔC_t 7R) were calculated for wheat and wheat + 1R1R. These values were utilized for a type 2 two-tailed T-test as well as to calculate fold variation in expression ($2^{-\text{mean } \Delta\Delta \text{ Ct}}$).

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4.7. Supporting Information

Figure S1

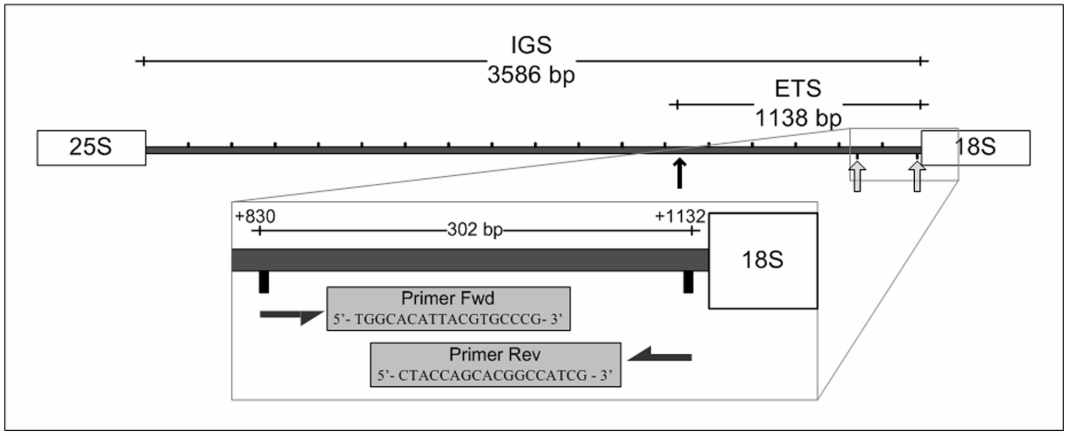

Figure S1 - Localization and size (bp) of the fragment expected from the amplification of a wheat-specific rDNA transcribed sequence in the ETS (External Transcribed Spacer) of Wheat rDNA 25S-18S intergenic region (IGS, accession number X07841). ⬆ - transcription initiation site, ⇧ - primers location (For +830, Rev +1132).

Chapter V

Size matters in Triticeae polyploids: larger genomes have higher remodelling

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REVIEW / SYNTHÈSE

Size matters in Triticeae polyploids: larger genomes have higher remodeling

Miguel Bento, J. Perry Gustafson, Wanda Viegas, and Manuela Silva

Abstract: Polyploidization is one of the major driving forces in plant evolution and is extremely relevant to speciation and diversity creation. Polyploidization leads to a myriad of genetic and epigenetic alterations that ultimately generate plants and species with increased genome plasticity. Polyploids are the result of the fusion of two or more genomes into the same nucleus and can be classified as allopolyploids (different genomes) or autopolyploids (same genome). Triticeae synthetic allopolyploid species are excellent models to study polyploids evolution, particularly the wheat–rye hybrid triticale, which includes various ploidy levels and genome combinations. In this review, we reanalyze data concerning genomic analysis of octoploid and hexaploid triticale and different synthetic wheat hybrids, in comparison with other polyploid species. This analysis reveals high levels of genomic restructuring events in triticale and wheat hybrids, namely major parental band disappearance and the appearance of novel bands. Furthermore, the data shows that restructuring depends on parental genomes, ploidy level, and sequence type (repetitive, low copy, and (or) coding); is markedly different after wide hybridization or genome doubling; and affects preferentially the larger parental genome. The shared role of genetic and epigenetic modifications in parental genome size homogenization, diploidization establishment, and stabilization of polyploid species is discussed.

Key words: genome restructuring, Triticeae, synthetic hybrids, polyploids.

Résumé : La polyploïdisation est l'une des forces motrices les plus importantes de l'évolution chez les plantes et joue un rôle important dans la spéciation et la création de diversité. Elle mène à un ensemble d'altérations génétiques et épigénétiques qui génèrent ultimement des plantes et des espèces avec une plasticité génomique accrue. Les polyploïdes résultent de la fusion de deux génomes ou plus au sein d'un même noyau et sont classifiés en allopolyploïdes (des génomes différents) et autopolyploïdes (le même génome). Les allopolyploïdes synthétiques chez les Triticées constituent d'excellents modèles pour étudier l'évolution des polyploïdes. Cela est particulièrement le cas pour le triticale, un hybride entre le blé et le seigle, lequel présente divers niveaux de ploïdie et combinaisons génomiques. Dans cette synthèse, les auteurs réexaminent les données portant sur l'analyse génomique de triticales hexaploïdes et octoploïdes, ainsi que différents blés hybrides synthétiques, en les comparant à d'autres espèces polyploïdes. Cette analyse révèle d'importants changements dans la structure des génomes chez le triticale et les blés hybrides dont : la disparition de bandes parentales majeures et l'apparition de nouvelles bandes. De plus, les données montrent que ces réarrangements structuraux dépendent des génomes parentaux, du niveau de ploïdie et du type de séquence (répétitive, à faible nombre de copies et codante/non-codante). Ils diffèrent de façon importante selon qu'ils suivent un croisement interspécifique ou un doublement chromosomique et ils touchent de manière préférentielle le génome parental le plus grand. Les auteurs discutent des rôles partagés que jouent les changements génétiques et épigénétiques dans l'homogénéisation de la taille des génomes parentaux ainsi que dans l'établissement et la stabilisation des espèces polyploïdes

Mots-clés : restructuration des génomes, Triticées, hybrides synthétiques, polyploïdes.

[Traduit par la Rédaction]

Introduction

Polyploidy is a major mode of evolution in plants, which involves two or more genomes being joined into the same nucleus. It has been estimated that 30%–70% of plant species are of polyploid origin, an assessment that is approaching 100% if paleopolyploids are included (Wendel 2000; Wolfe 2001). Polyploids are classified into autopolyploids

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5. Size matters in Triticeae polyploids: larger genomes have higher re-modeling

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Key words: genome restructuring, Triticeae, synthetic hybrids, polyploids.

5.1. Abstract

Polyploidization is one of the major driving forces in plant evolution and is extremely relevant to speciation and diversity creation. It leads to a myriad of genetic and epigenetic alterations that ultimately generate plants and species with increased genome plasticity. Polyploids are the result of the fusion of two or more genomes into the same nucleus and can classified as allopolyploids (different genomes) or autopolyploids (same genome). Triticeae synthetic allopolyploid species are excellent models to study polyploids evolution, particularly the wheat/rye hybrid triticale, which includes various ploidy levels and genome combinations.

In this review we reanalyze data concerning genomic analysis of octoploid and hexaploid triticale, and different synthetic wheat hybrids, in comparison with other polyploid species. This analysis reveals high levels of genomic restructuring events in triticale and wheat hybrids, namely: major parental band disappearance, and also the appearance of novel bands. Furthermore, the data also shows that restructuring depends on parental genomes, ploidy level, sequence type (repetitive, low-copy, and/or coding), is markedly different after wide hybridization or genome doubling, and affects preferentially the larger parental genome. The shared role of genetic and epigenetic modifications in parental genome size homogenization, diploidization establishment and stabilization of polyploid species is discussed.

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Résumé

La polyploïdisation est l'une des forces motrices les plus importantes de l'évolution chez les plantes et joue un rôle important dans la spéciation et la création de diversité. Elle mène à un ensemble d'altérations génétiques et épigénétiques qui génèrent ultimement des plantes et des espèces avec une plasticité génomique accrue. Les polyploïdes résultent de la fusion de deux génomes ou plus au sein d'un même noyau et sont classifiés en allopolyploïdes (des génomes différents) et

autopolyploïdes (le même génome). Les allopolyploïdes synthétiques chez les Triticées constituent d'excellents modèles pour étudier l'évolution des polyploïdes. Cela est particulièrement le cas pour le triticale, un hybride entre le blé et le seigle, lequel présente divers niveaux de ploïdie et combinaisons génomiques. Dans cette synthèse, les auteurs réexaminent les données portant sur l'analyse génomique de triticales hexaploïdes et octoploïdes, ainsi que différents blés hybrides synthétiques, en les comparant à d'autres espèces polyploïdes. Cette analyse révèle d'importants changements dans la structure des génomes chez le triticale et les blés hybrides dont : la disparition de bandes parentales majeures et l'apparition de nouvelles bandes. De plus, les données montrent que ces réarrangements structuraux dépendent des génomes parentaux, du niveau de ploïdie et du type de séquence (répétitive, à faible nombre de copies et codante/non-codante). Ils diffèrent de façon importante selon qu'ils suivent un croisement interspécifique ou un doublement chromosomique et ils touchent de manière préférentielle le génome parental le plus grand. Les auteurs discutent des rôles partagés que jouent les changements génétiques et e´pige´ne´tiques dans l'homogénéisation de la taille des génomes parentaux ainsi que dans l'établissement et la stabilisation des espèces polyploïdes.

Mots-clés: restructuration des génomes, Triticées, hybrides synthétiques, polyploïdes.
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5.2. Introduction

Polyploidy is a major mode of evolution in plants, which involves two or more genomes being joined into the same nucleus. It has been estimated that 30 to 70% of plants species are of polyploid origin, an assessment that is approaching 100% if paleopolyploids are included (Wendel 2000; Wolfe 2001). Polyploids are classified into autopolyploids and allopolyploids based on the origin of the component genomes, and can be represented by many different ploidy levels. An autopolyploid results from doubling of a diploid genome, and an allopolyploid is

formed by the combination of two or more different, but usually related, genomes through hybridization between distinct species or genera. Gene redundancy in polyploids is obvious, and leads to new expression patterns, which can generate developmental novelty and the appearance of new phenotypes, producing species with a higher degree of genome plasticity, when compared with their progenitors (Chen 2007). Furthermore, loss of self-incompatibility, gain of asexual reproduction and higher levels of heterozygosity can be fixed in allopolyploids (Comai 2005). All those modifications/changes can increase fitness, which may explain the widespread occurrence of polyploids in plants.

Newly synthesized polyploids, with precise known progenitors, are excellent materials to study the emergence of early and late evolutionary genetic and epigenetic events. This approach has been widely applied in many species, such as wheat (*Triticum* ssp.), *Arabidopsis*, *Brassica*, cotton (*Gossypium* ssp.) and triticale (X-*Triticosecale* Wittmack) (Dong et al. 2005; Liu et al. 2001; Ma et al. 2004; Ma and Gustafson 2006; Madlung et al. 2005; Ozkan et al. 2001; Salmon et al. 2005). In synthesized polyploids genetic and/or epigenetic changes were observed, although their rate, type and degree are markedly different between distinct polyploids (Chen 2007; Ma and Gustafson 2008). Allopolyploid genomes experience two different phases: a Revolutionary phase, occurring immediately after hybridization, responsible for rapid genetic and epigenetic changes; and an Evolutionary phase that corresponds to long term events, such as slow changes in DNA sequences and functional alterations over time (Feldman and Levy 2005; Levy and Feldman 2002).

Triticale, the first synthesized amphidiploid cereal, is a chromosome-doubled inter-generic hybrid that can be obtained by the cross of distinct wheat species (*Triticum* spp., AA, AABB, and AABBDD) and rye (*Secale cereale* L., RR), producing various genome combinations and ploidy levels, such as tetraploid AARR, hexaploid AABBRR and octoploid AABBDDRR. When compared with other allopolyploids, triticale is a very complex genome due to its high ploidy level, large genome size and the distant relationships between parental genomes. However, because of its short history and accumulated pedigree knowledge, it becomes a very useful model species to study evolutive processes mediated by polyploidization. Synthetic allotetrapolyploids involving *Aegilops* sp., and *Triticum* sp. have also been analyzed in comparison to their parental species in an attempt

to simulate natural wheat allopolyploids. The aims of this review are to summarize findings obtained so far in Triticeae polyploid species and compare results with other allopolypoid species.

5.3. Polyploid genomic analysis

Overall, genomic sequence changes have been extensively studied in Triticeae polyploids involving different octoploid and hexaploid triticales, the corresponding F1 hybrids, and their respective parental genomes to assess polyploidization-induced genome readjustments using molecular marker techniques such as amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP) analysis (Ma et al. 2004; Ma and Gustafson 2006). AFLP analysis was used to study different synthetic wheat allotetraploids between *Aegilops* sp. and *Triticum* sp., F1 hybrids, and their homozygous diploid parents (Dong et al. 2005; Shaked et al. 2001). Large-scale AFLP studies were implemented to obtain an unbiased genome-wide estimation of the occurrence of genomic sequence variation using different restriction enzymes, namely *Eco*RI–*Mse*I primers which amplify repetitive sequences (Dong et al. 2005; Ma et al. 2004; Ma and Gustafson 2006; Shaked et al. 2001), and *Pst*I–*Mse*I primers, which predominantly target low-copy sequences most present in distal gene-rich regions, since *Pst*I is highly sensitive to the cytosine status (Milla and Gustafson 2001; Young et al. 1999). Furthermore, coding sequence variation induced by polyploidization in Triticeae was investigated using cDNA-probed RFLP analyses (Ma et al. 2004).

Utilizing a wide series of primers combinations, octoploid triticale amphidiploids, and their wheat and rye parental genomes were analyzed using inter-retrotransposons amplified polymorphism (IRAP), retrotransposons-microsatellite amplified polymorphism (REMAP), and inter simple sequence repeat (ISSR) techniques (Bento et al. 2008). IRAP, REMAP and ISSR are PCR-based molecular marker techniques initially designed to identify different barley (*Hordeum vulgare* L.) cultivars (Kalendar et al. 1999) combining primers designed for: long terminal repeats (LTR) retrotransposons, which have a very important

role in genome evolution and speciation due to their dynamics and potential mobility (Vitte and Panaud 2005); and/or microsatellites, that constitute polymorphic loci present throughout nuclear DNA, preferentially associated with retrotransposons in cereals (Ramsay et al. 1999). Primers designed to evaluate LTRs point outwards and amplify retrotransposon flanking sequences, thus allowing for the detection of retrotransposon insertional polymorphisms (Kalendar and Schulman 2006). Recently a more specific molecular marker system involving SSR (Single Sequence Repeat), originally designed to study unique sequences containing microsatellites in the wheat genome (Roder et al. 1998), was used to study variation induced by polyploidization in triticale with different combinations of wheat and rye parents (Tang et al. 2008).

Table 1. Summary of genomic alterations detected in triticale using distinct methodologies of genomic analysis.

	AFLP [(a)]			**RFLP** [(b)]			**IRAP/REMAP/ISSR** [(c)]		
	No Change	**Loss**	**Novel**[(d)]	**No Change**	**Loss**	**Novel**[(d)]	**No Change**	**Loss**	**Novel**[(d)]
Wheat specific	9332 **(76.9)**	2799 **(23.1)**		1883 **(97.3)**	53 **(2.7)**		51 **(89.5)**	6 **(10.5)**	
Rye Specific	2500 **(34.2)**	4808 **(65.8)**		395 **(38.4)**	633 **(61.6)**		26 **(46.4)**	30 **(53.6)**	
Shared	2283 **(82.2)**	495 **(17.8)**		197 **(97.5)**	5 **(2.5)**		58 **(100)**	0 **(0)**	
Novel			1535 **(9.8)**			250 **(9.2)**			6 **(4.2)**
Total	14115 **(63.5)**	8102 **(36.5)**		2475 **(78.2)**	691 **(21.8)**		135 **(78.9)**	36 **(21.1)**	

Note: Percentage are displayed in (parentheses).
(a) AFLP results are a compilation of the results published by Ma et al. (2004) and Ma and Gustafson (2006).
(b) RFLP results presented in Ma and Gustafson (2004).
(c) IRAP/REMAP/ISSR results presented in Bento et al. (2008).
(d) Percentages are calculated in relation to the number of triticale observed bands.
% novel = novel/(no change + novel)x100.

The above molecular marker systems were crucial to disclosing genomic modifications induced by polyploidization, allowing the detection of extensive changes, accessed by alterations in banding profiles. AFLP and RFLP analyses allowed for a genome wide-range evaluation offering the possibility to differentiate between genome euchromatic and heterochromatic fractions. Whereas, IRAP,

REMAP and ISSR, which are unaffected by DNA methylation (Kalendar and Schulman 2006) and specific for repetitive motifs such as retrotransposons (Vitte and Panaud 2005), allowed for the detection of rearrangements involving both repetitive and coding sequences (Bento et al. 2008).

5.4. Genome rearrangement events revealed by band losses

The results obtained by the analysis of *triticale* polyploids were re-evaluated and have been summarized in Table 1, and clearly disclose the high level of genome restructuring events associated with polyploid establishment (Bento et al. 2008; Kashkush et al. 2002; Ma et al. 2004; Ma and Gustafson 2006; Tang et al. 2008). Although there are several studies regarding the evaluation of genomic restructuring events in Triticeae hybrids, besides the studies of Dong et al. (2005) and Shaked et al. (2001) (reviewed in Table 2), none of them discriminate levels of parental-specific alterations (Feldman et al. 1997; Kashkush et al. 2002; Ozkan et al. 2001; Ozkan et al. 2003; Tang et al. 2008).

Table 2. Re-evaluation of collected data of percent parental band loss/elimination by sequence type in octoploid and hexaploid triticales.

			% elimination			
			Octoploid		Hexaploid	
	Sequence type	Parental genome	**CSxI**	**HxK**	**CxS**	**CxU**
AFLP [(a)]	Repetitive	**W**	24.8	25.2	44.0	47.4
		R	61.1	65.5	62.5	67.8
	Low-copy	**W**	8.7	6.6	14.8	15.6
		R	69.8	69.7	61.1	68.8
RFLP [(b)]	Coding	**W**	2.2	1.2	5.5	2.4
		R	64.8	60.8	62.2	57.9
IRAP/REMAP ISSR [(c)]	Repetitive motifs- flanking regions	**W**	10.5			
		R	53.6			

Note: **W**, wheat; **R**, rye (2n=2x); **CS,** 'Chinese Spring' (2n=6x); **I**, 'Imperial'; **H** 'Holdfast' (2n=6x); **K**, 'King II'; **C,** 'Cocorit 71' (2n=4x); **S**, 'Snoopy'; **U**, 'UC90'. Percentages were calculated separately for each parent and shared bands are not taken into consideration (results for eliminated shared bands are not shown). % elimination = (specific type of parental bands eliminated)/(total of specific type of parental bands detected)x100.

(a) AFLP results are a compilation of the results published by Ma et al. (2004) and Ma and Gustafson (2006).

(b) RFLP results are presented in Ma et al. (2004).

(c) IRAP-REMAP-ISSR results are presented in Bento et al. (2008).

The data analysis is based on the comparison between parental lines and polyploid gel profiles. Thus, all the bands present in parental gel profiles are considered parental bands and shared bands are the ones present in both parental lines. Conserved bands are bands present in parental profiles and maintained in the polyploid profile while absent bands are bands present in the parental profiles that are missing in the polyploid, indicating the occurrence of a rearrangement event in the polyploid. On the other hand, novel bands are the ones that are present in the newly formed polyploid being absent in parental gel profiles, indicating also the occurrence of genome rearrangements.

The overall examination of the published results reveal that the variation detected in triticale is significantly higher than the observed in other synthetic polyploids, namely in wheat species complexes and in *Brassica* (Song et al. 1995). Such marked differences in polyploid behavior could be due to triticale being of intergeneric origin, which may have lead to additional enhanced modifications to parental genomes, in order to stabilize the newly formed polyploid. This hypothesis is reinforced by studies in natural and newly synthesized wheat interspecific polyploids, which suggest lower levels of parental genome restructuring in comparison to triticale (Dong et al. 2005; Feldman et al. 1997; Liu et al. 1998; Ozkan et al. 2001). Similarly, genetic distance is very important when analyzing hybrids within the same genus, for example crosses between *B. rapa* and *B. nigra* revealed a higher genome variation than those between the more closely related species *B. rapa* and *B. oleracea* (Song et al. 1995). The same was observed in hybrids among members of the Triticeae where an interspecific cross between *Ae. sharonensis* x *Ae. umbellulata* revealed lower variation (6.7%) than an intergeneric cross between *Ae. longissima* x *T. urartu* (11.8%) (Shaked et al. 2001) or *T. turgidum* x *Ae. tauschii* (17.2%) (Dong et al. 2005). The rearrangements detected in triticale through AFLP/RFLP and IRAP/REMAP/ISSR analysis (Bento et al. 2008; Kashkush et al. 2002; Ma et al. 2004; Ma and Gustafson 2006; Tang et al. 2008) established the disappearance of bands from both parental origins, and the emergence of novel bands, absent in progenitor's banding profiles. The appearance of novel bands was also described in *Brassica* polyploids (Song et al. 1995). Not surprisingly, the triticale genome analyses clearly demonstrated that the disappearance of parental bands was much more frequent than the appearance of novel bands. Frequencies of band disappearance (Table1) varied

between 36.5% (detected by AFLP) and 21.1% (detected by IRAP/REMAP/ISSR), while the appearance of novel bands was 9.8% (detected by AFLP), 9.2% (detected by RFLP), and 4.2% (detected by IRAP/REMAP/ISSR) (Bento et al. 2008; Ma et al. 2004; Ma and Gustafson 2006). Similar results were reported in other polyploids involving *Triticum*, *Brassica,* and *Spartina* (Dong et al. 2005; Kashkush et al. 2002; Salmon et al. 2005; Shaked et al. 2001; Song et al. 1995). Sequence restructuring therefore seems to be a widespread phenomenon associated with newly formed polyploids (Leitch and Bennett 2004). The exception to genome downsizing was reported in cotton (*Gossypium*) polyploids (Liu et al. 2001), where genomic changes were not detected by AFLP, although a reduction in C-DNA values was observed when these polyploids where compared to parental genomes (Bennett 1977; Bin and Kadir 1976).

5.5. Genome rearrangement frequencies in triticale depend on sequence types and wheat ploidy levels

Sequence rearrangements are not restricted to repetitive and non-coding sequences as coding sequences, regulatory elements and promoter regions appear to also be affected by polyploidization (Bento et al. 2008; Ma et al. 2004; Tang et al. 2008), although in different levels. In triticale repetitive sequences were found to be more frequently rearranged than low-copy and coding sequences (Ma et al. 2004). Published data indicated that 42%, 31%, and 22% of bands were lost from repetitive, low-copy and coding sequences, respectively (values presented in (Ma and Gustafson 2008). Wheat-specific sequence rearrangements are also highly affected by the kind of sequence being analyzed. In octoploid and hexaploid triticale the level of wheat-specific band loss varied, being approximately 25% and 46%, 7% and 15% and 1.5% and 4% for repetitive, low-copy, and coding sequences, respectively (Table 2). In contrast to the marked differences of wheat-specific band losses, rye-specific bands appear to be lost at similar frequencies, independently of the type of sequence, ranging between 57.9% and 69.8% (Table 2).

The data published by Ma et al. (2004) and Ma and Gustafson (2006) showed that the level of band losses in triticale was distinct for each parental genome (Table

2). Triticale genotype analyses demonstrate that the level of rye parental genome band elimination is higher than the observed for wheat genome. The maximum frequency of wheat-specific band elimination was 47.4% (detected by AFLP in hexaploid triticale), whereas rye-specific band elimination ranged between 53.6% (detected in octoploid triticale through IRAP/REMAP/ISSR analyses) and 69,8% (detected in octoploid triticale by AFLP). Differences between percentages of rye- and wheat-specific band elimination in hexaploid triticales were 18.5% by AFLP analysis and 62.6% in octoploid triticale by RFLP analysis.

(Gill 1991) suggested that the rye paternal genome being exposed to the adverse environment of maternal wheat cytoplasm in newly formed hybrids could explain its preferential restructuring in triticale. (Ma and Gustafson 2008) also suggested that the instability of rye-wheat hybrids, maybe due to nuclear-cytoplasmatic interactions. However, the Triticeae studies of Dong et al. (2005) and Shaked et al. (2001) contradict the paternal preferential elimination hypothesis since maternal genome elimination was observed in synthetic wheat (Table 3). When *Ae. speltoides* was crossed as female and as male, in a study designed to ascertain the effects of cytoplasm on the pattern and rate of sequence elimination, no cytoplasm effects were detected (Ozkan et al. 2001).

On the other hand, as triticale results from an intergeneric hybridization between a polyploid (wheat) and a diploid species (rye), we can consider that wheat has already been subject to genetic and/or epigenetic modifications during its evolution, thus being more adapted to the polyploidy condition than rye genome. A detailed analysis of data presented in Table 2 reveals a plausible correlation between genome rearrangement frequencies and wheat ploidy levels. However, such correlations were absent in the cross between *T. turgidum* and *Ae. tauschii* (using enzymes not sensible to methylation), as more genome modifications were detected in the maternal tetraploid genome than in the paternal diploid genome (Table 3).

A higher global genome variation was observed in hexaploid (40%) than in octoploid triticale (around 30%) (Ma and Gustafson 2008), reinforcing previous data (Boyko et al. 1984) showing that DNA content reduction was also higher in hexaploid than in octoploid triticale (28-30% and 9%, respectively). Although the elimination level of rye-specific bands appears similar both in hexaploid and octoploid triticale, the elimination rate of wheat-specific bands is much higher in

hexaploid triticale (for details see Table 2). The average elimination rate of wheat-specific repetitive sequences in hexaploid triticale was 45% and 25% in octoploid triticale, revealing a higher buffering capacity of hexaploid wheat genome to avoid large numbers of sequence rearrangements in triticale. Such correlations between differences between parental ploidy level and genome alterations rate have not been established in other species such cotton and wheat (A*egilops* x *Triticum*) polyploids (Liu et al. 1998; Ozkan et al. 2001).

Table 3. Collected data from Dong et al. (2005) and Shaked et al (2001) on percentage parental band loss/elimination using AFLP (repetitive sequence type) in *Aegilops x Aegilops*, *Aegilops x Triticum* and *Triticum x Aegilops* polyploid genotypes.

Polyploiyd genotype	Parental genome	% elimination of total bands
As x Au	As	14
	Au	0,5
Al x Tu	Al	12,2
	Tu	11,4
Tt x At	Tt	21
	At	12,3

Notes: As, *Ae. sharonensis* (2n=2x) ; **Au**, *Ae. umbellulata* (2n=2x); **Al**, *Ae. longissima* (2n=2x); Tu, *T. urartu* (2n=2x); **Tt**, *T. turgidum* (2n=4x); **At**, *Ae. tauschii* (2n=2x).

The results indicate that each triticale parental genome is subjected to distinct regulatory systems. In the wheat-origin genome, each type of sequence seems to have different relevancies in genome adaptation through polyploidization and the elimination of different sequences is apparently controlled accordingly. Contrastingly, the rye-origin genome appears to be highly restructured upon polyploidization independently of sequence type considered.

5.6. Parental genomes are differently restructured during hybridization and polyploidization

The data obtained for two hexaploid and two octoploid triticales, their respective parental lines, and correspondent F_1 hybrids (Ma and Gustafson 2006), clearly demonstrated the occurrence of two major stages of restructuring events. First during the formation of the wide hybrid, followed by a second after chromosome doubling of the hybrid. The re-analyzed results are summarized in Table 4,

revealing during the first stage an immediate and drastic response to hybridization, while in the second stage a continuous process of changes occur at a slower rate (Ma and Gustafson 2006).

Similar results, presented in Table 5, were described by Shaked et al. (2001) in *Aegilops* x *Aegilops* and *Aegilops* x *Triticum* and were also reported in *Aegilops x Triticum* and *Spartina* F1 hybrids, although not so pronounced (Ozkan et al. 2001; Salmon et al. 2005).

Table 4. Number and percentage (in parentheses) of bands lost before and after chromosome doubling detected by (Ma and Gustafson 2006) in wheat-rye hybrids

		Octoploid						Hexaploid					
		CSxI			**HxK**			**CxS**			**CxU**		
Sequence type	**Parental genome**	**F1**	**T**	**total**	**F1**	**T**	**total**	**F1**	**T**	**Total**	**F1**	**T**	**total**
Repetitive	**W**	104	156	1094	50	65	535	96	156	627	155	93	602
		(9.5)	**(14.3)**	**(23.8)**	**(9.3)**	**(12.1)**	**(21.5)**	**(15.3)**	**(24.9)**	**(40.2)**	**(25.7)**	**(15.4)**	**(41.2)**
	R	161	69	403	124	29	232	136	46	298	102	35	228
		(40.0)	**(17.1)**	**(57.1)**	**(53.4)**	**(12.5)**	**(65.9)**	**(45.6)**	**(15.4)**	**(61.1)**	**(44.7)**	**(15.4)**	**(60.1)**
Low-copy	**W**	18	45	939	8	12	616	26	40	526	20	30	427
		(1.9)	**(4.8)**	**(6.7)**	**(1.3)**	**(1.9)**	**(3.2)**	**(4.9)**	**(7.6)**	**(12.5)**	**(4.7)**	**(7.0)**	**(11.7)**
	R	284	138	646	165	45	314	144	81	379	116	63	285
		(44.0)	**(21.4)**	**(65.3)**	**(52.5)**	**(14.3)**	**(66.9)**	**(38.0)**	**(21.4)**	**(59.4)**	**(40.7)**	**(22.1)**	**(62.8)**

Notes: W, wheat; **R**, rye; F1, bands eliminated in F1 hybrids; **T**, bands eliminated in triticales after chromosome doubling (results for shared bands are not presented). **CS**, 'Chinese Spring'; **I**, 'Imperial'; **H,** 'Holdfast'; **K**, 'King II'; **C**, 'Cocorit 71'; **S**, 'Snoopy'; **U**, 'UC90'.
* % of total bands lost in hybrid plus lost in triticale.

The enhanced modifications levels observed when rye genome interacts either with hexaploid or tetraploid wheat genomes appear to be mainly the result of adjustments occurring immediately after hybridization. Curiously, the rate of band elimination observed after F1 hybrid chromosome doubling is very similar both for wheat and rye repetitive sequences, ranging between 12 and 17%, for most triticales excepting one hybrid analyzed by Ma and Gustafson (2006) in which the level of wheat repetitive bands lost was almost 25%. Moreover, Ma and Gustafson (2006) noted, in two sets of wheat-rye hybrids that hexaploid wheat repetitive sequences had a higher buffer capacity and less changes in comparison to tetraploid wheat genome (24.8% and 25.2% vs. 44% and 47.4%, respectively).

In all wheat-rye F1 hybrids and correspondent triticale studied (Ma and Gustafson, 2006), a preferential elimination of bands associated to repetitive rather than low-

copy sequences was observed concerning the wheat parental genome. Conversely, rye-origin repetitive and low-copy sequences were altered in similar level in both the hybrid and after chromosome doubling. Therefore it is clear that bands associated with coding sequences, are comparatively more eliminated in rye than in wheat. Such preferential genome elimination can drastically reduce homoeologous gene copies avoiding gene redundancy, favoring a "diploid" behavior and further polyploid stabilization, as proposed (Feldman et al. 1997).

Table 5. Number and percentage (in parentheses) of bands lost before and after chromosome doubling detected by (Shaked et al. 2001) in *Aegilops* x *Aegilops* and *Aegilops* x *Triticum* hybrid genotypes.

		As x Au			**Al x Tu**			
Sequence type	Parental genome	**F1**	**A**	**Total[(a)]**	**F1**	**A**	**Total[(a)]**	**Global**
Repetitive[(b)]	**As**	20	4	171	-	-	-	
		(11.7)	**(2,3)**	**(14)**	**-**	**-**	**-**	25
	Au	0	1	202	-	-	-	**(6,7)**
		(0)	**(0.5)**	**(0.5)**	**-**	**-**	**-**	
	Al	-	-	-	12	10	180	
		-	**-**	**-**	**(15.0)**	**(27.5)**	**(12,2)**	41
	Tu	-	-	-	1	18	166	**(11,8)**
		-	**-**	**-**	**(0.6)**	**(10,8)**	**(11,4)**	

Notes: As, *Ae. sharonensis*; **Au**, *Ae. umbellulata*; **Al**, *Ae. Longíssima*; **Tu**, *T. urartu*; **F1**, bands eliminated in F1 hybrids; **A**, bands eliminated in allotetraploids after chromosome doubling.
(a) % of total bands lost in hybrid plus lost in allotetraploid.
(b) Results only for non-methylation-sensitivite enzymes.

5.7. Size matters in triticale genome rearrangements: larger genomes are more affected

As described earlier, major genomic restructuring events were identified in triticale at higher frequencies than in any other polyploid studied, which affected variation in repetitive, low-copy, and coding sequences present in the genome. Analysis of triticale confirmed early suggestions on the mechanisms involved in polyploidization adjustment, but also revealed new concerns. The idea that sequence elimination is a major event involved in the stabilization of newly formed polyploids was reinforced by the results reviewed, as the overall number of sequences lost confirmed previous descriptions on genome size decrease in

triticale (Boyko et al. 1984). In Ozkan et al. (2003) and Eilam et al. (2008) an extensive list of genome downsizing examples in *Aegilops* x *Triticum* hybrid genotypes was presented. Moreover, it was recently shown that the absence of rye-origin bands in wheat-rye hybrid genotypes resulted from sequence elimination rather than from changes on primer annealing sites (Bento et al. 2010).

Table 6. Parental genome preferential restructuring in Triticeae polyploid systems.

	Maternal genome		**Paternal genome**		**Higher elimination**	**Source**
	Ploidy 1C(Mbp)	**elimination rate (%)**	**Ploidy 1C(Mbp)**	**elimination rate (%)**		
Ae. sharonensis x	2n=2x=14		2n=2x=14		Maternal	(Shaked et al., 2001)
Ae. umbellulata	6909	14%	4949	0.5%		
Ae.longíssima x	2n=2x=14		2n=2x=14		Maternal	(Shaked et al., 2001)
T. urartu	5929	12.2%	4827	11.4%		
T. turgidum x	2n=4x=28		2n=2x=14		Paternal	(Ma and Gustafson, 2004, 2006)
S. cereale	12030	27,6%	8110	64%		
T. aestivum x	2n=6x=42		2n=2x=14		Paternal	(Ma and Gustafson, 2004, 2006; Bento et al. 2008)
S. cereale	16979	14,2%	8110	66,2%		
T. turgidum x	2n=4x=28		2n=2x=14		Maternal	(Dong et al., 2005)
Ae. tauschii	12030	21.0%	5027	12.3%		

The higher degree of paternal genome elimination observed in triticale is not the general rule that has been observed in other newly formed polyploids. In Table 6 preferential parental genome elimination is presented for some Triticeae polyploids, showing that the maternal genome can also show preferential sequence elimination, contradicting Gill (1991) hypothesis. The results compiled clearly demonstrated that the genome suffering more modifications during polyploidization was always the larger one (comparing DNA contents per haploid genome), independently of their maternal or the paternal status. Thus, the results collected in this review clearly point out, for the first time, the tendency in cereal wide hybridization for parental genome size homogenization, which preferentially affects the larger genome, to stabilize the newly formed polyploid species. Large scale rearrangement events are also observed, being probably the loss of telomeric heterochromatin a mechanism to obtain a more balanced nucleotype in triticale (Jouve et al. 1989). Bernardo et al. (1988) demonstrated a clear negative effect of rye heterochromatin on triticale meiotic pairing and the loss of telomeric

heterochromatic blocs are related with yield increase in hexaploid triticale (Gustafson and Bennett 1982). This phenomenon can be the outcome of the relation between nuclear DNA content and the speed of DNA replication (Francis et al. 2008). In fact, the larger parental genome in a hybrid nucleus may not be able to complete the cell cycle by the time of telophase and/or cell wall formation, thus inducing DNA elimination through breakage-fusion-bridges as previously observed in the early endosperm development of wheat-rye hybrids (Bennett and Gustafson 1982; Gustafson and Bennett 1982). Moreover, those sequence elimination events will certainly preferentially affect late replication repetitive fractions of the genome, namely the dense rye heterochromatic sub-telomeric domains (Bennett 1977; Neves et al. 1997).

Table 7. DNA 1C-value and cell cycle time (CCT) in triticale and wheat and rye parental species.

Species	**1C-value[(a)] (pg)**	**CCT[(b)] (h)**
Secale cereale 'UC90'	8.3	12.0
Triticum turgidum 'Cocorit'	12.28	12.0
x Triticosecale 'Cocorit' x 'UC90'	16.8	12.0

(a) Plant DNA C-values Database, Royal Botanic Gardens, Kew, UK.
(b) Kidd *et al*. 1987.

That hypothesis is reinforced by the effect of DNA C-values in cell cycle duration on correlations between DNA amount, nuclear volume, and cell cycle length in angiosperms ((Van't Hof and Sparrow 1963) and in triticale (Kaltsikes, 1971; Bennett and Kaltsikes, 1973). Recently, the analysis of cell cycle duration in 110 monocots and eudicots species were plotted against the respective nuclear DNA C-values (Francis et al. 2008) and a highly significant regression was observed for all species analyzed, independently of their ploidy level. However, Kidd et al. (1987) presented values for hexaploid triticale in comparison with parental species (Table 7) and surprisingly demonstrated that, although polyploidization leads to an increase on genome size, Cell Cycle Time (CCT) values are constant both for progenitors and the polyploid species. They also showed the maintenance of stable cell cycle lengths in hexaploid wheat where the parental CCT values are 11.4 h in *Ae. tauschii,* and ranges from 11.0 h to 13.9 h in *T. turgidum*, but doesn't

exceed 14.0 h in the allopolyploid *T. aestivum*. The clear correlation between nuclear DNA amounts and cell cycle length appears to be associated both with genome heterochromatic fraction dimension (reviewed in (Redi et al. 2001) and with the speed of DNA replication (Francis et al. 2008).

This is where genome restructuring meets epigenetic remodeling of parental genomes allocated to the same nuclear background. In fact, more than just genome rearrangements are necessary for the adjustment of both parental genomes following polyploidization, chromatin remodeling also mediates required changes when two species share a common hybrid nucleus. Viegas et al. (2002) proposed a model explaining chromatin-imprinting control of nucleolar dominance in polyploid species, based in the importance of genome size in such interactions. With the Viegas et al. (2002) model, differences in genome size due to repetitive DNA sequence variation allocated in heterochromatin domains should induce the need for greater elimination events in the larger rye genome, to properly "accommodate" in the hybrid nucleus. Following such hypothesis, more intimate associations between heterochromatic domains should also occur modifying expression patterns of neighboring genes. Epigenetic functional fine-tuning of parental genomes together with preferential rearrangements of the larger parental genome will therefore certainly assist polyploid genome downsizing. Genetic and epigenetic modifications seem therefore crucial to establish the diploid-like behavior and speciation of polyploid genomes, as proposed by Ma and Gustafson (2005).

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Chapter VI

Conclusion and Perspectives

6. Conclusion and perspectives

The most relevant aim of this project was to contribute for a deeper understanding of processes underlying parental genomes adjustment associated with polyploidization events. The selection of the model species to be studied was therefore crucial, taking into consideration the need of guaranteed inbred parental and polyploid lines. Triticale was elected as the polyploid model species to be analyzed due to its synthetic origin, associated to the existence of a line with excellent characteristics present in the USDA-Sears collection. This line constitutes a stable octoploid triticale (2n=8x, AABBDDRR) with at least 35 selfing generations, produced by crossing isogenic lines of wheat (*Triticum aestivum* 'Chinese Spring', 2n=6x, AABBDD) and rye (*Secale cereale* 'Imperial' 2n=2x, RR) (Ma et al., 2004). In order to assess genome interactions dynamics when complete chromosome sets of distinct species are present in a polyploidy, and also when only specific chromosomes are introgressed in a host genome, we took advantage of the seven wheat lines with the addition of each rye chromosome pair also produced by Sears in 1954 (wheat-rye addition lines). Wheat-rye addition lines resulted from backcrossing triticale to parental wheat line followed by selfing and selection for several generations (Sears, 1954).

Large plant genomes are mainly composed of repetitive sequences, which in Triticeae can represent up to more than 90% of the genome. The majority of those repetitive sequences are represented by tandem or interspersed repeats (Dvorak, 2009). Microssatelites are tandem repeats present in high copy number and dispersed throughout most plant genomes (Wang et al., 1994). In Triticeae the majority of interspersed repetitive sequences are transposable elements, namely retrotransposons which are ubiquitous in all organisms and highly involved in genes and genomes evolution (Bennetzen, 2000). To perform a wide genomic analysis of hybrid/polyploid genotypes, retrotransposons and microsatellites were selected as marker sequences, using PCR based techniques - Inter Retrotransposons Amplified Polymorphism (IRAP), Retrotransposons Microsatellite Amplified Polymorphism (REMAP), and Inter Simple Sequence Repeat (ISSR) developed by Kalendar et al. in 1999 and already used to

distinguish cultivars/varieties from distinct species (Baumel et al., 2002; Pereira et al., 2005; Guo et al., 2006; Smykal, 2006; Branco et al., 2007).
In this work we demonstrate that such techniques are very effective tools in genomic analysis as each primer combinations used allowed wheat and rye genome characterization. Triticale presented a reproducible banding profile distinct from the parental species and most primer combinations used contributed to disclose genomic rearrangements associated with polyploidization (Bento et al., 2008). Using the same techniques, opposite results were observed in the analysis of the natural allopolyploid *Spartina anglica* which did not reveal genomic rearrangements when IRAP, REMAP and ISSR patterns from parental genomes were compared with the polyploid (Baumel et al., 2002). Parental genome additive patterns were also obtained in *S. anglica* through AFLP analysis (Salmon et al., 2005). Thus, the lack of parental genome rearrangement seems to be the rule in this natural allopolyploid species.

In triticale the assessment of retrotransposons and microsatellite related sequences using ten different primer combinations, revealed 51% rearranged bands, in comparison with the parental genomes. The banding profiles obtained also allowed the identification of restructured sequences parental origin, proving that rye genome is considerably more restructured than the wheat genome (Bento et al., 2008), as previously described in wheat-rye F1 hybrid and triticale through AFLP and RFLP analysis (Ma et al., 2004; Ma and Gustafson, 2006). Global levels of genomic restructuring events reported in hexaploid (39,9%) and octoploid (30,6%) triticale using AFLP and RFLP analysis (Ma et al., 2004; Ma and Gustafson, 2006) and also through AFLP in synthetic wheat polyploids (between 13 and 20%) (Dong et al., 2005; Shaked et al., 2001) were however substantially lower in comparison to the values we detected in triticale using REMAP, IRAP and SSR techniques. Such obvious differences between frequencies of genomic rearrangements detected with distinct molecular markers must result from the target sequences analyzed, since the AFLP technique allows a broad genome analysis whereas REMAP and IRAP assess particularly labile repetitive sequences like retrotransposons.
Additionaly, most genomic rearrangements detected by IRAP, REMAP and ISSR in triticale represent bands losses, with only a minor frequency (4,2%) of novel

bands. Bands loss was also the preferential restructuring modification observed through AFLP and RFLP techniques in synthetic polyploid wheats (Shaked et al., 2001; Dong et al., 2005; Khasdan et al., 2010), and triticale (Ma et al., 2004; Ma and Gustafson, 2006; Tang et al., 2008).

However, we aimed to deeper understand genomic rearrangements at the sequence level through the exact characterization of modified bands. Sequencing of the wheat-specific band Mob-11-1200W absent in triticale revealed a nested structure of copia-like retrotransposons elements, namely Claudia and Barbara. Conversely, the rye-specific band Mob-111-1000R absent in triticale presented homology with the sequence coding to a protein belonging to a major family of inducible defense response proteins. Moreover, the novel triticale specific sequence Mob-110-100T was mainly composed of microsatellite sequences. Restructured bands analysis therefore clearly demonstrates that genomic rearrangements associated with polyploidization are widespread, affecting both repetitive and coding sequences (Bento et al., 2008). Tang et al. (2008) simultaneously showed the involvement of regulatory elements and promoter regions in triticale rearrangement events resulting from hybridization and/or polyploidization, corroborating that all types of sequences are implicated in genomic adjustment induced by such evolutive processes.
A broader assessment of restructured parental genome sequences in triticale will require further sequencing of all IRAP, REMAP and ISSR amplification products, probably recurring to new generation sequencing techniques. Such analysis will certainly markedly amplify the already high level of genome adjustments described in this work since in all evaluations performed, bands with the same size were assumed to correspond to identical sequences, although they can have distinct nucleotide sequences and also different genomic origins.

Although the knowledge we obtained with the characterization of restructured bands was very relevant, we further questioned about events underlying triticale genome restructuring through the study of internal fragments of wheat and rye-origin rearranged sequences as parental band loss in triticale could either result from sequences modification or elimination. The detection in triticale of internal sequences from Mob-11-1200W (unpublished results) and Mob-111-1000R

internal segments (Bento et. al., 2010) clearly proved that their absence in the polyploidy results only from alterations affecting one or both primer annealing sites. Similar experiments involving SSR markers in triticale also revealed that internal fragments of modified bands were present in the newly formed polyploidy (Tang et al., 2008).

Interestingly, the follow-up of internal fragments from rye-origin band (Mob-111-1000R) revealed their complete absence in wheat-rye addition lines (Bento et al., 2010). This is a clear indication that while in triticale genome modifications may be restricted to some nucleotides, in wheat-rye addition lines a drastic elimination of long nucleotide sequences occurs.

The new round of genomic restructuring events disclosed in wheat-rye addition lines was very extensive as all rye-origin bands as well as triticale novel bands are absent in those genotypes. Preferential rye sequence elimination detected in wheat-rye addition lines must occur in the generations following triticale x wheat backcross (BC), as we still detected such bands after the BC (Bento et al., 2010). Our data is in accordance with previous cytological observations involving rye chromosomes anomalies described in generations following triticale x wheat BC (Lukaszewski and Gustafson, 1983). Although previous results also demonstrated the preferential restructuring of rye repetitive sequences in wheat-rye addition lines (Alkhimova et al., 1999; Szakacs and Molnar-Lang, 2010), our analysis unraveled for the first time the deletion of parental sequences. It must however be emphasized the observation of wheat-origin bands absent in triticale immediately after BC, as well as in addition lines. Thus, our results prove that smaller rye chromatin introgressions in wheat background are correlated with higher genomic readjustments.

The major question on preferential restructuring/elimination of rye genome sequences in triticale, remains however unexplained. A detailed re-analysis of our data and that from previous studies oriented to the quantification of genomic restructuring events in synthetic Triticeae hybrids/polyploids (Bento et al., 2008; Dong et al., 2005; Ma et al., 2004; Ma and Gustafson, 2006; Shaked et al., 2001), allowed the formulation of the first model to explain preferential parental genomes adjustments (Bento et al., 2011). This model demonstrates that parental genomes with higher relative dimensions are more restructured in hybrid/polyploidy

situations, probably representing a pathway to solve parental genomes conflicts mediated by discrepant cell cycle durations (Francis et al., 2008). Major genomes size differences result from repetitive sequences number mostly represented by heterochromatin fractions with late replication (Bennett, 1977). A preferential elimination of repetitive sequences from rye-origin, which represents the larger parental genome in triticale, could then contribute to enhance cell cycle synchronization (Kidd et al., 1987).
Characterization of C-values in natural and synthetic *Aegilops* and *Triticum* allopolyploids and in triticale (Eilam et al., 2008; Francis et al., 2008) reinforces the model presented as genome downsizing was already correlated with polyploidization (Leitch and Bennett, 2004). However future C-values determinations must be performed in wheat-rye addition lines by flow cytometry allowing the assessment of rye preferential genome elimination detected,

Although no chromosome mapping yet exists of specific restructured bands in triticale or in wheat-rye hybrids, detected by IRAP, REMAP and ISSR in this project or by RFLP and AFLP in previous works, our analysis using the Nikita/(CT)9G REMAP amplification reaction in *in situ* hybridization showed a widespread chromosome distribution of such sequences. In rye genome the pervasiveness of transposable related sequences has already been described for pSc20H, showing however a reduced intensity in sub-telomeric domains (Ko et al., 2002) where the sequences we mapped are more frequent.

Besides the characterization of genomic changes in wheat-rye interactions we also considered very relevant to contribute for a deeper understanding of their effect on gene expression patterns, through the analysis of 45S rDNA. Such sequences are highly repetitive coding sequences clustered at the Nucleolus Organizer Region (NOR) and usually presenting preferential parental silencing in hybrids and polyploid organisms, a process designated as nucleolar dominance. That process was initially described in *Crepis* spp hybrids in Navashin (1934) pioneer work and is certainly the most well studied epigenetic alteration associated with polyploidization. Navashin also proved that NORs silencing in hybrids was reversed after backcrossing, emphasizing in this way the absence of any potential association between rDNA silencing and restructuring of those sequences.

A chromatin imprinting model was proposed by Viegas et al. in 2002, indicating that preferential rDNA silencing observed in polyploids was associated with the parental genome with larger dimensions, probably resulting from the establishment of new heterochromatic domains in a smaller hybrid nuclear volume. What happens to the "dominant" NORs remained however elusive. In the wheat-rye system it is well established that rye-origin NORs are silenced (Cermeño et al., 1984; Lacadena et al., 1984; Neves et al., 1997) but nothing was known regarding modulation of wheat-origin dominant NORs activity. Our studies on transcriptional dynamics of wheat-origin dominant NORs in the wheat line with the addition of rye 1R nucleolar chromosome disclosed additionally a marked up-regulation of wheat NORs. Such modification on the activity of dominant wheat NORs was moreover correlated with chromatin organization remodeling of such *loci* (Silva et al., 2008). The mutual amphiplasty phenomenon we unraveled in nucleolar dominance process, markedly contributes to substantiate the chromatin imprinting model previously described. Future, comparative studies on wheat dominant NORs transcription patterns in the remaining wheat lines with the addition of non-nucleolar chromosomes are critical to further evaluate the extension of novel chromatin imprinted patterns. Ribosomal chromatin dynamics is modulated by specific epigenetic marks, namely DNA methylation and histone H3 and H4 post-translational modifications, as methylation and acetylation (Neves et al., 2005; Earley et al., 2006). Peng and Karpen (2007) moreover demonstrated a direct association between changes in rDNA organization, epigenetic marks and production of extrachromosomal arrays of rRNA genes. Taking into consideration massive modifications on DNA methylation patterns observed in TE and neighbor sequences in synthetic allopolyploids (Yaakov and Kashkush, 2011) we are tempted to speculate that the vast genomic reorganization events previously described also result from changes in chromatin organization patterns of parental genomes in hybrid/polyploid conditions.

Knowledge already gathered on the organization of specific genome repetitive regions of Triticeae species (Dvorak, 2009), along with the advent of large scale sequencing, in particular entire genome sequencing initiatives as the International Wheat Genome Sequencing Consortium, will certainly allow the future characterization of Triticeae polyploids genomic and epigenomic landscapes,

contributing to understand structural and functional implications underlying plant genome evolution.

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List of abbreviations

List of abbreviations

AFLP	---	Amplified Fragment Length Polymorphism
A	---	Adenine
Ag	---	Silver
BC	---	BackCross
BLAST	---	Basic Local Alignment Search Tool
Bp, Kbp	---	base pairs, kilobases
C	---	Cytosine
CCT	---	Cell Cycle Time
cDNA, C-DNA	---	complementar DNA
Ct	---	threshold Cycle
CTAB	---	CetylTrimethylAmmonium Bromide
Cy3	---	Cyanine 3
DAPI	---	4',6-DiAmidino-2-PhenylIndole
DNA	---	DeoxyriboNucleic Acid
dNTP	---	deoxyriboNucleotide TriPhosphate
DR	---	Direct Repeat
DRM2	---	de novo cytosine methyltransferase 2
dUTP	---	deoxyUracyl TriPhosphate
EB	---	Enzyme Buffer
ETS	---	Expressed Sequences Tags
FISH	---	Fluorescent In Situ Hybridization
FITC	---	Fluorescein IsoThioCyanate
G	---	Guanine
Gag	---	Core structural protein genes or Capsid Protein gene
Gc	---	Gametocidal
GISH	---	Genomic In Situ Hybridization
hrs, min, sec	---	hours, minutes, seconds
HDA6	---	histone deacetylase 6
HRGP	---	Hydroxyproline-Rich GlycoProteins
INT	---	Integrase
IR	---	Inverted Repeat
IRAP	---	Inter Retrotransposons Amplified Polymorphism
ISSR	---	Inter Simple Sequence Repeat
Kg, g, mg, µg, ηg, pg	---	kilogram, gram, milligram, microgram, nanogram, picogram
LINE	---	Long Interspersed Nuclear Elements
LTR	---	Long Terminal Repeat
MBD10	---	Methylcytosine Binding Domain 10
MBD6	---	Methylcytosine Binding Domain 6
Mbp	---	Mega base pair
miRNA	---	microRNA
MITE	---	Miniature inverted-repeat transposable elements

M, mM	---	Molar, milliMolar
mRNA	---	messenger RNA
NCBI	---	National Center for Biotechnology Information
NOR	---	Nucleolar Organizing Regions
ºC	---	degree Celsius
PBS	---	Primer Binding Site
PCR	---	Polymerase Chain Reaction
PlantSat	---	Plant Satellite DNA database
Pol	---	Retrotransposons Polyprotein Region
PPT	---	PolyPurine Tract
PR	---	Protease
pSc200	---	Secale cereale tandem repeat sequence
pTa71	---	Triticum aestivum 45S rDNA sequence
rDNA, rRNA	---	ribosomal DNA
REMAP	---	Retrotransposons Microsatellite Amplified Polymorphism
RFLP	---	Restriction Fragment Length Polymorphism
RNA	---	RiboNucleic Acid
RT	---	Reverse Transcriptase
SINE	---	Short Interspersed Nuclear Elements
SSAP	---	Sequence-Specific Amplified Polymorphism
SSR	---	Simple Sequence Repeats or Microsatellite
T	---	Thymine
TAS	---	Telomere-Associated Sequences or Telomere-Associated Repeats
TE	---	Transposable Elements
TIGR	---	The Institute for Genomic Research
TIR	---	terminal inverted repeats
Tm	---	melting Temperature
TRIM	---	Terminal-repeat Retrotransposons In Miniature
U	---	enzyme Unit
USDA	---	United States Department of Agriculture
UTR	---	Untranslated Regions
V	---	Volt
Vol/vol or v/v	---	volume per volume
Δ	---	difference
ηmol	---	nanomole
(l or L), ml, ml, µl	---	litres, millilitres, microlitres, microlitres
µm	---	Micrometre

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