UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

DEPARTAMENTO DE GENÉTICA

ANALYSIS OF A SECONDARY CONSTRICTION IN CHROMOSOME 5R OF RYE AND ITS NEOCENTROMERIC ACTIVITY

ANÁLISIS DE UNA CONSTRICCIÓN SECUNDARIA EN EL CROMOSOMA 5R DE CENTENO Y SU ACTIVIDAD NEOCENTROMÉRICA

TESIS DOCTORAL DE:

MARÍA CUACOS MARCOS

BAJO LA DIRECCIÓN DE:

JUAN MANUEL VEGA MELERO

Madrid, 2013

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Memoria
que para optar al grado de Doctora en Biología
presenta

MARÍA CUACOS MARCOS

VºBº del Director **La Autora**

JUAN MANUEL VEGA MELERO **MARÍA CUACOS MARCOS**

Madrid 2013

This PhD thesis was funded by the following projects:

- Centrómeros y cromosomas artificiales de cereales (Centromeres and artificial chromosomes in crops). AGL2011-28542.
PI: Juan Manuel Vega Melero.

- Genómica funcional de los centrómeros de cereales (Functional genomics of cereal centromeres). CCG10-UCM/GEN-5559.
PI: Juan Manuel Vega Melero.

- Análisis citogenético y genómico (Citogenetic and genomic analysis). Grupo UCM, Santander Central Hispano-Universidad Complutense, Ref: 910452-1064.
PI: Juan Luis Santos Coloma.

- Embriogénesis de polen y doble-haploides para mejora de especies de interés agronómico (Polen and double-haploids embryogenesis for agronomical-important plant breeding). DGICYT-MEC, AGL2008-04255.
PI: María del Carmen Risueño Almeida.

- Análisis citogenético y genómico (Citogenetic and genomic analysis). Grupo UCM. Universidad Complutense-Comunidad de Madrid, Grupo: 910452.
PI: César Benito Jiménez.

- Análisis estructural y funcional de los centrómeros de cereales (Structural and functional analysis of crops centromeres), BFU 2006-10921/BMC.
PI: Juan Manuel Vega Melero.

María Cuacos Marcos was a predoctoral grant holder from Complutense University (Madrid).

Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning.

Albert Einstein (1879-1955)

Aprende del pasado, vive el presente, ten esperanza para el futuro.
Lo importante es no dejar de hacerse preguntas.
(Traducción libre)

The most exciting phrase to hear in science, the one that heralds
new discoveries, is not 'Eureka!' but 'That's funny...'

Isaac Asimov (1920-1992)

La frase más excitante que se puede oír en ciencia, la que anuncia nuevos descubrimientos, no es "¡Eureka!" sino "Es extraño..."
(Traducción libre)

AGRADECIMIENTOS

En primer lugar, me gustaría dar las gracias al Dr. Juan Manuel Vega y a la Dra. María Jesús Puertas, por la oportunidad de realizar en su grupo mi tesis doctoral. A Juanma le agradezco su optimismo y la confianza que siempre ha tenido en este proyecto, el empeño y las interminables horas destinadas al cuidado de las plantas, y su valiosa aportación en el diseño experimental y la discusión de resultados. A Chechu le agradezco enormemente las horas oficiales y "extraoficiales" dedicadas a la tesis, su compromiso hasta el final, su experiencia y esmero y sus incesantes consejos para que redactara ciencia y no novelas.

Quiero dar las gracias a todos los miembros del Departamento de Genética, y muy especialmente a su directora, la Dra. Concepción Romero, por combinar tan acertadamente el trato profesional y personal y por su permanente disponibilidad, a pesar de tener mil asuntos entre manos. Gracias también al Dr. Juan Luis Santos, entre otras cosas, por contribuir al desarrollo científico del departamento organizando los seminarios. Gracias a los técnicos: Mª Carmen Moreno, por tener siempre listos los medios, encargarse de pedidos y de cualquier cosa que hiciera falta; y José Barrios, por estar siempre dispuesto a reconciliarnos con los equipos informáticos.

Fuera del departamento, universalmente conocida en la facultad, quisiera agradecer a Rosa su infinita capacidad para lidiar con los asuntos de doctorado y estar siempre pendiente hasta del más mínimo detalle.

I acknowledge all the people that contributed to this work either with seeds (Dr. Moshe Feldman and Dr. Tomás Naranjo), with antibodies (Dr. Andreas Houben, Dr. Kelly Dawe, Dr. Fangpu Han and Dr. Zhukuan Cheng) or with probes (Dr. Angeles Cuadrado and Dr. Peter Langridge). Also special thanks to Dr. Elena Benavente for providing greenhouse space at the ETS Ingenieros Agrónomos (Universidad Politécnica de Madrid).

I would like to thank Dr. Andreas Houben and his entire group, for letting me work in their lab (even!) two times, for their kindness, for the highly motivating working atmosphere and their permanent willingness to discuss about science. Particularly thanks to Katrin Kumke for excellent technical assistance and arranging plant material. And big thanks to Dr. Veit Schubert for the wonderful SIM images.

Gracias a mis compañeras y compañeros de laboratorio: a Miriam y Mónica, por haber guiado mis primeros pasos en el mundo de la citogénetica y siempre estar dispuestas para resolver cualquier duda; a Diana y Adri, por haber colaborado en el aislamiento de anteras (¡y muchas cosas más!) especialmente en los últimos meses de experimentación; y a Manolo, por su contribución en el laboratorio.

Gracias a todos los demás compañeros del departamento, por haber amenizado estos años de trabajo. Gracias a Bea, porque sin ti los descansos no hubieran sido lo mismo; a Patri, por los viajes y la vida fuera de la universidad, por sonrisas y besos de abuela; a Mónica, por tu escucha siempre pausada, tu excelente calidad como investigadora y como persona; a Javi, por tus acertadas preguntas, tu pasión por la ciencia y tu capacidad para provocar risas y sonrisas; a Ceci y Alberto, por haber compartido la facultad en días y horas intempestivas; a Diego, por tu calidez y cariño; a Alejandra, por tu naturalidad y armonía; a Natalia, por ser una más y alegrar nuestras comidas; a Naike, por tu inocencia y simpatía; a Nohelia, por dejar huella en mi corazón; a Celia, por tu espontaneidad y buen humor; a todos los demás, pequeños y no tan pequeños, que están o han estado en el departamento, por cada buenos días; y muy especialmente, gracias a Silva, porque nunca olvidaré una conversación en forestales que sentó las bases que me permitirían progresar como científica.

De otra manera muy distinta quiero agradecer con mucho cariño a mis amigas, Ester, Rocío, Eva, Sandra… por estar siempre ahí, por haber compartido estos años y tantas experiencias, por haber crecido juntas, porque me hacéis sentir dichosa y afortunada, porque sois una parte fundamental en mi vida.

Desde lo más profundo de mi corazón, gracias a mi familia. Gracias a mis padres, por aceptar y comprender que haya elegido una profesión tan… incomprensible, cuando lo que pretendo es ganarme el pan con ella. Gracias por vuestro amor incondicional, porque sois mi mejor ejemplo, de bondad, de compromiso, de trabajo y tesón, porque os debo lo que soy. Gracias a mi hermano, por hacer que me sienta orgullosa de él, y por haberse sentido a veces orgulloso de mí; Juancar, eres muy importante en mi vida. Gracias a mis abuelos, por derrochar amor, por cada gesto y cada palabra. Gracias a Ana, por ser la mejor compañera de piso que podía tener en esta última etapa, por prepararme la cena tantas veces, por alegrarme cada noche al volver a casa; porque además de prima, eres una gran amiga.

Lieber Stefan, vor einiger Zeit dachte ich, dass ich Dir nicht für 1000 Dinge danken könnte. Nun ist es fast unmöglich - ich schulde Dir so unendlich viel! Vielen lieben Dank dafür, dass... Du mir im Labor geholfen hast, Du Dich um meine Pflanzen gekümmert hast, Du mit so viel Einsatz meine Dissertation Korrektur gelesen hast, Du mir viele unbezahlbare Ratschläge gegeben hast, Du mir die Leidenschaft für die Forschung zurückgegeben hast. ...Ich Dich kennenlernen durfte, ich so sein konnte, wie Du es bist, ich mit Dir entdecken konnte, was wahre Liebe ist. …Du mein Leben verändert hast, Du mit mir diese anstrengenden Zeiten überwunden hast, bis jetzt auf mich gewartet hast, und nun alles weitere auf uns zukommen kann. Dies ist nicht das Ende, es ist gerade erst der Anfang!

Y… gracias a las personas que pasaron por mi vida y que ya no están, porque de alguna manera siguen estando.

La ciencia te permite no dejar de asombrarte, de maravillarte, de aprender…
y por eso me apasiona.

María, 2013

TABLE OF CONTENTS

TABLE OF CONTENTS

RESUMEN 1

ABSTRACT 10

INTRODUCTION 13

1. The centromere. 14

1.1. Centromeric DNA. 14

1.2. Constitutively- and transiently-associated centromeric proteins. 15

1.3. Centromeric and pericentromeric chromatin: structure and function 16

1.4. Epigenetic determination of centromeres 17

2. Neocentromeres. 18

2.1. 'De novo' neocentromeres. 18

2.1.1. Clinical and induced neocentromeres in humans. 18

2.1.2. Induced neocentromeres in *Drosophila*. 19

2.1.3. Induced neocentromeres in yeast. 20

2.1.4. 'De novo' neocentromeres in plants. 21

2.2. Terminal neocentromeres. 21

2.2.1. Terminal neocentromeres in maize 21

2.2.2. Terminal neocentromeres in rye 22

2.3. Interstitial neocentromere in chromosome 5R of rye 23

AIMS OF THE STUDY 25

MATERIALS AND METHODS 27

Plant material 28

Methods 29

1. Plant culture conditions 29

2. Pesticide treatment 29

3. Cytogenetic methods. 30

3.1. Material selection 30

3.2. Fluorescence *in situ* hybridization (FISH) 30

3.2.1. Slide preparation: Spreading and Squash 30
3.2.2. Hybridization 31
3.2.3. Probes used: isolation and labelling 32
3.3. Immunostaining. 33
3.3.1. Slide preparation 33
3.3.2. Immunolocalization 34
3.3.3. Antibodies 34
4. Microscopy 36

RESULTS 37
1. A secondary constriction in the long arm of chromosome 5R (5RL) of rye 38
1.1. Identification of chromosome 5R by FISH 38
1.2. General features of the 5RL constriction 39
2. Analysis of the 5RL constriction in rye 41
2.1. Occurrence of the 5RL constriction in different rye species and cultivars 41
2.2. Occurrence of the 5RL constriction in the interspecific and intercultivar F1 45
2.3. Occurrence of the 5RL constriction in the interspecific F2. 47
3. Neocentromeric activity in chromosome 5R of rye 49
3.1. 5R neocentromere in a wheat-5RL monotelosomic addition line 49
3.2. 5R neocentromere in a wheat-5RL ditelosomic addition line. 51
3.3. 5R neocentromere in wheat-5R monosomic and disomic addition lines 52
3.4. 5R neocentromere in diploid rye. 55
3.5. Terminal neocentromeres in diploid rye. 55
4. Characterization of the 5RL constriction with neocentromeric activity. 57
4.1. DNA sequences 57
4.2. Centromeric proteins and pericentromere-associated histone modifications. 57
4.3. Antibodies atypically labelling the 5RL constriction in rye 62
5. Influence of Diazinon on the frequency of the 5R neocentromere 63
5.1. Neocentromeric frequencies in a wheat-5RL monotelosomic addition line 63
5.2. Neocentromeric frequencies in a wheat-5RL ditelosomic addition line 65
5.3. Neocentromeric frequencies in wheat-5R mono- and disomic addition lines 67
6. Effect of Diazinon on the spindle at metaphase I 68

DISCUSSION 70
1. Occurrence of a secondary constriction in chromosome 5R of rye 71
2. The 5RL constriction shows neocentromeric activity 74
3. The properties of the constriction could determine the neocentromeric activity 76
4. Typical centromeric proteins are not detected at the 5R neocentromere 78
5. Active 5R neocentromere in diploid rye 81
6. A pesticide promotes the neocentromere due to alterations of the spindle. 81
7. Concluding remarks 84

CONCLUSIONS 86

REFERENCES 88

APPENDIX 99

Resumen

RESUMEN

INTRODUCCIÓN

El centrómero es la estructura del cromosoma eucariótico que permite el reparto de la información genética a las células hijas. Citológicamente se visualiza como una constricción, y está constituido por el ADN y las proteínas centroméricas. Las secuencias de ADN centroméricas son muy variables entre especies, pero tienen en común la asociación con una variante de la histona H3 denominada cenH3 (De Rop y col. 2012). Por ello, se considera que el centrómero está determinado epigenéticamente.

Los neocentrómeros son regiones del cromosoma distintas del verdadero centrómero en estructura, secuencia y localización, que en determinadas ocasiones pueden mostrar algunas de las características centroméricas. Hasta la fecha, los neocentrómeros descritos se pueden dividir en dos grupos:

- Neocentrómeros que sustituyen al centrómero en fragmentos acéntricos (Burrack y Berman 2012). Se han descrito en cromosomas humanos, *Drosophila*, levaduras y dos casos en plantas. Cuando el cromosoma pierde el centrómero (bien por rotura o por inactivación) puede formarse un neocentrómero en otra región que asegura la transmisión de ese fragmento. En estos neocentrómeros se detecta cenH3, además de otras proteínas que forman un cinetocoro funcional y se visualizan como una constricción. Son activos en mitosis, aunque también se han descrito algunos casos de transmisión en meiosis.

- Neocentrómeros meióticos (o terminales) de plantas (Guerra y col. 2010). Fueron los primeros descritos, y se visualizan como extensiones de la heterocromatina subtelomérica hacia los polos. Los mejor descritos son los de maíz y centeno, donde la activación de los neocentrómeros tiene un control genético. Son activos simultáneamente con el centrómero, no contienen cenH3 y no forman un cinetocoro funcional aunque interaccionan con los microtúbulos del huso.

Existe un tercer tipo de neocentrómero, localizado en el brazo largo del cromosoma 5R (5RL) de centeno. Se ha visto en determinados genotipos: centeno haploide, híbridos trigo centeno, y en líneas de adición trigo-centeno que incluyan el cromosoma 5R o el brazo largo del mismo (Schlegel 1987; Manzanero y col. 2000b, 2002; Cuacos y col. 2011). Este neocentrómero es activo sólo en meiosis, donde interacciona con microtúbulos y puede dirigir el movimiento del cromosoma hacia los polos celulares; además, mantiene las cromátidas hermanas unidas en anafase I. Carece de secuencias centroméricas o teloméricas que pudieran justificar su actividad pero presenta proteínas (no identificadas) desde metafase I hasta anafase II (Manzanero y col. 2002). Una característica importante es que la actividad neocentromérica aparece en una constricción secundaria del cromosoma 5R. Esta constricción se ha observado en los genotipos descritos anteriormente, además de en líneas consanguíneas de centeno diploide (Lamm 1936; Müntzing y Akdik 1948). La secuencia repetida pSc119.2 (Bedbrook y col. 1980; McIntyre y col. 1990) se localiza en la constricción, que coincide con la banda C 5RL1-3 (Mukai y col. 1992; Cuadrado y col. 1995). El neocentrómero se observó con frecuencias variables en los distintos trabajos publicados, apuntando a una posible influencia ambiental en su activación. En el inicio de esta tesis se descubrió que plantas tratadas con un pesticida comercial, cuyo compuesto activo es un derivado organofosforado, presentaban una frecuencia alta de células con el neocentrómero del cromosoma 5R.

OBJETIVOS

En esta tesis doctoral se plantean tres objetivos:

1. Analizar la constricción del cromosoma 5RL de centeno y determinar las causas de su aparición.

La presencia de la constricción es un elemento clave para la actividad neocentromérica. En centeno, algunas variedades muestran la constricción y otras no. Por ello se utilizarán distintos centenos diploides para estudiar en detalle el comportamiento de esta región.

Primero se determinará si las siguientes especies y cultivares de centeno presentan la constricción del 5RL, y con qué frecuencia: *Secale ancestrale*, *S. cereale* cv. Imperial, *S.*

cereale cv. IPK, *S. cereale* cv. I89, *S. cereale* cv. Paldang, *S. cereale* cv. Khorasan, *S. cereale* cv. Merced y *S. cereale* cv. Transbaikal. Dado que en algunos trabajos la constricción se observó en líneas consanguíneas, se tomarán datos de la frecuencia de quiasmas y la presencia de univalentes como indicadores de la consanguinidad de las plantas (relación demostrada, por ejemplo, por Rees [1955]).

Para analizar si la constricción está controlada genéticamente o intervienen otros factores, se realizarán cruzamientos entre especies y cultivares de centeno con constricción y sin ella y se examinarán la F1 y la F2.

2. Estudiar la actividad neocentromérica del cromosoma 5R.

El neocentrómero se había descrito en líneas de adición trigo-5RL monotelosómicas y ditelosómicas (que incluyen una copia o dos, respectivamente, del brazo largo del cromosoma 5R), y en una línea de adición trigo-5R monosómica (que incluye una copia completa del cromosoma 5R). En este trabajo se estudiará, además de estas líneas, una línea de adición trigo-5R disómica (con dos copias completas del cromosoma 5R). Así, se determinará si la activación del neocentrómero depende de que el cromosoma 5R se encuentre en situación de univalente o de bivalente y si puede influir el hecho de que el centrómero esté completo o truncado en las líneas que contienen sólo el brazo largo. Además, se analizará si la actividad neocentromérica aparece en los cultivares de centeno anteriormente indicados.

La identificación de las proteínas asociadas con este neocentrómero es un paso clave para comprender su estructura y función. Para ello, se realizará inmunolocalización de anticuerpos que detecten proteínas centroméricas, incluyendo cenH3. Además, para comprobar la posible regulación epigenética del neocentrómero, se utilizarán anticuerpos que detectan modificaciones de histonas asociadas con la función centromérica y con regiones de heterocromatina.

3. Evaluar la influencia del pesticida en la frecuencia del neocentrómero intersticial del cromosoma 5R.

Plantas de las cuatro líneas de adición se tratarán con un pesticida comercial que puede inducir el neocentrómero añadiendo el producto granulado en los tiestos y regando a continuación para que las plantas lo absorban. Espigas en meiosis se recolectarán antes del tratamiento, para tener un control, y después de añadir el pesticida. Se realizarán

tratamientos sucesivos para evaluar el posible efecto de dosis crecientes de pesticida; asimismo, el material se recogerá en distintos intervalos desde el último tratamiento para determinar si la influencia del pesticida en la frecuencia del neocentrómero disminuye con el tiempo.

Por otro lado, se ha descrito que los carbamatos comúnmente presentes en herbicidas comerciales actúan en mitosis alterando el sistema de microtúbulos y sus centros organizadores (Hepler y Jackson 1969; Coss y Pickett-Heaps 1974; Yemets y col. 2008). Para determinar si el pesticida organofosforado tiene un efecto similar en meiosis, se realizará inmunodetección con un anticuerpo anti-tubulina en meiocitos de plantas control y tratadas.

DISCUSIÓN Y CONCLUSIONES

1. **La formación de la constricción secundaria del cromosoma 5R depende de factores genéticos y posiblemente también epigenéticos.**

La constricción está influida por la homocigosis de las plantas, de tal manera que en las plantas más consanguíneas (con una media de quiasmas baja) la constricción del cromosoma 5RL es visible en un mayor porcentaje de células. En la F1, procedente de cruces interespecíficos e intercultivares entre centenos con y sin la constricción, la frecuencia de células con la constricción disminuye, en consonancia con una reducción de la homocigosis. En cambio en la F2, procedente de autofecundación, la frecuencia de células con la constricción aumenta. Estos datos indican que hay un componente genético controlando la formación de la constricción.

Por otro lado, al cruzar una especie que presenta la constricción en la mayoría de los meiocitos en todas las plantas (*Secale cereale* cv. Imperial) con una especie sin la constricción (*Secale ancestrale*), ésta se vio en la mayoría de las plantas de la descendencia cuando *S. cereale* actuó como madre, es decir, en el citoplasma de esta especie, lo cual no sucedió en el citoplasma de *S. ancestrale*. Además, nunca se observaron bivalentes heteromórficos, lo que indica que el cromosoma 5R de un centeno que presentaba la constricción puede perderla cuando se transfiere a un citoplasma distinto, y viceversa. El diferente comportamiento de la constricción dependiendo del parental que actúa como

madre puede explicarse por la influencia de genes marcados por imprinting. En *Arabidopsis thaliana* se ha encontrado que algunos de estos genes codifican para proteínas que remodelan la cromatina (Gehring y col. 2011) y un sistema similar podría existir en centeno. Por tanto, los factores que inducen la formación de la constricción podrían tener también una naturaleza epigenética.

2. **La constricción del cromosoma 5RL puede actuar como un neocentrómero en las líneas de adición trigo-5R y -5RL y en centeno diploide durante meiosis.**

La actividad neocentromérica se vio en todas las líneas de adición estudiadas y también en centeno diploide, por lo que se demuestra que el neocentrómero no depende de la ploidía ni de que el cromosoma tenga un centrómero completo (5R) o truncado (5RL).

En las líneas de adición, el neocentrómero intersticial del cromosoma 5R es capaz de mantener las cromátidas hermanas unidas en anafase I, interaccionar con microtúbulos y dirigir el movimiento del cromosoma hacia los polos.

3. **El neocentrómero carece de proteínas y marcas epigenéticas típicas del centrómero, incluida la histona cenH3.**

Se ha comprobado que el neocentrómero del 5R carece de la histona cenH3, considerada el determinante centromérico (De Rop y col. 2012). Asimismo, las proteínas CENP-C y MIS12 no se localizan en la constricción. Se buscó la presencia de otras proteínas del cinetocoro (como NDC80 y Shugoshina) pero los anticuerpos disponibles no funcionaron en este material. La ausencia de cenH3 en el neocentrómero podría estar revelando una regulación de la actividad neocentromérica independiente de cenH3 durante meiosis.

Se analizaron dos marcas de centrómeros activos: H3S28ph y H2AT133ph (fosforilación de la histona H3 en la serina 28, y de la histona H2A en la treonina 133, respectivamente). Estas modificaciones de histonas se detectaron en los centrómeros de trigo y centeno pero estaban ausentes en la constricción. La marca asociada con heterocromatina H3K27me3 (trimetilación de la histona H3 en la lisina 27) apareció en las regiones distales de los cromosomas de trigo y de centeno, estando ausente en el centrómero y también en la constricción del 5RL. Es posible que este patrón revele un estado “abierto” de la cromatina que pudiera ser compatible con la transcripción de

secuencias localizadas en el centrómero y en la constricción, y que estos ARN puedan participar en la estructura y función de ambas regiones.

4. **Las características de la constricción pueden determinar su actividad neocentromérica.**

Los centrómeros de la mayoría de las especies aparecen en regiones de heterocromatina, con secuencias repetidas que se organizan formando una constricción (Henikoff y col. 2000). Estas propiedades, también presentes en el neocentrómero intersticial del cromosoma 5R, parecen ser permisivas con una actividad cinética. Además, la importancia de la heterocromatina para la actividad neocentromérica se ha demostrado en levaduras (Ishii y col. 2008), en *Drosophila* (Olszak y col. 2011) y en plantas (Guerra y col. 2010); y para el mantenimiento de la cohesión de cromátidas hermanas en numerosas especies (Bernard y col. 2001; Alonso y col. 2010).

5. **La actividad neocentromérica del 5R se da en las líneas de adición con una frecuencia baja, pero esta frecuencia aumenta mediante el tratamiento de las plantas con un pesticida comercial. El pesticida altera los microtúbulos del huso, posiblemente facilitando la interacción de éstos con la constricción.**

En todas las líneas de adición analizadas se detectó una frecuencia de neocentrómeros basal, generalmente baja (en torno a un 5% de las células). En cambio, en las plantas tratadas con el pesticida, la frecuencia de células con el neocentrómero del cromosoma 5R aumentó significativamente hasta valores de un 45%.

El pesticida causó alteraciones en los microtúbulos. En algunas células, el centro organizador de los microtúbulos apareció dividido en lugar de estar focalizado en el polo celular; en otras células, los haces de microtúbulos aparecían difusos y no bien orientados. Es posible que estas alteraciones faciliten la interacción de los microtúbulos con la constricción del 5RL, y de esta manera aumente la frecuencia de células con el neocentrómero.

6. **El neocentrómero podría contribuir a la orientación del telocromosoma 5RL en metafase I en las líneas de adición.**

El análisis de las frecuencias de orientación del univalente y del bivalente 5RL ha revelado una posible función del neocentrómero. En la línea monotelosómica el univalente

podía orientarse sintélica o anfitélicamente en metafase I. En las plantas tratadas, el 5RL se orientó sintélicamente con una frecuencia significativamente mayor que en las plantas control; es decir, cuando el pesticida indujo la actividad neocentromérica el univalente migró a un polo completo, en lugar de separar cromátidas en anafase I, con una frecuencia mayor. Además, en la línea ditelosómica se encontraron células donde el bivalente 5R no estaba orientado hacia los polos (ni el centrómero ni el neocentrómero de cada cromosoma 5R mostraban tensión hacia los polos). Esta configuración se encontró con una frecuencia significativamente inferior en las plantas tratadas que en las plantas control. Por tanto, es posible que el neocentrómero tenga un papel activo en la orientación del telocromosoma en las líneas de adición.

APORTACIONES FUNDAMENTALES DE LA TESIS

En esta tesis se ha estudiado un neocentrómero en el cromosoma 5R de centeno. Los neocentrómeros son herramientas de gran utilidad para estudiar la función del centrómero, puesto que sus diferencias y semejanzas con los centrómeros endógenos permiten definir los requisitos mínimos para que una región del cromosoma adquiera actividad cinética.

El hecho de que el neocentrómero aparezca en una región de heterocromatina que forma una constricción demuestra la importancia de este tipo de secuencias para la función centromérica y neocentromérica. Si bien se han encontrado neocentrómeros en regiones de eucromatina (especialmente los neocentrómeros humanos), que indican que las secuencias repetidas no son necesarias para esta actividad, la mayoría de los centrómeros descritos hasta la fecha aparecen en regiones heterocromáticas compuestas por secuencias repetidas. Además, la heterocromatina puede jugar un papel importante en el mantenimiento de la cohesión de cromátidas hermanas en anafase I.

Se ha demostrado que el neocentrómero del cromosoma 5R carece de cenH3, así como de otras proteínas del cinetocoro. La histona cenH3 está considerada el determinante epigenético que define el centrómero; sin embargo, el papel exacto que juega en la determinación centromérica no está claro. Una hipótesis es que únicamente indica el lugar en el cromosoma donde se tiene que localizar la actividad centromérica. De ser así, en el caso del cromosoma 5R, la posición del neocentrómero viene definida por la existencia de la

constricción secundaria. Por otro lado, existe al menos un caso bien documentado de actividad centromérica en ausencia de cenH3. Se trata del nematodo *Caenorhabditis elegans*, que posee cromosomas holocéntricos, pero durante meiosis se comportan como monocéntricos. En este caso, la actividad cinética se localiza en el extremo del bivalente opuesto al quiasma y carente de cenH3. Además, en este organismo la mutación de cenH3 causa graves alteraciones en mitosis pero la meiosis transcurre con normalidad. Este ejemplo, junto con el neocentrómero del 5R, podría revelar la existencia de una regulación diferente de la actividad centromérica independiente de cenH3 durante meiosis.

Un resultado importante de esta tesis es la observación del neocentrómero del 5R en centeno. Hasta la fecha, el neocentrómero sólo se había descrito en líneas de adición, en híbridos trigo-centeno y en centeno haploide. El hecho de que se haya encontrado en centeno diploide demuestra el potencial de la región de la constricción para activarse como un neocentrómero; y el estudio en profundidad de este resultado puede ser muy útil para entender la regulación de la activación neocentromérica.

Por último, en este trabajo se ha descubierto y caracterizado un pesticida comercial que actúa como inductor del neocentrómero del cromosoma 5R. Se ha demostrado que éste fue el factor ambiental responsable de las frecuencias variables del neocentrómero descritas previamente por Manzanero y col. (2000b, 2002). Por tanto, este producto puede utilizarse como herramienta para inducir y estudiar el neocentrómero del cromosoma 5R.

Abstract

ABSTRACT

A neocentromere is a chromosomal locus different from the centromere that acquires centromeric activity under specific circumstances. They are of great interest because their similarities and differences with canonical centromeres shed light into the minimum requirements for a chromosomal region to acquire kinetic activity. Chromosome 5R of rye shows a neocentromere in the long arm (5RL) in wheat-5R and wheat-5RL addition lines. It can be active during the first meiotic division interacting with the spindle, leading the chromosome movement to the poles and keeping sister chromatids together at anaphase I. The neocentromere arises at a secondary constriction, which is visible in the above mentioned addition lines and in some varieties of diploid rye. In the present work, both the 5RL constriction and its neocentromeric activity were analyzed. In addition, the influence of an organophosphate pesticide on the neocentromeric frequency was evaluated.

The 5RL constriction is visible in the addition lines during meiosis and with low frequency during mitosis. In rye, the presence of the constriction positively correlated with high consanguinity in the plants. Only *Secale cereale* cv. Imperial showed the 5RL constriction in all the plants analyzed whereas other varieties showed it with variable frequencies and others never. After crossing plants with and without the 5RL constriction, less occurrence of the constriction was observed in the progeny. This frequency increased in the self-pollinated F2. Heteromorphic bivalents were never found. In the interspecific cross between *S. cereale* and *S. ancestrale*, the constriction appeared in the hybrid plants differently depending on which species was the maternal plant. Thus, the formation of the constriction seems to be genetically and epigenetically regulated.

The 5R neocentromere was observed in all the addition lines analyzed. The frequency of cells showing the 5R neocentromere was low (usually less than 5%) but after treating the plants with a commercial pesticide this frequency significantly increased up to 45%. Treated plants showed disturbances in the meiotic spindle, which could facilitate the interaction of microtubules with the 5RL constriction and, in this way, promote the 5R neocentromere. For the first time, the 5R neocentromere was observed in diploid rye.

After immunostaining with typical centromeric proteins and pericentromere-associated histone modifications, cenH3, CENP-C, MIS12, H3S28ph and H2AT133ph were detected in wheat and rye centromeres but they were not detected at the 5RL constriction. Only H3K27me3 was absent simultaneously in the centromere and in the constriction. The lack of cenH3 in the neocentromere suggests a cenH3 independent regulation of the neocentromeric activity during meiosis.

Introduction

INTRODUCTION

1. The centromere

The centromere is the chromosomal locus where spindle microtubules attach to distribute sister chromatids or homologous chromosomes to the cell poles. Cytologically, centromeres are visualized as the primary constriction in condensed metaphase chromosomes (except for holokinetic chromosomes, in which the centromere is distributed nearly all along the chromosome and does not form a primary constriction [Guerra et al. 2010; Heckmann & Houben 2013]). In the present work, the term centromere will be used to designate the chromosomal region composed of centromeric DNA and constitutively associated proteins; and the term kinetochore to define the multiprotein complex that assembles transiently on the centromere during cell divisions.

The function of the centromere implies three key processes: i) interaction with the spindle and chromosome movement, ii) checkpoint for the transition metaphase-anaphase and iii) sister-chromatid cohesion, maintained at the centromere until anaphase (mitosis) or until anaphase II (meiosis) (reviewed in Allshire 1997).

Centromeric function is conserved amongst eukaryotes and so are centromere-associated proteins, but a remarkable variability exists for centromeric DNA among different species and even between chromosomes from the same organism. This disparity was called the ‘centromere paradox’ (Henikoff et al. 2001). Nowadays it is accepted that the centromere is not determined by the underlying DNA. Instead, the centromere is specified by epigenetic mechanisms. A key element for this identity is the histone variant cenH3, which replaces H3 in centromeric nucleosomes (De Rop et al. 2012).

1.1. Centromeric DNA

The simplest centromere reported corresponds to *Saccharomyces cerevisiae.* It is composed of 125 bp and constitutes the only case where the centromere is intrinsically specified by the DNA sequence (Clarke & Carbon 1980). The absence of repetitive DNA sequences within these centromeres led to the designation 'point centromeres', also present in other species related phylogenetically (Meraldi et al. 2006).

In contrast, regional centromeres are complex structures mainly composed of highly repetitive DNA sequences (Csink & Henikoff 1998). In *Schizosaccharomyces pombe* repetitive sequences cover 40 to 100 kb within the centromere (Clarke et al. 1993), in *Drosophila* the functional centromeric region was confined to 420 kb (Murphy & Karpen 1995), and human centromeres comprise megabases of ~171 bp tandemly repeated alpha-satellite arrays (Murphy & Karpen 1998).

Plant centromeres are typically composed of both satellite and retrotransposon-derived sequences which span up to several megabases (Jiang et al. 2003). Centromere-associated satellites are highly divergent and species-specific; contrary, centromere-associated retrotransposons are relatively conserved among species. Particularly in grasses, a family of long terminal repeats (LTR) retrotransposons from the CRM clade (Ty3/gypsy), called Centromeric Retrotransposons (CR) is present in a variety of species (Houben & Schubert 2003; Neumann et al. 2011). The centromere-specific retrotransposon Bilby from the Ty1/copia family was described in rye (Francki 2001). Centromere-specific satellites have been described in cereals such as sorghum (Miller et al. 1998), rice (Dong et al. 1998), maize (Ananiev et al. 1998) and barley (Hudakova et al. 2001) but have not been found in wheat or rye centromeres.

1.2. Constitutively- and transiently-associated centromeric proteins

A number of proteins are associated with the centromeric region, both constitutively (‘centromeric’ proteins) or transiently during cell divisions (‘kinetochore proteins’).

One of the first centromeric proteins identified was cenH3 (CENtromeric histone H3). Initially named as CENP-A (CENtromere Protein A, Earnshaw & Rothfield 1985) it was identified in several organisms but reported with different names; in this work it is presented as cenH3 as the proposed consensus name by Talbert et al. (2012). cenH3 substitutes canonical histone H3 in centromeric nucleosomes (Palmer et al. 1987, 1991) and is characterized by a highly variable N-terminal region (Sullivan et al. 1994), which was suggested to co-evolve with centromeric satellites (Henikoff et al. 2001). cenH3 is considered as the epigenetic mark defining centromeres (see section 1.4).

More than 20 proteins constitutively associated with centromeric DNA have been identified in humans (Perpelescu & Fukagawa 2011) and homologous of some of them were identified in plants, such as CENP-C (Dawe et al. 1999), MIS12 (Li & Dawe 2009) and

NDC80 (Du & Dawe 2007) in maize. CENP-C is a conserved constitutive protein associated with centromeric DNA and necessary for correct kinetochore assembly (Fukagawa et al. 1999; Politi et al. 2002). In human, MIS12 and NDC80 are only assembled before cell divisions (Cheeseman & Desai 2008) whereas in plants they are permanently associated with the centromere.

Other transiently associated centromeric proteins include checkpoint effectors and motor-proteins (reviewed in Cheeseman & Desai 2008). An additional set of proteins is involved in maintaining sister-chromatid cohesion at the centromere (Dej & Orr-Weaver 2000), with an important role of Shugoshin protecting centromeric cohesion during the first meiotic division (Clift & Marston 2011).

1.3. Centromeric and pericentromeric chromatin: structure and function

Chromatin is usually classified into euchromatin (rich in transcriptionally active genes and less compacted throughout the cell cycle) and heterochromatin (more compacted and poor in genes, typically found at centromeres and telomeres). Initially considered as 'junk' or 'inert' DNA, it turned out that heterochromatin is an essential part of the chromosomes as it is important for several biological processes (reviewed in Grewal & Jia 2007).

Centromeric chromatin is flanked by pericentromeric heterochromatin, which is responsible for a variety of centromeric functions such as maintenance of sister-chromatid cohesion (Topp & Dawe 2006; Grewal & Jia 2007).

Histones can be post-translationally modified as a mechanism of genome regulation. Centromeric and pericentromeric regions display a distinct histone modification pattern, which differs from typical eu- and heterochromatin (Sullivan & Karpen 2004) although it varies between plants and non-plant species (Fuchs & Schubert 2012). Thus, human and *Drosophila* centromeres are composed of interspersed cenH3 and H3K4me2 (histone H3 dimethylated at lysine residue 4) nucleosomes and pericentromeric regions are commonly marked by H3K9me2 and H3K9me3 (histone H3 di- and trimethylated, respectively, at lysine residue 9) (Sullivan & Karpen 2004); contrary, maize centromeres are typically marked by H3K9me2 and H3K9me3 (Jin et al. 2008; Shi & Dawe 2006) and strong differences between centromeric and pericentromeric regions are not found (Gent et al. 2012).

Other post-translational histone modifications, particularly phosphorylation, are associated with active centromeres. This is the case for H3S10ph and H3S28ph (histone H3 phosphorylated at serines 10 and 28, respectively) in plants (Houben et al. 1999; Nasuda et al. 2005); as well as H2AT133ph (histone H2A phosphorylated at threonine 133) which was reported in maize as a novel mark for active centromeres (Dong & Han 2012).

Additionally, centromeric and pericentromeric sequences can be transcribed and corresponding non-coding RNAs play a role in the centromere structure and function, as they are involved in several processes such as recruiting proteins and promoting changes in chromatin (reviewed in Hall et al. 2012).

1.4. Epigenetic determination of centromeres

Initial studies transfecting alpha-satellite DNA into human cell cultures to create artificial chromosomes revealed that centromeric proteins were recruited and chromosomes were mitotically stable (Haaf et al. 1992; Larin et al. 1994; Harrington et al. 1997). This led to the conclusion that alpha-satellite DNA was sufficient to confer centromeric function. However, further experiments demonstrated that a primary DNA sequence was not necessary for centromere activity (with the sole exception of species with point centromeres); rather, centromeres are epigenetically specified. Firstly, there is an intriguing sequence variability in the centromeric DNA among species. Secondly, dicentric chromosomes (chromosomes with two centromeres) have been reported where only one of the centromeres is active and the other remains inactive (reviewed in Stimpson et al. 2012). The inactivation occurs by unknown mechanisms and is not influenced by the underlying (centromeric or not) DNA, but only the active centromere is cenH3-positive, assembles a functional kinetochore and is stably transmitted whereas the inactivated centromere loses typical centromere-associated proteins, including cenH3, and the primary constriction is not further visible. Remarkably, the inactivated centromere can be ‘reactivated’ after separation from the active centromere by intrachromosomal recombination (Han et al. 2009). Thirdly, acentric fragments resulting from chromosome breakage can be successfully transmitted via neocentromere formation. In these cases, cenH3 is incorporated in a hitherto non-centromeric region and triggers the recruitment of additional proteins to assemble a functional kinetochore which directs chromosome movement (reviewed in Burrack & Berman 2012).

These results support the non-sequence-dependent epigenetic determination of centromeres with a key role of cenH3, which is present in active centromeres of standard and dicentric chromosomes and in one type of neocentromeres.

2. Neocentromeres

A neocentromere is a chromosomal locus with kinetic activity outside the proper centromere. They are of great interest due to the ability of a non-centromeric locus to bind microtubules and lead the chromosome movement to the poles. Their similarities and differences with canonical centromeres can shed light into the minimal requirements for a chromosomal region to acquire kinetic activity.

Neocentromeres have been described in a variety of species ranging from yeast and insects to mammals and plants. All neocentromeres reported display different features but can be classified into two categories (Table 1): i) neocentromeres that arise in acentric chromosomes to enable its transmission, which have been found in humans and *Drosophila* and named 'de novo' or 'rescue', and ii) 'classic', 'knobs' or 'terminal' neocentromeres which are only active during plant meiosis and appear as a stretching of the terminal heterochromatin to the poles due to interaction with the spindle. In the present work, a third type of neocentromere is described and characterized, displaying features of both types of neocentromeres.

2.1. 'De novo' neocentromeres

2.1.1. Clinical and induced neocentromeres in humans

Neocentromeres in humans are usually isolated from clinical samples after cytogenetic screening, particularly from patients with developmental delay. The first human neocentromere was reported by Voullaire et al. (1993) in the C-band 10q25 of a truncated chromosome 10 that had lost the centromere. This region showed no alpha-satellite DNA, but it formed a primary constriction, it was positive for CREST (acronym for Calcinosis, Raynaud's syndrome, Esophageal dysmotility, Sclerodactyly, Telangiectasia syndrome) antiserum which detects centromeric proteins (Moroi et al. 1980) and most important the acentric fragment was mitotically stable both in the patient and in cultured cells.

Since then, over 100 neocentromeres have been reported in humans (Marshall et al. 2008). They are usually associated with large rearrangements in the chromosome that produce an acentric fragment (marker chromosome) and the neocentromere ensures the transmission of that fragment. Alternatively, they may also occur in intact chromosomes substituting the canonical centromere when it has been inactivated (Marshall et al. 2008; Hasson et al. 2011).

Human neocentromeres can arise in autosomes and sex chromosomes (Marshall et al. 2008; Liehr et al. 2010; Klein et al. 2012) typically in euchromatic regions devoid of alpha-satellite DNA (Hasson et al. 2011) unaffecting the transcription of genes within that region (Marshall et al. 2008). As common features, human neocentromeres form a primary constriction, incorporate cenH3 and bind most centromeric proteins tested (excluding the DNA-associated protein CENP-B) (Saffery et al. 2000). Once established, these neocentromeres are active during mitosis and some cases of transmission during meiosis were reported (Amor & Choo 2002).

Neocentromeres have been induced in human cells. Overexpression of cenH3 results in its misincorporation in ectopic loci and the recruitment of other centromeric proteins such as CENP-C, but it is not sufficient to form a functional kinetochore and neocentromeric activity is not detected (Van Hooser et al. 2001; Gascoigne et al. 2001). On the contrary, it was recently shown that artificial tethering of the cenH3 chaperone HJURP (Holliday Junction-Recognizing Protein [Dunleavy et al. 2009; Foltz et al. 2009]) was sufficient to recruit kinetochore proteins and form a ‘de novo’ centromere (Barnhart et al. 2011).

2.1.2. Induced neocentromeres in *Drosophila*

Centromeric activity in non-centromeric regions has been induced in *Drosophila* by three methods.

First, irradiation mutagenesis produced acentric chromosome fragments that were stably transmitted in both mitosis and meiosis via neocentromere formation (Williams et al. 1998; Maggert & Karpen 2001). The proximity to the endogenous centromere before breakage seemed to be a requirement for the neocentromere activation (Maggert & Karpen 2001), thus the authors proposed a mechanism of spreading of centromeric markers to adjacent regions. However, the capacity of forming neocentromeres in *Drosophila* is not

limited to the pericentromeric regions, as a distal heterochromatic block detached from the chromosome showed similarly neocentromeric activity (Platero et al. 1999).

Second, overexpression of cenH3 caused its stable misincorporation in non-centromeric regions and, opposite to the results obtained in human cells (Van Hooser et al. 2001; Gascoigne et al. 2011), a functional kinetochore was established (Heun et al. 2006). Further analyses revealed that these neocentromeres were more frequent at the boundaries of eu- and heterochromatin (Olszak et al. 2011).

The third method was tethering cenH3 ectopically with a LacI/LacO system (Mendiburo et al. 2011). This ectopic cenH3 was sufficient to recruit additional cenH3 molecules and to assemble a functional kinetochore, which in turn provided transmission stability even after elimination of the initially targeted cenH3.

2.1.3. Induced neocentromeres in yeast

Neocentromeres occurred in *S. pombe* after depletion of the endogenous centromere (Ishii et al. 2008). These neocentromeres occurred within subtelomeric regions but not at internal loci, particularly at the boundaries of eu- and heterochromatin similarly to the induced neocentromeres in *Drosophila* by cenH3 overexpression (Olszak et al. 2011). Furthermore, mutations of heterochromatin-defining proteins reduced significantly the formation of neocentromeres (Ishii et al. 2008), indicating that heterochromatin plays a crucial role for the neocentromeric activity.

In the pathogen yeast *Candida albicans* neocentromeres were induced by replacing the endogenous centromere with a selectable marker (Ketel et al. 2009). Similarly to humans, neocentromeres could appear at any chromosomal location; however, these regions showed low density of genes and repetitive sequences were present. Interestingly, the neocentromeric loci were not fixed and, under selective conditions, they could shift their position up to several kilobases.

Recently, ‘centromere-like regions’ (CLR) have been found in *S. cerevisiae* based on cenH3 overexpression (Lefrançois et al. 2013). This cenH3-containing ectopic foci recruited centromeric proteins, formed functional kinetochores and enhanced the transmission of chromosome and even plasmids. These centromere-like structures typically occurred in intergenic regions close to the endogenous centromere.

2.1.4. ‘De novo’ neocentromeres in plants

Two examples of ‘de novo’ formed neocentromeres are documented in plants.

The first one was described in a barley chromosome added to wheat after induced chromosome breakage (Nasuda et al. 2005). A region devoid of barley and wheat centromeric sequences formed a functional neocentromere, as revealed by presence of cenH3, CENP-C, CBF5 and SKP1 (barley homologous of yeast kinetochore proteins [ten Hoopen et al. 2000]), H3S10ph and H3S28ph. This neocentromere appeared close to the endogenous centromere; thus, the hypothesis of an extension of centromeric markers, proposed for *Drosophila* neocentromeres (Maggert & Karpen 2001), could explain these results.

The second one was found in a maize acentric chromosome added to oat (Topp et al. 2009). Similarly, this neocentromere lacked centromeric sequences but showed association with cenH3 and was stably transmitted through mitosis and meiosis. This neocentromere in different lines contained variable amounts of cenH3 and less amount of cenH3 correlated with lower transmission ratios, suggesting that a period to accumulate cenH3 might be necessary for the stabilization of this neocentromere.

2.2. Terminal neocentromeres

Terminal plant neocentromeres appear as extensions of the telomeric regions to the poles in intact chromosomes. They are only active during meiosis and have been documented in several plant species including a moss (Dawe & Hiatt 2004). The best characterized are the terminal neocentromeres of maize and rye.

2.2.1. Terminal neocentromeres in maize

In maize, a mutation in chromosome 10 (Ab10) promotes that large subterminal heterochromatic domains (knobs) acquire neocentromeric activity and are strongly pulled polewards (Rhoades & Vilkomerson 1942). Neocentromeres can arise in all maize chromosomes. A 180 bp tandemly repeated sequence is present in all knobs whereas the 350 bp (TR-1) repeat is present in some of them (Peacock et al. 1981; Ananiev et al. 1998; González-Sánchez et al. 2007). Neocentromeric activity is independently regulated by (at least) two *trans*-acting genes (Hiatt et al. 2002; Mroczek et al. 2006). cenH3, CENP-C and

the checkpoint protein MAD2 (Yu et al. 1999) are not found at knobs (Dawe & Hiatt 2004). This terminal neocentromeres interact with spindle microtubules in a lateral way instead of the typical end-on interaction of microtubules with canonical centromeres (Yu et al. 1997). Neocentromeres movement was proposed to be mediated by microtubule-based motors (Hiatt et al. 2002).

Maize neocentromeres have been related to a process of meiotic drive. This means that knobs and associated genes are accumulated preferentially in the single functional megaspore of the tetrad (Rhoades 1942) and this is at least partially controlled by the locus *smd1* (suppressor of meiotic drive) (Dawe & Cande 1996; Mroczek et al. 2006).

2.2.2. Terminal neocentromeres in rye

Terminal neocentromeres appear in rye at both meiotic divisions in inbred lines (Katterman 1939; Prakken & Müntzing 1942; Rees 1955), in cross pollinated varieties (Kavander & Viinikka 1987; Manzanero & Puertas 2003) and in interspecific hybrids (Jones 1969). In these situations, chromosome arms are directed to either the same or the opposite pole of the centromere. All chromosomes may display neocentromeres but they are more frequent in chromosomes with large terminal heterochromatic blocks (Manzanero & Puertas 2003).

Truncation analyses showed that neocentromere activation requires a *cis*-acting centromere, because terminal heterochromatin of truncated acentric chromosomes does not form neocentromeres (Puertas et al. 2005). Two tandemly repeated sequences are found within the stretched regions of neocentromeres (Manzanero & Puertas 2003): pSc34 and pSc74 (Bedbrook et al. 1980). No centromeric proteins were described at these regions. Neocentromeres can bind microtubules in an end-on fashion, similarly to canonical centromeres, although the bundle of microtubules attached to the neocentromeres is always thinner (Puertas et al. 2005).

Hayward (1962) reported that these neocentromeres could have a polygenic control. Segregation analyses performed by Puertas et al. (2005) suggested that this activity is controlled by two *trans*-acting genes.

2.3. Interstitial neocentromere in chromosome 5R of rye

A third type of neocentromere arises in an interstitial constriction in the long arm of chromosome 5R (5RL) of rye. This secondary constriction is visible in haploid rye (Schlegel 1987), inbred lines of diploid rye (Lamm 1936; Müntzing & Akdik 1948), wheat-rye and wheat-Triticale hybrids (Schlegel 1987) and addition lines involving chromosome 5R (Schlegel 1987; Manzanero et al. 2000b, 2002; Cuacos et al. 2011).

The 5R neocentromere was described initially by Schlegel (1987) in meiosis of haploid rye, wheat-rye hybrids and in a wheat-5R monosomic addition line. Manzanero et al. (2000b, 2002) characterized it in meiosis of wheat-5RL monotelo- and ditelosomic addition lines demonstrating that the haploid condition was not inducing the neocentromere. The constriction appeared remarkably stretched due to the orientation centromere-neocentromere to opposite poles and sister chromatids were kept together at anaphase I in this region or between the centromere and the constriction. Neither the neocentromere nor the constriction were found at mitosis (Schlegel 1987; Manzanero et al. 2000b, 2002).

Rye centromeric (Bilby [Francki 2001], CCS1 [Cereal Centromeric Sequence 1, Aragón-Alcaide et al. 1996]), telomeric (pAtT4 from *Arabidopsis thaliana* [Richards & Ausubel 1988]) and the subtelomeric sequence pSc200 (Vershinin et al. 1995), as well as the 180 bp repetitive sequence from maize terminal neocentromeres are not detected at the 5RL constriction by fluorescence *in situ* hybridization (Manzanero et al. 2002). Only the repetitive sequence pSc119.2 (Bedbrook et al. 1980; McIntyre et al. 1990) is localized at the constriction, which corresponds to the C-band 5RL1-3 (Mukai et al. 1992; Cuadrado et al. 1995). Proteins are accumulated at the constriction from metaphase I to anaphase II as revealed by silver staining, but these proteins were not identified (Manzanero et al. 2002). Immunostaining with an anti-α-tubulin antibody revealed that a thin bundle of microtubules was bound to the constriction in an end-on fashion, even in the absence of neocentromeric activity (Manzanero et al. 2002).

This interstitial neocentromere arose with variable frequencies in different studies. In haploid rye the frequency of stretched 5R chromosomes was up to 70% (Schlegel 1987). In the addition lines, Manzanero et al. (2000b, 2002) detected around 25% of cells with the 5R neocentromere in one year, but the frequency was highly reduced in the following years. Thus, the authors suggested that an environmental factor may promote the 5R neocentromeric activity.

	Origin	Substitutes centromeric function	Genomic location	Visible Constriction	Presence of cenH3	Centromeric proteins and histone modifications	Active in mitosis / meiosis	Maintains sister chromatid-cohesion	References
Humans	Chromosome breakage/ Centromere inactivation	Yes	Mostly euchromatin. Few cases reported in heterochromatin	Yes	Yes	All tested centromeric proteins besides CENP-B	Yes / Yes	Yes	(Marshall et al. 2008)
Drosophila	Chromosome breakage	Yes	Pericentromeric heterochromatin	NR	Yes	ZW10	Yes / Yes	NR	(Williams et al. 1998; Maggert & Karpen 2001)
S. pombe	Centromere depletion	Yes	Subtelomeric heterochromatin	NR	Yes	CENP-C, MIS12, HP1, H3K9me	Yes / Yes	NR	(Ishii et al. 2008)
C. albicans	Centromere substitution	Yes	Different locations; preferentially low-gene density and repetitive-sequences-containing regions	NR	Yes	NR	Yes / NR	NR	(Ketel et al. 2009)
Barley	Chromosome breakage	Yes	Pericentromeric heterochromatin	No	Yes	CENP-C, CBF5, SKP1, H3S10ph, H3S28ph	Yes / Yes	NR	(Nasuda et al. 2005)
Maize	Chromosome breakage	Yes	Unlinked to the centromeric region	No	Yes	NR	Yes / Yes	NR	(Topp et al. 2009)
Maize (terminal)	Genetic control. Presence of Ab10	No	Subtelomeric heterochromatin	No	No	No (tested CENP-C, Mad2)	No / Yes	No	(Guerra et al. 2010)
Rye (terminal)	Genetic control. Influenced by presence of terminal heterochromatin	No	Subtelomeric heterochromatin	No	No	No	No / Yes	No	(Guerra et al. 2010)

Table 1. Summary of described neocentromeres. They can be grouped into two categories: 'de novo' (do substitute the centromere) and terminal (do not substitute the centromere)

AIMS OF THE STUDY

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Neocentromeres are excellent tools to analyze the centromeric function when it appears at ectopic loci. Chromosome 5R of rye can show a neocentromere at meiosis in wheat-5R and wheat-5RL addition lines in a secondary constriction in the long arm (5RL). Proteins are accumulated in this region but their nature is unknown. The occurrence of the neocentromere within a constriction is reminiscent of the primary constriction found at canonical centromeres. The properties of this region seem to be decisive for the neocentromeric activity. This neocentromere was found with variable frequencies in different years (Manzanero et al. 2000b, 2002), suggesting the influence of environmental factors in its activation.

In the present work, both the secondary (5RL) constriction and its neocentromeric activity will be analyzed in an attempt to elucidate the causes of this activity. For this purpose, the following specific objectives are proposed:

1. Analyze the occurrence of the 5RL constriction in different species and cultivars of diploid rye.

2. Test the potential genetic control of the 5RL constriction by crossing different rye species and cultivars with and without constriction.

3. Characterize the neocentromeric activity of the 5RL constriction.

4. Evaluate the effect of a commercial pesticide in the neocentromeric activation.

5. Determine the protein composition and the chromatin environment at the neocentromere.

Materials and Methods

MATERIALS AND METHODS

PLANT MATERIAL

Several diploid rye (2n = 2x = 14) cultivars (cv.) and species were analyzed (Table 2).

Table 2. Rye species and cultivars analyzed.

Material	Origin
***Secale cereale* cv. Imperial**	University of California Riverside
Secale cereale* cv. IPK	IPK Gatersleben (Genebank reference number R1150)
***Secale cereale* cv. I89**	'Imperial' rye cultivated in our lab since 1989
Secale cereale* cv. Paldang	University of Seoul, cultivated in our lab since 1990
Secale cereale* cv. Khorasan	Iran
***Secale cereale* cv. Merced**	USA
***Secale cereale* cv. Transbaikal**	Siberia
Secale ancestrale	IPK Gatersleben (Genebank reference number R62)

*This varieties can carry B chromosomes.

Wheat-5RL monotelosomic and ditelosomic addition lines, and wheat-5R monosomic and disomic addition lines were used. Plants of these genotypes were selected in the offspring of stocks produced by E. R. Sears (Driscoll & Sears 1971). Monotelo- and ditelosomic lines (seeds kindly provided by M. Feldman) involve the addition of one or two copies, respectively, of the long arm of chromosome 5R (5RL) of *Secale cereale* cv. Imperial to *Triticum aestivum* (2n = 6x = 42) cv. Chinese Spring. Mono- and disomic lines (seeds kindly provided by T. Naranjo and obtained from A. Lukaszewski) carry one or two copies, respectively, of the entire chromosome 5R.

METHODS

1. Plant culture conditions

Seeds were germinated around November on wet filter paper, some days later transferred to soil and cultivated in a greenhouse without controlled conditions (greenhouse space kindly provided by E. Benavente). Wheat was maintained in the greenhouse until collection while rye was shifted outside to a shady area around March for better growth. Spikes from the appropriated stages (morphological criterion) were collected during springtime.

For immunostaining fresh material was used; otherwise material was fixed in 3:1 (v/v) ethanol:acetic acid in vacuum at 400 mmHg for 10 minutes. Fixative was exchanged in the following days two-three times until chlorophyll was removed and the solution remained clear. For short-term storage material was maintained at 4°C and for long-term storage fixative was replaced by 70% ethanol and stored at -20°C.

2. Pesticide treatment

Plants were treated adding per pot 75 mg of the commercial granulated pesticide Diazinon (COMPO) according to manufacturer's instructions (30 g/m^2) and watered afterwards. When possible, spikes at meiosis from the same plant were collected before treatment as control material.

Two types of treatments were applied:

- Consecutive treatment. Plants were treated one to six consecutive times and spikes were collected afterwards.
- Different collecting times. Spikes were collected one to ten days after the last treatment.

3. Cytogenetic methods

3.1. Material selection

ANTHERS

Meiotic studies were performed with male meiocytes from anthers.

Rye and wheat flowers enclose three synchronic anthers. The meiotic stage was determined by anther squash in 1% acetocarmine under the light microscope. Anthers containing meiocytes at the appropriated stage were collected in ice-cold water for immunostaining or in 3:1 (v/v) ethanol:acetic acid for FISH and stored at 4°C.

ROOTS

To obtain root tips for mitotic analyses, seeds were germinated in Petri dishes on wet filter papers in darkness. 1-2 cm long roots were isolated, collected in distilled water (dH_2O), kept on ice for 48 h to accumulate metaphases, fixed in 3:1 (v/v) ethanol:acetic acid and stored at 4°C.

3.2. Fluorescence *in situ* hybridization (FISH)

3.2.1. Slide preparation: Spreading and Squash

Meiotic and mitotic chromosomes were prepared by Spreading or Squash, respectively.

Prior to slide preparation, anthers and roots were digested with an enzyme mixture to degrade the cell walls allowing a better penetration of probes and antibodies. Fixed material was washed three times with dH_2O and three times with 10 mM citric acid-sodium buffer (citrate buffer, pH 4.6) for 5 minutes each. Maceration was done in 0.1% (w/v) each of cytohelicase, pectolyase and cellulase (Sigma) in 10 mM citrate buffer (pH 4.6) at 37°C for one to two hours depending on the material. Enzymatic reaction was stopped by washing the material twice with citrate buffer (pH 4.6) and twice with dH_2O for 5 minutes each. Macerated anthers and roots were kept on ice while preparing the slides.

SPREADING

Meiotic chromosomes from macerated anthers were prepared by Spreading according to Zhong et al. (1996). This procedure was chosen because it involves no mechanical pressure to distribute the cells on the slides and therefore the three-dimensional information is preserved.

Anthers were placed individually on an ethanol-cleaned slide and covered by 7 µl of 60% acetic acid, softly squashed and covered by 7 µl more of 60% acetic acid. After 2 minutes, 7 µl more of 60% acetic acid were added, the mix was homogenized and the slide was placed on a 40°C hot plate. During 2 minutes, the suspension was moved with a needle parallel to the slide by surface tension. The treatment with hot acetic acid removes the cell cytoplasm and promotes the cell deposition on the slides. Then, 200 µl of ice-cold 3:1 (v/v) ethanol:acetic acid were distributed around the suspension three times, slides were transferred to 60% acetic acid for 10 minutes, washed four-five times in 100% ethanol, air dried and used directly or stored at 4°C for several months.

SQUASH

Macerated root tips were placed on an ethanol-cleaned slide with a drop of 45% acetic acid and covered with a cover slip. A lancet was first used to tap gently and break the tissue. Then, protecting the preparation with filter paper, strong pressure was applied with the thumb without moving the cover slip. Slides were frozen in liquid nitrogen and, after removing the cover slip with a razorblade, they were collected and kept in 100% ethanol for 10 minutes, air dried and used directly or stored at 4°C for several months.

3.2.2. Hybridization

FISH was carried out according to Manzanero et al. (2002) with minor modifications.

Slides were washed twice in 2xSSC (Saline-Sodium Citrate: 0.3 M sodium chloride, 0.03 mM trisodium citrate, pH 7.0) for 5 minutes, treated with 0.1% pepsin (Sigma) in HCl (1 N) at 37°C for six minutes, washed twice in 1xPBS (phosphate buffered saline pH 7.4) and once in 2xSSC, 5 minutes each. Post-fixation was performed to reduce the loss of material in 4% (w/v) paraformaldehyde (Merck) in 1xSSC (pH 8.0) for 10 minutes. Then, slides were washed three times for 5 minutes in 2xSSC, dehydrated in an ethanol series (70%, 90% and 100%, three minutes each) and air dried for one hour.

20 µl per slide of hybridization mixture containing 4 µl 50% dextran sulfate, 10 µl 100% deionized formamide, 2 µl 20xSSC and 4 µl digoxigenin-, biotin- or DNP-labelled probes (final probe concentration 2 ng/µl) were denatured in boiling water for 10 minutes, kept on ice for at least 7 minutes, added to each slide and covered with a cover slip. Chromosomal DNA was denatured by placing the slides on a hot plate at 68°C for 2 minutes. Hybridization reaction was kept at 37°C over night in a moisture chamber.

Next day, all washing steps were performed with gentle shaking. First, slides were washed three times in 2xSSC for 5, 10 and 15 minutes each. In the first wash cover slips were removed. Subsequently, slides were washed once in 1xSSC for 5 minutes at room temperature (RT), once in 1xSSC for 30 minutes at 37°C and twice in 2xSSC at RT. To reduce unspecific binding, a blocking step was included using 4B (0.5% (p/v) powder skimmed milk in 4xSSC) for 35 minutes at 37°C.

Probes were detected by the following fluorochrome-conjugated antibodies: Fluorescein conjugated anti-DIG antibody (Roche) (10 ng/µl) or Cy5 conjugated anti-DIG antibody (Jackson ImmunoResearch) (8.5 ng/µl) to detect digoxigenin-11-dUTP; Cy3 conjugated Streptavidin (GE Healthcare) (15 ng/µl) to detect biotin-16-dUTP; Fluorescein conjugated anti-DNP antibody (Life Technologies) (10 ng/µl) to detect DNP-11-dUTP. 40 µl of hybridization mixture diluted in 4B solution were applied per slide, covered with parafilm and incubated one hour at 37°C in a moisture chamber. To remove the excess of antibody, slides were washed in darkness three times for 5 minutes with detection buffer (0.2% (v/v) Tween 20 in 4xSSC), twice at 37°C and once at RT. Finally, slides were washed briefly in dH_2O, counterstained with a 4',6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories) solution and stored in darkness at 4°C.

3.2.3. Probes used: isolation and labelling

The following sequences were used as probes:

pSc119.2 (McIntyre et al. 1990). Cloned in pUC18, the sequence length is 611 bp with a 118 bp monomer length. It is a subclone from pSc119 (Bedbrook et al. 1980). This probe labels subtelomeric and interstitial regions in wheat and rye.

pSc200 (Vershinin et al. 1995). Cloned in pUC18, the sequence length is 521 bp with a 379 bp monomer length. This probe labels subtelomeric regions in rye. Probe kindly provided by A. Cuadrado.

Bilby (Francki 2001). Cloned in pAWRC.1, this sequence is part of a Ty1-copia retrotransposon with a size of 2250 bp. This probe is specific for rye centromeres. Probe kindly provided by P. Langridge.

UCM600 (González-García et al. 2011). Isolated in our laboratory and cloned in pCRII-TOPO (Invitrogen), this sequence contains a 592 bp fragment from the rye-specific dispersed repetitive family R173 (Rogowsky et al. 1992). This probe is dispersed throughout the rye chromosomes excluding telomeric and pericentromeric regions.

Plasmids were maintained in bacterial permanent cultures (1:1 (v/v) 50% glycerol:Ampicilin-containing Luria-Bertani (LB) culture medium mixture) at -80°C. Plasmid DNA was obtained with a 'High Pure Plasmid Isolation Kit' (Roche) according to manufacturer's instructions. Concentration was evaluated with a NanoDrop ND-1000 (NanoDrop Technologies, Thermo Fisher Scientific). DNA was labelled by nick translation with the following dUTP analogues and ratios:

0.25 : 2.75	dTTP : biotin-16-dUTP (Roche)
2 : 1	dTTP : digoxigenin-11-dUTP (Roche)
1 : 1	dTTP : DNP-11-dUTP (Perkin Elmer)

UCM600 and pSc200 were biotin-, Bilby was digoxigenin- and pSc119.2 was digoxigenin- and DNP-labelled. Nick Translation was carried out in 20 µl reaction volume including 0.5 to 1 µg of plasmid DNA, 0.1 mM dNTPs and 4 µl of Nick Translation Mix (Roche) containing DNase, DNA-polymerase I and nick translation buffer for two hours at 15°C. Reaction was stopped by heating up to 65°C for 10 minutes.

3.3. Immunostaining

3.3.1. Slide preparation

Immunostaining was carried out according to Manzanero et al. (2000a) with minor modifications.

Anthers or roots were fixed for 20 minutes in ice-cold freshly prepared 4% (w/v) paraformaldehyde in 1xPBS or 1xMTSB (Microtubule-Stabilizing Buffer: 50 mM PIPES, 5 mM $MgSO_4$, 5 mM EGTA, pH 6.9). Mild vacuum for about 1 minute was applied three to four

times during fixation to improve fixative penetration. MTSB was used to detect tubulin only or simultaneously with other proteins, as this buffer protects microtubules from degradation. The buffer chosen for fixation was later used for the complete procedure. After fixation, material was washed three times for 15 minutes in 1xPBS/1xMTSB on ice, digested in a mixture of 2.5% (w/v) each of pectinase, cellulase and pectolyase (Sigma) in 1xPBS/1xMTSB at 37°C for 20 minutes and washed two times for 15 minutes in 1xPBS/1xMTSB on ice.

For slide preparation, anthers were homogenized with a needle on Polysine slides (Thermo Scientific) in 7 µl of 1xPBS/1xMTSB containing 1% Triton X-100. After covering with a cover slip, the tissue was broken by softly tapping with a lancet and, protecting with a filter paper, squashed by pressing with the thumb. Then, slides were frozen in liquid nitrogen, the cover slips were removed using a razorblade and slides were collected in 1xPBS/1xMTSB for direct use.

3.3.2. Immunolocalization

Blocking was performed adding 30 µl blocking solution (4% BSA [bovine serum albumin] and 0.1% Tween 20 in 1xPBS/1xMTSB) per slide, covering with a parafilm and incubating one hour at 37°C in a moisture chamber. Then, 30 µl of the primary antibody diluted in Antibody Buffer (1% BSA and 0.1% Tween 20 in 1xPBS/1xMTSB) were added per slide, covered with parafilm and incubated over night at 4°C in a moisture chamber.

Next, parafilm was carefully removed and slides were washed three times for 5 minutes in 1xPBS/1xMTSB. Next, 30 µl of the secondary antibody diluted in Antibody Buffer were applied per slide, covered with parafilm and incubated one hour at 37°C in a moisture chamber. After washing three times for 5 minutes in 1xPBS/1xMTSB, DNA was counterstained with DAPI in Vectashield (Vector Laboratories) and slides were stored in darkness at 4°C.

3.3.3. Antibodies

Primary antibodies and working dilutions are indicated in Table 3. The following secondary antibodies and dilutions were used:

- Alexa 488-conjugated goat **anti-mouse** IgG (1:400) (Invitrogen).
- Cy3-conjugated goat **anti-rabbit** IgG (1:200) (Jackson ImmunoResearch).

Table 3. Primary antibodies.

Antibody	From	Detecting	Dilution	Company / Reference
anti α-tubulin	Mouse	α-tubulin	1:300	Clone DM 1A, Sigma
anti cenH3	Rabbit	Rabbit cenH3	1:200	Nagaki et al. (2004)[1]
anti CENP-C	Rabbit	Maize CENP-C	1:100	Dawe et al. (1999)[2]
anti MIS12	Rabbit	Maize MIS12	1:100	Li & Dawe (2009)[2]
anti NDC80	Rabbit	Maize NDC80	1:100	Du & Dawe (2007)[2]
anti Shugoshin	Rabbit	Rice Shugoshin	1.100	Wang et al. (2011)[3]
anti H3K4me2	Rabbit	Histone H3 dimethylated (Lys 4)	1:100	Millipore[1]
anti H3K9me1	Rabbit	Histone H3 monomethylated (Lys 9)	1:100	Millipore[1]
anti H3K9me2	Rabbit	Histone H3 dimethylated (Lys 9)	1:100	Millipore[1]
anti H3K27me1	Rabbit	Histone H3 monomethylated (Lys 27)	1:100	Millipore[1]
anti H3K27me3	Rabbit	Histone H3 trimethylated (Lys 27)	1:100	Millipore[1]
anti H3S10ph	Rabbit	Histone H3 phosphorylated (Ser 10)	1:100	Millipore[1]
anti H3S28ph	Rabbit	Histone H3 phosphorylated (Ser 28)	1:100	Millipore[1]
anti H3T3ph	Rabbit	Histone H3 phosphorylated (Thr 3)	1:100	Millipore[1]
anti H3T11ph	Rabbit	Histone H3 phosphorylated (Thr 11)	1:100	Millipore[1]
anti H2AT133ph	Rabbit	Histone H2A phosphorylated (Thr 133)	1:1000	Dong & Han (2012)[4]

[1]Kindly provided by A. Houben.

[2]Kindly provided by K. Dawe.

[3]Kindly provided by Z. Cheng.

[4]Kindly provided by F. Han.

4. Microscopy

Fluorescence images were acquired with an Olympus BX61 fluorescence microscope equipped with a CCD DP7 camera (Olympus Optical Co.) or an ORCA-ER CCD camera (Hamamatsu). To improve image resolution and to reduce out-of-focus signals, 3D-deconvolution was applied. Image stacks of ten optical sections per cell were gathered, and the maximum intensity projections were processed with the program AnalySIS (Soft Imaging System). Images were optimized for best contrast and brightness with Adobe Photoshop CS2.

To achieve an optical resolution of approximately 100 nm Structured Illumination Microscopy (SIM) was applied using a C-Apo 63x/1.2W Korr objective of an Elyra microscope system and the software ZEN (Zeiss).

Results

RESULTS

1. A secondary constriction in the long arm of chromosome 5R (5RL) of rye

1.1. Identification of chromosome 5R by FISH

The chromosome 5R of rye can be microscopically identified in several plant materials due to a secondary constriction in the long arm (5RL constriction). However, in wheat-rye addition lines the high number of chromosomes and in some diploid rye species the absence of the 5RL constriction hinder its identification. Thus, FISH with the probes pSc119.2, pSc200 and UCM600 was used for its detection.

In diploid rye, pSc119.2 and pSc200 allow to distinguish between all rye chromosomes when used simultaneously in FISH. In chromosome 5R, large subterminal clusters of pSc119.2 and pSc200 are found in the short arm, whereas in the long arm pSc119.2 clusters interstitial (coincident with the 5RL constriction when it is visible) and subterminal, and pSc200 clusters subterminal only in some varieties (Figs. 1 and 3a-c). Bilby was included as a specific mark for rye centromeres.

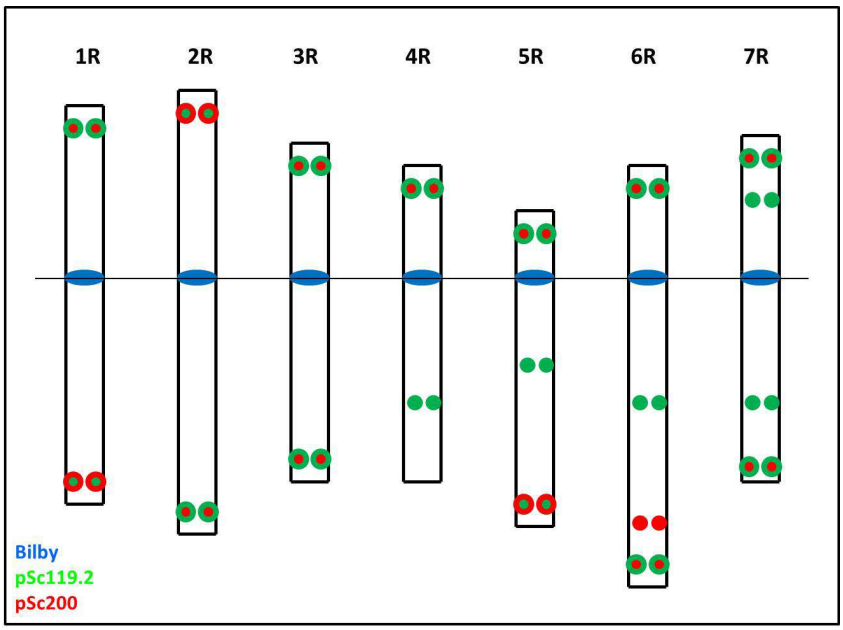

Fig. 1. FISH pattern of Bilby (blue), pSc119.2 (green) and pSc200 (red) in rye chromosomes. pSc200 is absent in the long arm of chromosome 5R in *Secale cereale* cv. Imperial.

In wheat-rye addition lines, UCM600 labels specifically the rye chromosome allowing its identification. In these lines, pSc119.2 and occasionally Bilby were included as FISH probes. The first labels several wheat chromosomes in addition to the rye 5R chromosome; the second is specific for rye centromeres (Figs. 2 and 3d-i). These two probes were commonly labelled in green; when they were used simultaneously, their different location in the chromosome allowed distinguishing them.

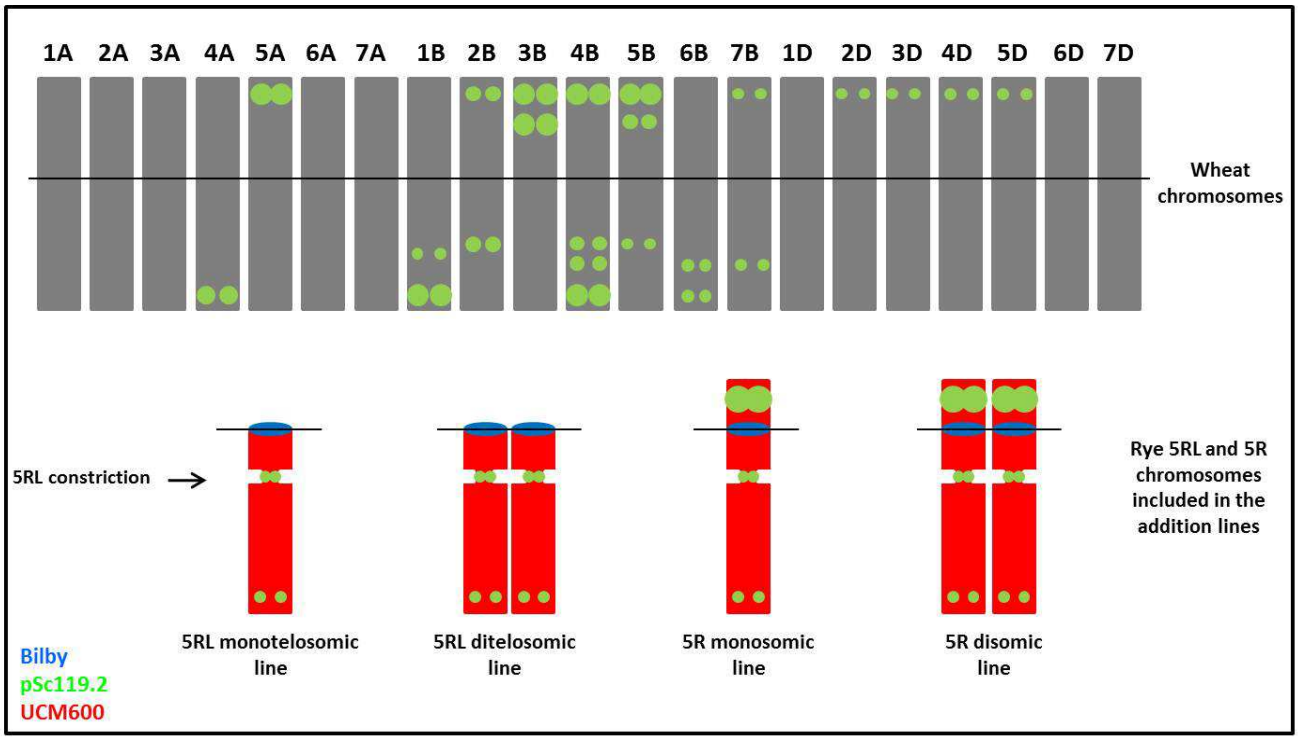

Fig. 2. FISH pattern of Bilby (blue), pSc119.2 (green) and UCM600 (red) in wheat-5R and wheat-5RL addition lines. Labelling of pSc119.2 in wheat chromosomes modified from Schneider et al. (2003). Black lines represent the centromeres. Size and shape of chromosomes do not correspond to reality.

1.2. General features of the 5RL constriction

The 5RL constriction is visible at meiosis, from diakinesis to anaphase I, in all the addition lines analyzed (wheat-5R monosomic and disomic; and wheat-5RL monotelosomic and ditelosomic), as well as in the diploid rye cv. 'Imperial' (Fig. 3a-g) but only rarely at mitosis. Roots from plants of the addition lines were analyzed and, occasionally, the constriction was visible in some cells (Fig. 3h, i) but this occurred with very low frequency.

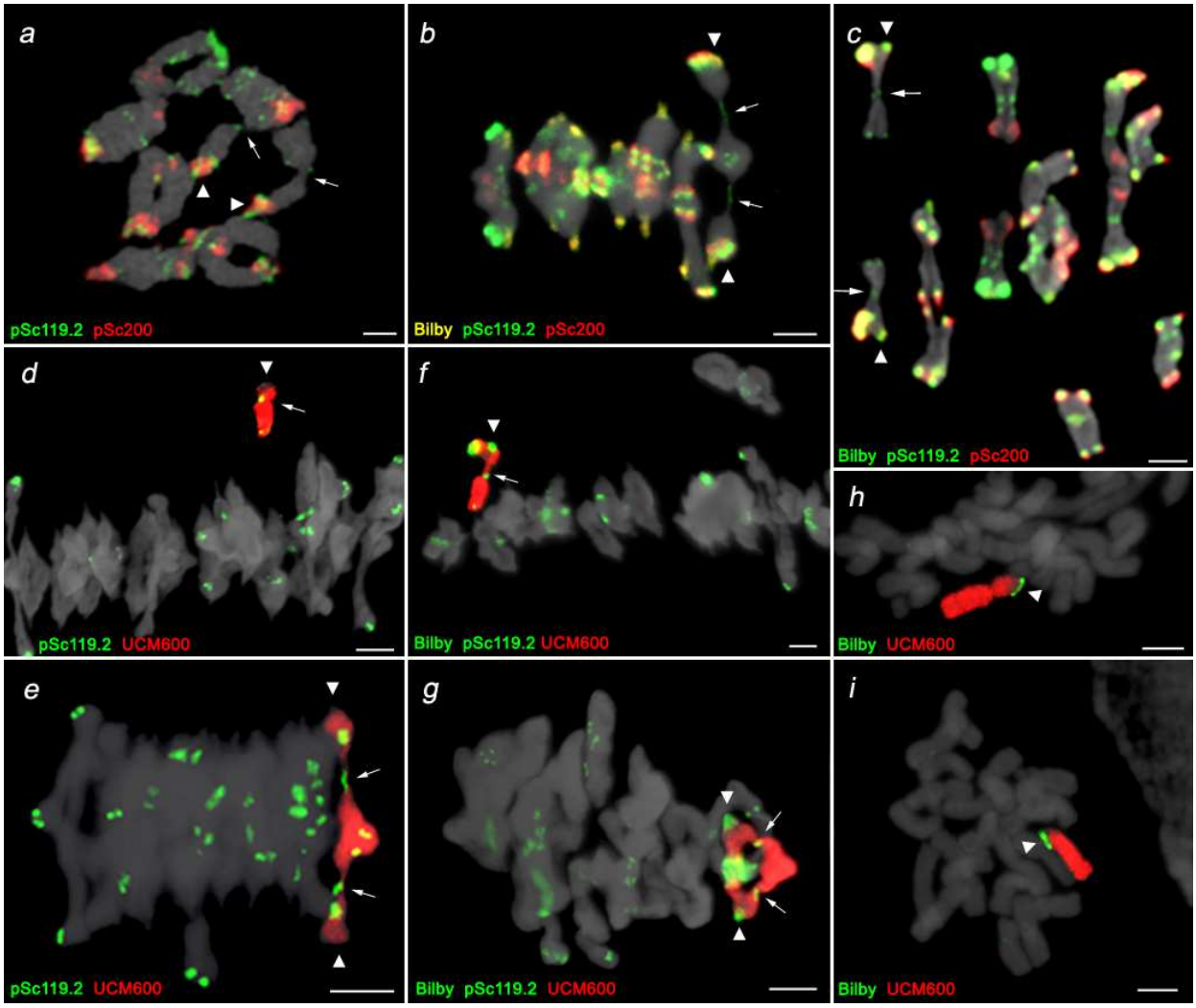

Fig. 3. Different situations where the 5RL constriction is visible after FISH. Probes are indicated in the pictures. Arrowheads point to the 5R centromere, arrows point to the 5RL constriction. Bars = 5 µm.
a-c. Rye meiosis: diakinesis (**a**), metaphase I (**b**) and anaphase I (**c**).
d-i. Meiosis and mitosis in the addition lines: metaphase I in monotelo- (**d**) and ditelosomic (**e**) wheat-5RL addition lines and in mono- (**f**) and disomic (**g**) wheat-5R addition lines; mitotic metaphase of the monotelosomic addition line where the constriction is visible (**h**) or not visible (**i**).

The appearance of the constriction varies strongly between species and cultivars and can even vary between meiocytes within the same anther. In some cells the constriction was subtle, visualized as a narrowing in the chromosome; in other cells the constriction was conspicuous, it appeared as a thin thread of chromatin. However, in all cases the constriction exhibited elastic properties, as the tension to the poles from both sides of the constriction caused the stretching of this region, elongating it up to several times the chromosome length.

2. Analysis of the 5RL constriction in rye

2.1. Occurrence of the 5RL constriction in different rye species and cultivars

The presence of the 5RL constriction in different diploid rye species and cultivars was evaluated (Table 4). The constriction was observed previously in inbred lines, i.e. in lines with high consanguinity, which are characterized by reduced chiasmata (see e.g. Rees 1955). Thus, to test whether the formation of the constriction was related to high consanguinity, the presence of univalents (unpaired chromosomes) and the number of chiasmata (scored as bound arms per cell) at metaphase I were registered. In addition, 'Imperial' plants produced by self-pollination (SP) and cross-pollination (CP) were analyzed.

Table 4. Occurrence of the 5RL constriction in the different rye species and cultivars analyzed. At least 50 cells were analyzed per plant. CP: plants originated by cross-pollination; SP: plants originated by self-pollination.

Cultivar or species	No. of plants analyzed	% of cells per plant showing the 5RL constriction	Mean chiasma frequency	% of cells with univalents
Secale cereale, cv. Merced	2	0, 0	13.65	0
Secale cereale cv. Transbaikal	2	0, 0	13.26	0
Secale ancestrale	3	0, 0, 0	13.21	0
Secale cereale cv. IPK	6	0, 0, 0, 0, 22, 42	13.08	0
Secale cereale cv. Paldang	5	0, 0, 1, 2, 5	12.95	2.4
Secale cereale cv. Khorasan	3	6, 6, 22	12.92	3.3
Secale cereale cv. I89	4	0, 0, 5, 16	12.06	1.6
Secale cereale cv. Imperial (CP)	4	36, 55, 80, 100	11.63	11.3
Secale cereale cv. Imperial (SP)	13	74, 88, 92, 92, 94, 98, 98,100, 100, 100, 100, 100, 100	10.87	24.3

S. cereale* cv. Merced and Transbaikal and *S. ancestrale never showed the 5RL constriction (*S. ancestrale* is shown in Fig. 4a). They displayed the highest chiasma frequency and univalents were never found.

In *S. cereale* cv. IPK the 5RL constriction appeared in 42% of meiocytes in one plant (Fig. 4b), in 22% in one descendant of this plant, and in 0% of meiocytes in four additional plants. Mean chiasma frequency was over 13 and univalents did not occur.

***S. cereale* cv. Paldang** showed the 5RL constriction in a low percentage of cells (<5%) in some plants only (one cell without the 5RL constriction is shown in Fig. 4c). Mean chiasma frequency was nearly 13 and univalents were rarely found.

***S. cereale* cv. Khorasan** in two plants 6% and in another plant 22% of cells showed the 5RL constriction. Similarly to 'Paldang', mean chiasma frequency was close to 13 and only few cells showed univalents.

In *S. cereale* cv. I89 the 5RL constriction occurred in 5% of meiocytes in one plant and in 16.67% in another plant (Fig. 4d). In two plants it was not found. Interestingly, one of them was a sister anther from the 16.67%-plant. Mean chiasma frequency was around 12 and univalents were found at low frequency.

***S. cereale* cv. Imperial** was the only rye cultivar which showed the 5RL constriction in all plants analyzed. Mean chiasma frequency was the lowest of all rye species and cultivars studied, and univalents were frequently observed. However, these plants showed differences depending on their origin: in plants produced by cross-pollination the 5RL constriction was less frequent than in plants produced by self-pollination. Moreover, the stretching of the constriction was more conspicuous in self-pollinated plants (Fig. 4f) than in cross-pollinated plants (Fig. 4e).

Based on the differences exhibited by 'Imperial', plants from three different generations (collected during the years 2009, 2010 and 2011) were studied in detail, considering if they were produced by self-pollination (SP) or cross-pollination (CP) (Table 5).

Plants coming from self-pollination showed high frequency of cells with the 5RL constriction (often 100%, e.g. plants 1 and 11) and it was usually conspicuous, whereas the frequency was lower and more variable in plants coming from cross-pollination (e.g. plants 2 and 9) and the constriction was frequently subtle.

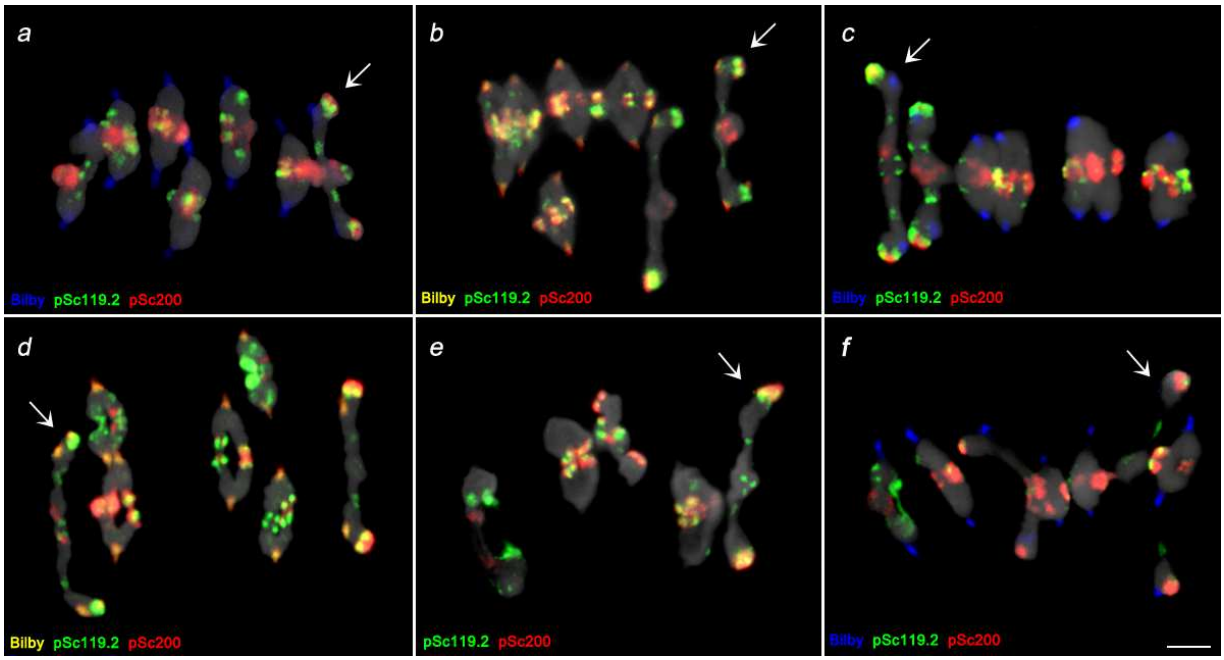

Fig. 4. Metaphase I meiocytes from *Secale* species and cultivars after FISH. Probes are indicated in the pictures. Arrows indicate the 5R bivalent. Bar = 5 µm.
a. *S. ancestrale* (no constriction). **b**. *S. cereale* cv. IPK (subtle constriction). **c**. *S. cereale* cv. Paldang (no constriction). **d**. *S. cereale* cv. I89 (subtle constriction). **e**. *S. cereale* cv. Imperial, cross-pollination (subtle constriction). Only five bivalents are visible. **f**. *S. cereale* cv. Imperial, self-pollination (conspicuous constriction).

When CP-plants were self-pollinated, the frequency of the 5RL constriction in the SP-progeny varied (Table 5; plants 4 and 5, 7 and 8, 10). This frequency could increase when the reproductive method was self-pollination (plant 10). This conclusion is not possible to extend to the SP-progeny from plants 3 and 6 because information from those mother plants is not available. However, considering the frequency of the 5RL constriction in other CP-plants (e.g. plant 1) it is likely that the frequency of the constriction in plants 3 and 6 was lower than in their SP-progeny.

When SP-plants were self-pollinated, the frequency of the 5RL constriction in the SP-progeny remained high (plants 14-18). On the contrary, if SP-plants were cross-pollinated, the frequency of the constriction in the CP-progeny could be reduced compared to the mother (mother plant 11 and progeny plant 13).

Figure 5 summarizes the effect of the reproductive method on the frequency of the 5RL constriction.

Table 5. Occurrence of the 5RL constriction in 'Imperial' rye depending on their origin: SP: self-pollination, grey shaded row; CP: cross-pollination, colourless row. A mother plant and the progeny are represented consecutively and linked by arrows. Plant names include: year in which they were collected (2009 (09), 2010 (10) or 2011 (11)), if they were produced by SP or CP and the plant number (preceded by the number of the mother plant, when it was known). The appearance of the constriction notably differed between plants; thus, it was distinguished subtle (*) or conspicuous (***) constriction. Plants were numbered to make easier the explanations. At least 50 cells were analyzed per plant. NA: Not available.

	Plant	Relationship with mother plant	% of cells with constriction	Appearance
1	10IMP(SP)-1		100	***
2	10IMP(CP)-1		55	*
3	10IMP(CP)-2		NA	NA
4	11IMP(SP)-2.1	Daughter of CP by SP	88	***
5	11IMP(SP)-2.2	Daughter of CP by SP	98	* and ***
6	10IMP(CP)-3		NA	NA
7	11IMP(SP)-3.1	Daughter of CP by SP	74	* and ***
8	11IMP(SP)-3.2	Daughter of CP by SP	100	* and ***
9	10IMP(CP)-4		36	*
10	11IMP(SP)-4.1	Daughter of CP by SP	92	***
11	09IMP(SP)-5		100	* and ***
12	10IMP(CP)-5.1	Daughter of SP by CP	100	***
13	10IMP(CP)-5.2	Daughter of SP by CP	80	***
14	10IMP(SP)-5.3	Daughter of SP by SP	100	***
15	10IMP(SP)-5.4	Daughter of SP by SP	100	***
16	10IMP(SP)-5.5	Daughter of SP by SP	98	***
17	10IMP(SP)-5.6	Daughter of SP by SP	94	***
18	11IMP(SP)-5.6.1	Daughter of SP by SP	92	***

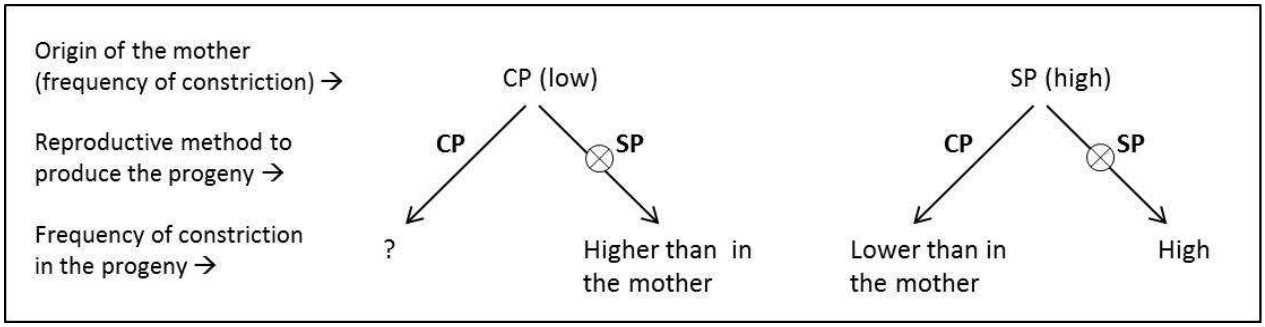

Fig. 5. Scheme representing the frequency of the 5RL constriction (high or low) depending on the reproductive method (CP: cross-pollination, SP: self-pollination).

Considering the data from all rye species and cultivars analyzed, a significant negative correlation is found between the mean chiasma frequency and the frequency of occurrence of the 5RL constriction ($r = –0.8463$, $p = 0.0000$). Thus, high consanguinity in the plants (indicated by lower chiasma frequency) seems to increase the formation of the constriction.

2.2. Occurrence of the 5RL constriction in the interspecific and intercultivar F1

In order to confirm this result, *S. cereale* cv. Imperial (IMP) (showing the highest percentage of metaphase I cells with the 5RL constriction of all analyzed materials) was crossed with *S. ancestrale* (ANC) (never showed the 5RL constriction), 'Paldang' (PAL) and 'IPK' (IPK) (both showed the 5RL constriction with variable frequency) (Table 6 and Fig. 6). These three genotypes presented higher chiasma frequencies than 'Imperial'. Every plant involved in each cross was analyzed individually to ensure the presence/absence of the constriction and to quantify the mean chiasma frequency. Crosses were made in the two possible directions, i.e. using 'Imperial' as female or male parent. In addition, some plants were used as female parent in one cross and as male parent in a different cross.

The mean chiasma frequency in the progeny was always higher than in the parental ‘Imperial’ and sometimes higher than in the other parental plant participating in the cross (Table 6). This suggests that consanguinity decreased in the progeny; however, the constriction was still visible in some cells.

The 5RL constriction occurred in the progeny with low frequency, but it was found in all types of crosses. In the F1 there is a significant negative correlation between the mean chiasma frequency and the frequency of constriction ($r = -0.5133$, $p = 0.0000$). However, the constriction was visible even in plants with high chiasma frequency, e.g. IPKxIMP-2 (20% of constriction; chiasma frequency 13.56), IMPxANC-8 (18%; 12.82) or PALxIMP-9 (54%; 12.74).

Differences among the crosses were found. The 5RL constriction appeared in the progenies of IMPxIPK and IPKxIMP, as well as IMPxPAL and PALxIMP. In general these frequencies were low (except for cross 11, where one plant showed the 5RL constriction in 54% of cells) and no differences depending on the direction of the cross were observed. This was not the case for *S. ancestrale* and ‘Imperial’. In the progeny from ANCxIMP the

constriction was never visible; on the contrary, it appeared in the progeny from IMPxANC although the frequency was always lower than in the parental IMP.

Table 6. Occurrence of the 5RL constriction in the parental plants participating in the crosses and in the F1 progeny. Plants coloured different from black indicate it is the same plant. n: number of plants analyzed in the progeny. IMP: *Secale cereale* cv. Imperial. PAL: *S. cereale* cv. Paldang. ANC: *S. ancestrale*. NA: Data not available.

Cross no.	**CROSS** (female x male)	**Female parent**		**Male parent**		**Progeny**		
		% 5RL constr.	Mean chiasma frequency	**% 5RL constr.**	Mean chiasma frequency	**% 5RL constr.**	Mean chiasma frequency	n
1	**ANC-1 x IMP-1**	**0**	13.21	**94**	10.80	**0, 0, 0**	12.81	3
2	**ANC-1 x IMP-2**	**0**	13.21	**100**	10.45	**0, 0, 0**	12.99	3
3	**IMP-3 x ANC-1**	**93**	11.15	**0**	13.21	**0, 5, 5, 10**	12.93	4
4	**IMP-4 x ANC-2**	**55**	11.77	**NA**	NA	**18, 28**	12.49	2
5	**IPK-1 x IMP-6**	**0**	13.05	**NA**	NA	**20, 24**	13.42	2
6	**IPK-3 x IMP-3**	**32**	13.03	**93**	11.15	**30, 32**	13.50	2
7	**IMP-5 x IPK-1**	**NA**	NA	**0**	13.05	**6, 8**	13.21	2
8	**IMP-1 x IPK-2**	**94**	10.80	**0**	13.20	**8**	12.60	1
9	**PAL-1 x IMP-7**	**0**	12.80	**98**	10.42	**4**	12.50	1
10	**PAL-1 x IMP-8**	**0**	12.80	**74**	11.84	**0**	13.38	1
11	**PAL-2 x IMP-9**	**0**	12.70	**36**	11.42	**4, 8, 54**	12.95	3
12	**IMP-10 x PAL-3**	**100**	11.77	**NA**	NA	**0, 0, 2, 4**	12.79	4

Interestingly, no heteromorphic bivalents were found in the F1 progeny of any cross. Thus, although one parental plant carried a 5R chromosome with, and the other parental plant without the constriction, in the progeny the 5RL constriction was present in both or in none of the homologous. This was not influenced by the cross direction.

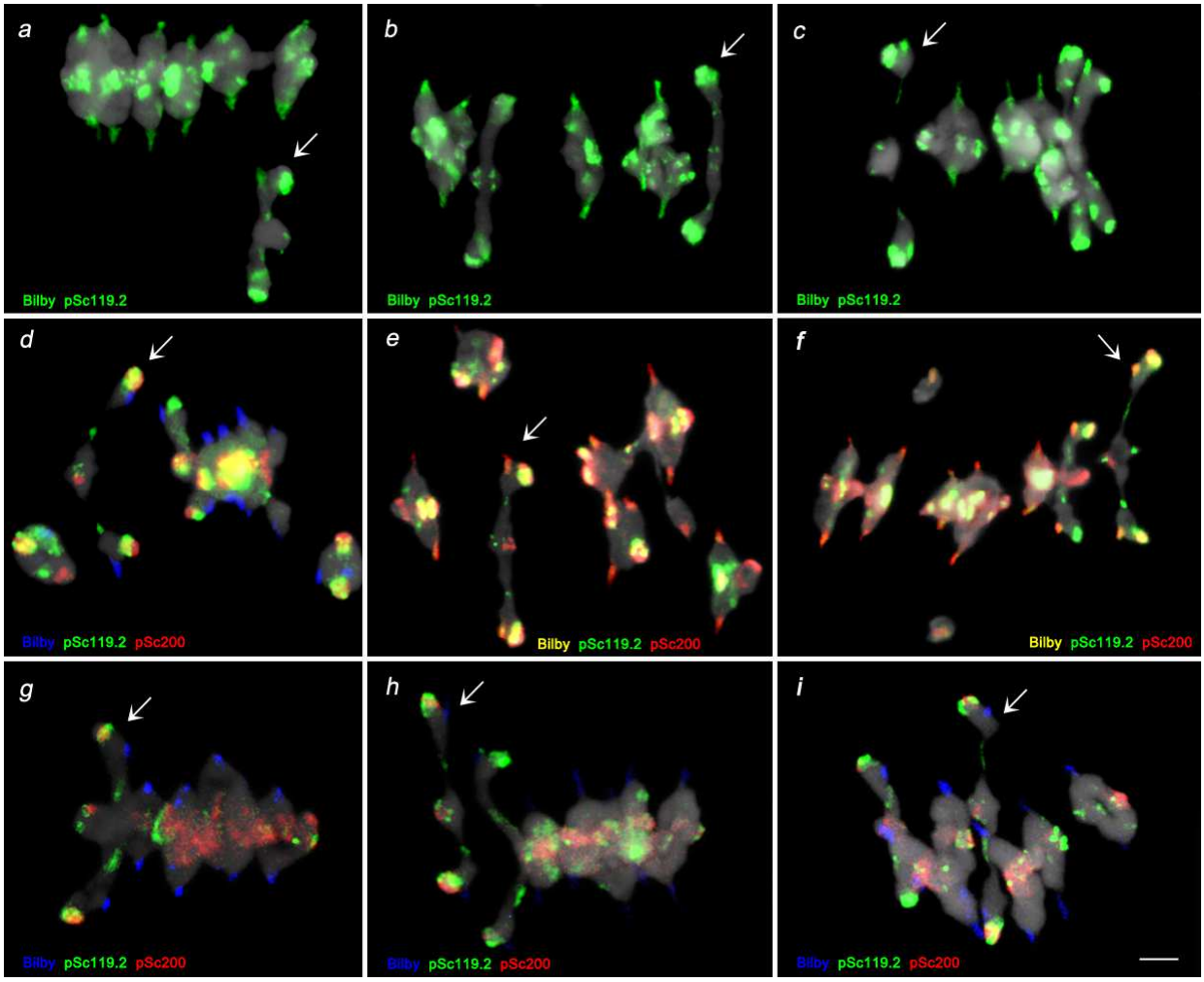

Fig. 6. 5RL constriction at metaphase I in the F1 meiocytes after FISH. Probes are indicated in the pictures. Arrows indicate the 5R bivalent. Bar = 5 µm.
a. ANCxIMP (no constriction). **b**. IMPxANC (subtle constriction). **c**. IMPxANC (conspicuous constriction). **d**. IPKxIMP (conspicuous constriction). **e**. IMPxIPK (subtle constriction). **f**. IMPxIPK (conspicuous constriction). **g**. IMPxPAL (no constriction). **h**. PALxIMP (subtle constriction). **i**. PALxIMP (conspicuous constriction).

2.3. Occurrence of the 5RL constriction in the interspecific F2

To test whether a recessive allele might control the formation of the 5RL constriction, F1 plants from *S. ancestrale* (ANC) and *S. cereale* cv. Imperial (IMP) were self-pollinated. Thirteen plants from each direction (IMPxANC, ANCxIMP) were analyzed in the F2.

When 'Imperial' was the female parent, eight plants showed the 5RL constriction conspicuous in 100% of cells and frequently stretched (Fig. 7a-c). In addition, it was visible even at diplotene-diakinesis (Fig. 8). In the F2, the constriction was more pronounced at these stages than in the F1 and in other materials where it is visible (addition lines, 'Imperial'

rye). Three plants showed the constriction in approximately 50% and last two plants in 6-8% of meiocytes.

Contrary, when *S. ancestrale* was the female parent, twelve plants either did not show the constriction or showed it with very low frequency (less than 5% of meiocytes) (Fig. 7d, e). When it was visible, it was subtle and never stretched. One exception was the plant ANCxIMP F2-10, which showed conspicuous constriction in 100% of cells (Fig. 7f).

Similarly to the F1 progeny, heteromorphic bivalents were not observed in the F2, i.e. either both or none of the 5R homologous showed the constriction (Fig. 7).

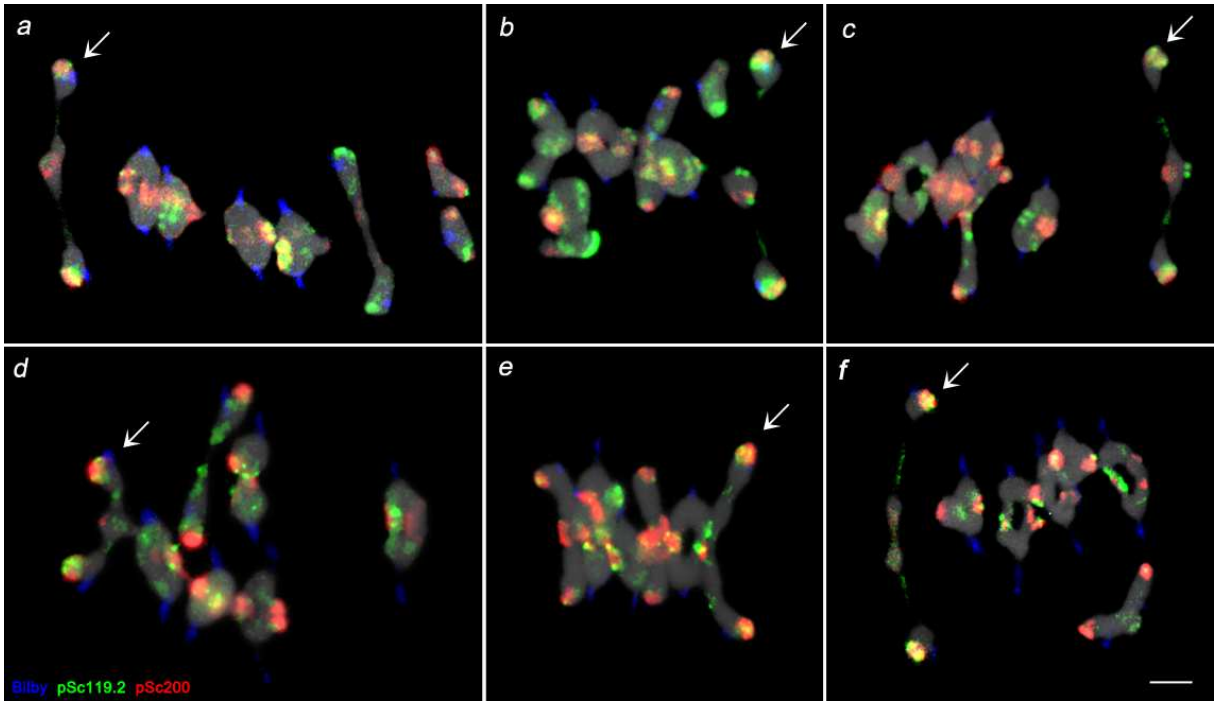

Fig. 7. 5R constriction at metaphase I in the F2 meiocytes after FISH. Probes are indicated in the figure. Arrows indicate the 5R bivalent. Bar = 5 µm.
a-c. IMPxANC F2 (conspicuous constriction). **d**, **e**. ANCxIMP F2 (no constriction). **f**. ANCxIMP F2-10 (conspicuous constriction).

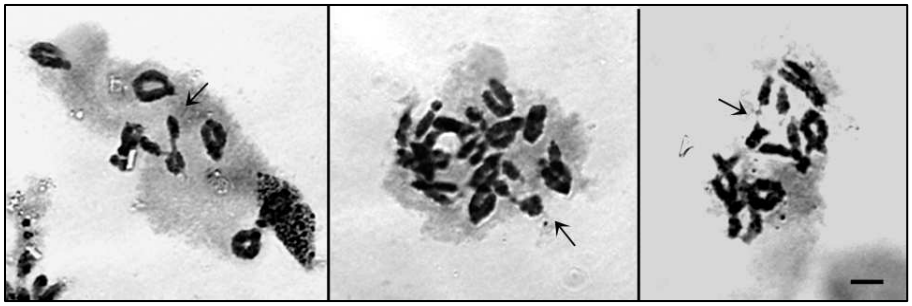

Fig. 8. 5R constriction at diplotene-diakinesis in the F2 meiocytes stained with acetocarmine. Arrows indicate the 5R bivalent. Bar = 5 µm.

3. Neocentromeric activity in chromosome 5R of rye

The 5RL constriction may acquire neocentromeric activity during the first meiotic division in all the addition lines analyzed: wheat-5R and 5RL monosomic, disomic, monotelosomic and ditelosomic. It was not found during meiosis II or mitosis, but for the first time it has been detected in diploid rye during meiosis.

The neocentromeric activity was revealed when the constriction showed tension to the cell poles and when it kept sister chromatids together at anaphase I. Due to the neocentromeric activity, the univalent or bivalent 5R or 5RL showed different morphologies.

3.1. 5R neocentromere in a wheat-5RL monotelosomic addition line

The different morphologies of the 5RL telochromosome (referred to as 5RL chromosome or 5R univalent) at metaphase I are shown in Figures 9 and 10.

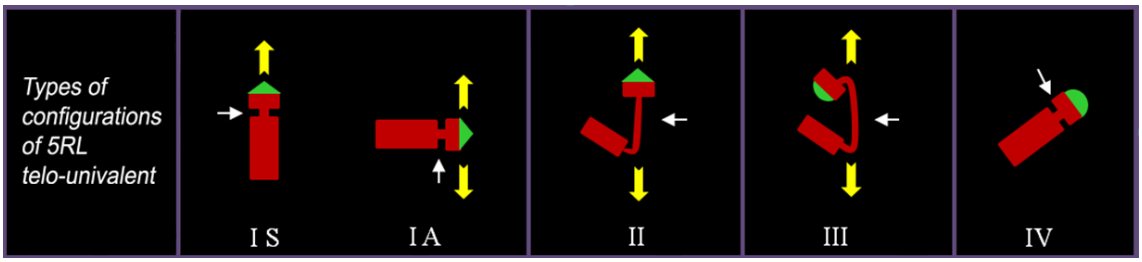

Fig. 9. Scheme of the 5RL univalent configurations at metaphase I in the monotelosomic addition line. Green triangles represent centromeres with tension to the poles; green semicircles, centromeres without tension to the poles. Yellow arrows indicate tension to the poles; white arrows indicate the position of the constriction.

TYPE I (active centromere, inactive neocentromere)
This type represents the situation in which only the centromere is active. The univalent might show syntelic (IS) or amphitelic (IA) orientation at metaphase I, migrating to one pole or separating chromatids at anaphase I, respectively. The constriction was always visible but not stretched (Figs. 9 and 10a, b).

TYPE II (active centromere, active neocentromere)
The constriction is stretched due to the centromere-neocentromere orientation to opposite poles (Figs. 9 and 10c, d). The stretching of the constriction was highly variable. Therefore,

the neocentromere was only considered active when the length of the constriction exceeded half the length of the complete 5RL univalent and the chromosome was properly orientated to the cell poles. This configuration showed the maximum stretching of the constriction, but broken chromosomes were not found.

TYPE III (inactive centromere, two active neocentromeres within the constriction)
The univalent is properly orientated at metaphase I showing tension at both ends of the constriction. The centromere seems to be inactive because it is not stretched to the pole (Figs. 9 and 10e).

TYPE IV (inactive centromere, inactive neocentromere)
The last type represents situations in which both the centromere and the neocentromere seem to be inactive. The univalent appeared without any orientation at metaphase I showing no tension to the cell poles (Figs. 9 and 10f).

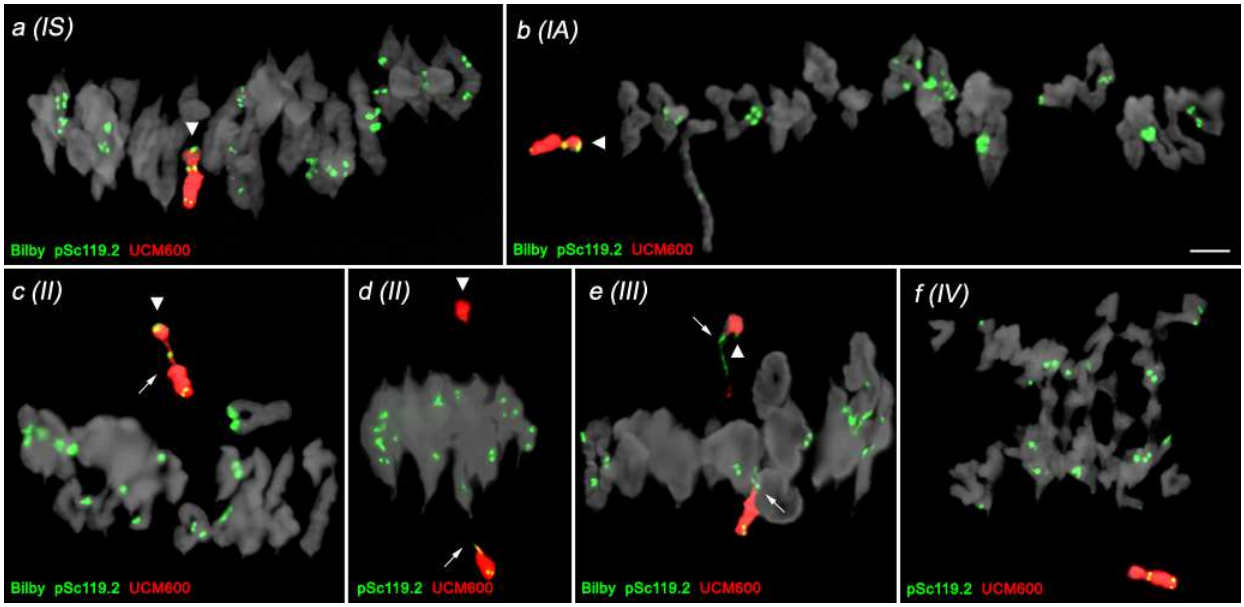

Fig. 10. 5RL univalent configurations at metaphase I in wheat-5RL monotelosomic addition line after FISH. Probes are indicated in the pictures. Arrowheads point to active 5R centromeres, arrows point to the 5R neocentromere. Bar = 5 µm.
a. 5RL in syntelic orientation (IS). **b**. 5RL in amphitelic orientation (IA). **c**, **d**. 5RL with the constriction stretched due to neocentromeric activity: centromere and neocentromere show tension to opposite poles (type II). **e**. 5RL orientated to the poles by two sites within the constriction (type III), centromere without tension. **f**. 5RL showing no orientation or tension to the cell poles at anaphase I, both the centromere and the neocentromere seem to be inactive.

3.2. 5R neocentromere in a wheat-5RL ditelosomic addition line

The two 5RL chromosomes pair in a rod bivalent at metaphase I, showing different morphologies depending on the activity of the neocentromere (Figs. 11 and 12).

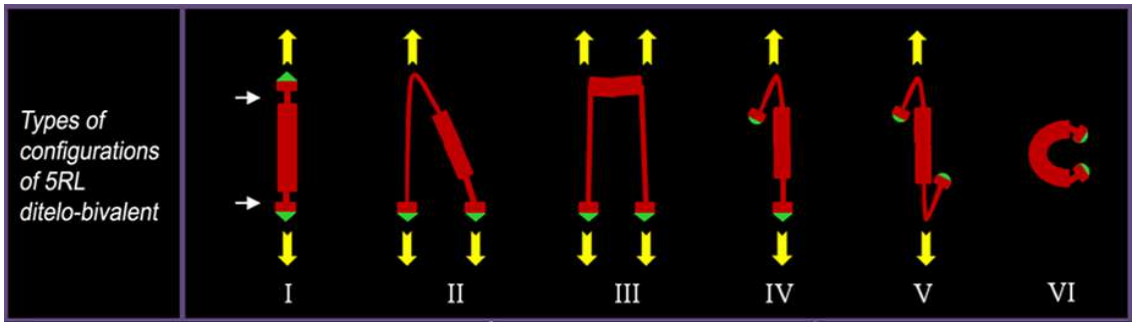

Fig. 11. Scheme of the 5RL bivalent configurations at metaphase I in the ditelosomic addition line (adapted from Manzanero et al. [2000b]). Green triangles represent centromeres with tension to the poles; green semicircles, centromeres without tension to the poles. Yellow arrows indicate tension to the poles; white arrows indicate the 5RL constriction in a rod bivalent without neocentromeres.

TYPE I (active centromere and inactive neocentromere in both 5RL chromosomes)
The most common bivalent type corresponds to the situation in which only the centromeres are active (Figs. 11 and 12a). The centromeric tension to the cell poles causes a slight stretching of the constriction due to its elasticity. This stretching can vary in the two homologous and it is not indicative for neocentromeric activity.

TYPE II (active centromere in both 5RL chromosomes and active neocentromere in one 5RL chromosome)
The bivalent is V-shaped resembling a trivalent. One neocentromere is active in one of the homologous stretching the constriction to one pole, whereas the two centromeres are co-orientated to the opposite pole (Figs. 11 and 12b).

TYPE III (active centromere and active neocentromere in both 5RL chromosomes)
The bivalent is U-shaped due to the co-orientation of the centromeres to one pole and the co-orientation of one neocentromere in each chromosome to the opposite pole (Figs. 11 and 12c, d). Type II and type III showed the maximum stretching of the constriction, but broken chromosomes were not registered.

TYPE IV (active centromere in one 5RL chromosome and active neocentromere in the homologous 5RL chromosome)
The bivalent is properly orientated at metaphase I by one centromere in one chromosome and one neocentromere in the homologous chromosome. The other centromere seems to be inactive because it is not stretched to the pole, thus the neocentromere might substitute the centromere (Figs. 11 and 12e, f).

TYPE V (inactive centromere and active neocentromere in both 5RL chromosomes)
The bivalent is orientated by active neocentromeres in both homologous, whereas the centromeres seem to be inactive (Figs. 11 and 12g). This type was the least frequent.

TYPE VI (inactive centromere and inactive neocentromere in both 5RL chromosomes)
The bivalent is not orientated and shows no tension to the cell poles (Figs. 11 and 12h).

In spite of all the bivalent orientations, segregation errors were rarely found. Only in some cells the two homologous migrated to the same pole at anaphase I (Fig. 12i).

3.3. 5R neocentromere in wheat-5R monosomic and disomic addition lines

In the monosomic addition line, the 5R univalent behaves similarly as the 5RL telochromosome. It can be amphitelically or syntelically orientated at metaphase I (Fig. 13a, b) and the constriction can be highly stretched due to the centromere-neocentromere orientation to opposite poles (Fig. 13c). Interestingly, in some anaphase I cells the univalent migrated to the neocentromeric pole (Fig. 13d).

In the disomic addition line the two 5R chromosomes pair in a ring or rod bivalent (Fig. 13f, g). Bivalent configurations resembling the ditelobivalent type III (both centromeres co-orientated to one pole and the 5R neocentromere in each chromosome co-orientated to the opposite pole) were found (Fig. 13h).

Also in these lines, the 5R neocentromere can keep sister chromatids together at anaphase I (Fig. 13e).

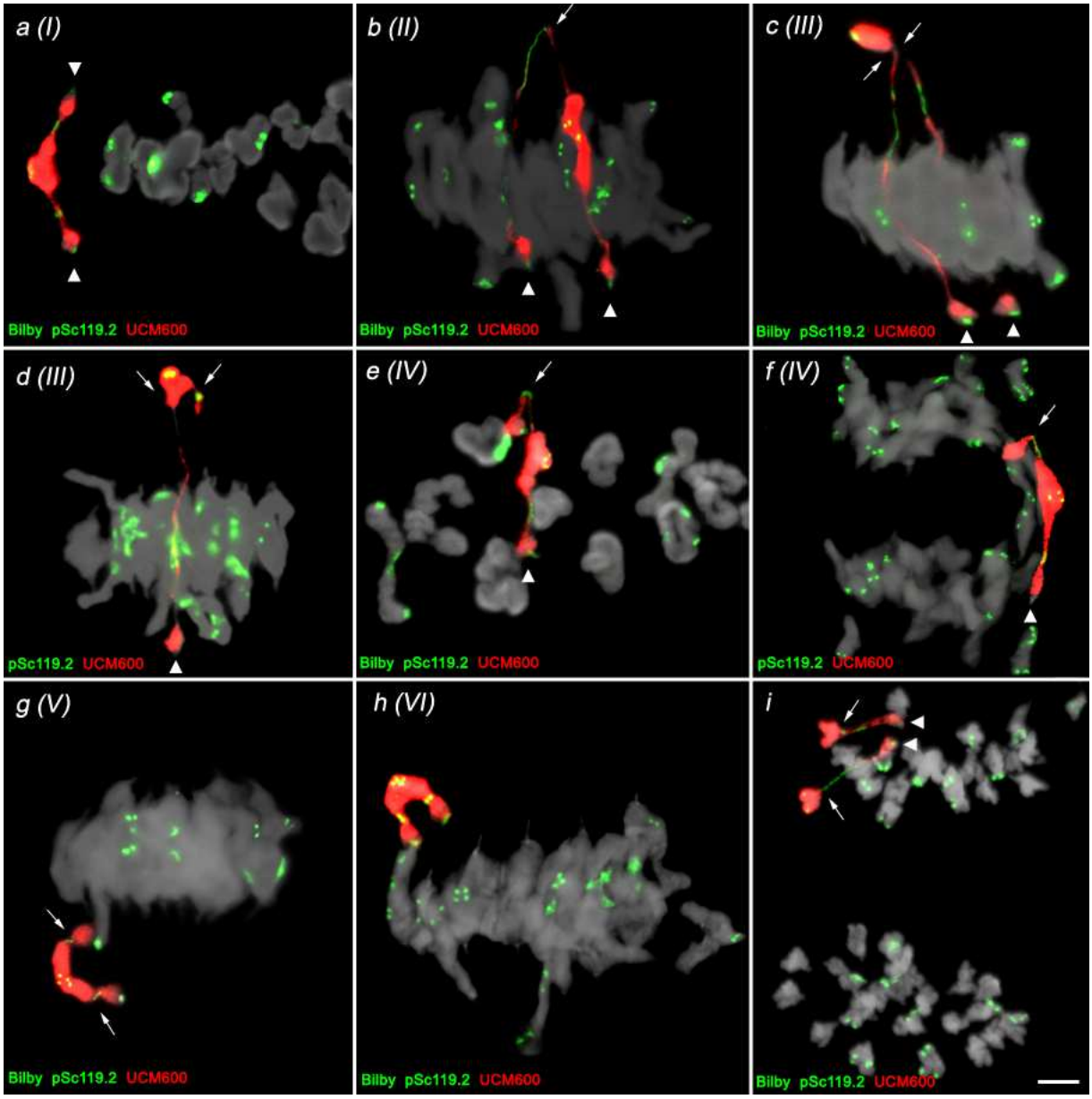

Fig. 12. 5RL bivalent configurations at metaphase I (**a-h**) and anaphase I (**i**) in wheat-5RL ditelosomic addition line after FISH. Probes are indicated in the pictures. Arrowheads point to active 5R centromeres, arrows point to the 5R neocentromere. Bar = 5 µm.

a. 5RL ditelobivalent orientated to the poles by the centromeres, the constriction is conspicuous (type I). **b**. V-shaped ditelobivalent where both centromeres are orientated to the same pole and one neocentromere to the opposite pole (type II). **c**, **d**. U-shaped ditelobivalent where both centromeres are orientated to the same pole, and the neocentromere in each 5R chromosome to the opposite pole (type III). **e**, **f**. 5RL ditelobivalent orientated by the centromere in one chromosome and by the neocentromere in the homologous chromosome, the other centromere seems to be inactive (type IV). **g**. Ditelobivalent orientated by one neocentromere in each 5R chromosome. Centromeres seem to be inactive (type V). **h**. Ditelobivalent showing no orientation or tension to the cell poles, centromere and neocentromere in the two homologous seem to be inactive. **i**. Anaphase I with two 5RL chromosomes migrating to the same pole; the constriction keeps sister chromatids together.

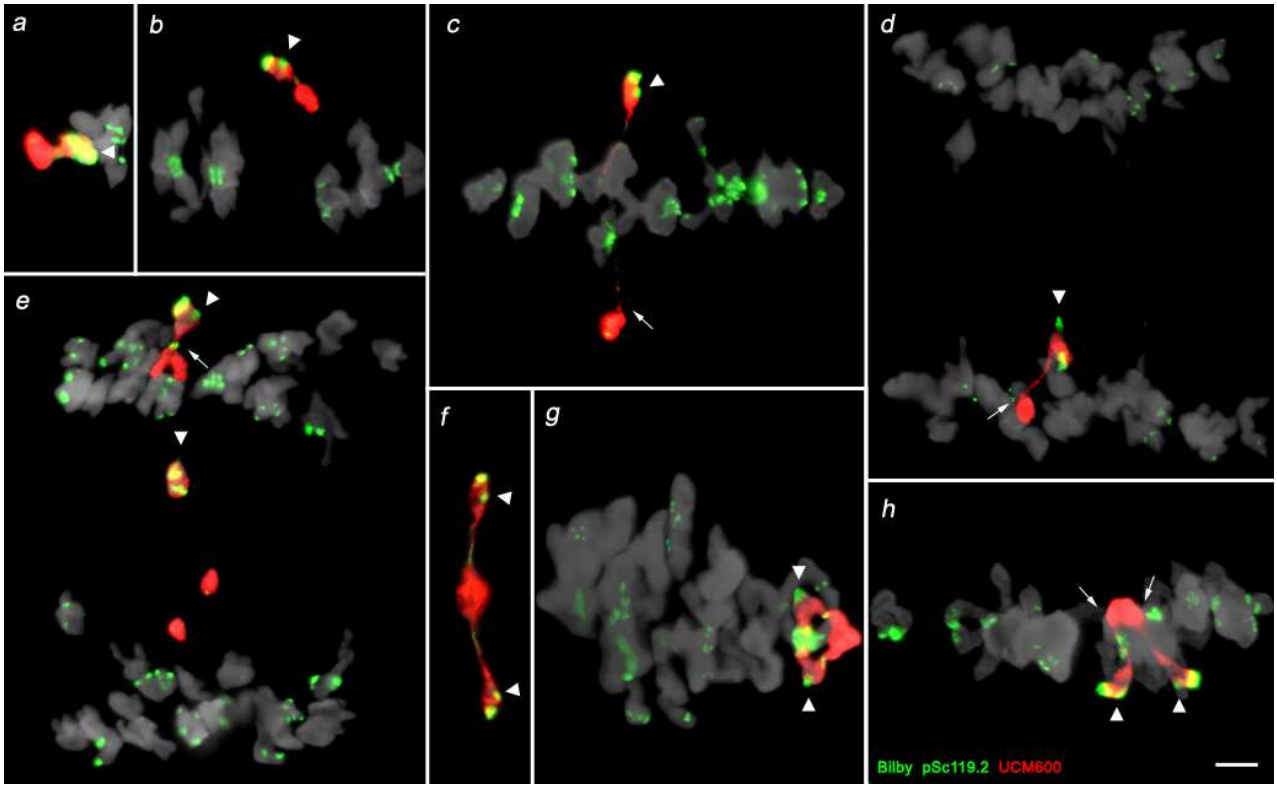

Fig. 13. 5R univalent and bivalent configurations in monosomic (**a-d**) and disomic (**e-h**) wheat-5R addition lines after FISH. Probes are indicated in the figure. Arrowheads point to active 5R centromeres, arrows point to the 5R neocentromere. Bar = 5 µm.

a. Inset of metaphase I, 5R univalent in amphitelic orientation. **b**. Inset of metaphase I, 5R univalent in syntelic orientation. **c**. Metaphase I, the 5R univalent is orientated to opposite poles by the centromere and the neocentromere. **d**. Anaphase I similar to c; the 5R chromosome has reached the pole of the neocentromere. **e**. Anaphase I. The 5R chromosome at the upper pole shows sister chromatids together at the constriction. The lagging 5R is broken at the constriction resulting in three fragments, one including the 5RS, the centromere and the proximal part of the 5RL, and the other two fragments correspond to the chromatids of the distal part of the 5RL. **f**. 5R rod bivalent with conspicuous constriction. **g**. Metaphase I, 5R ring bivalent showing the constriction in both long arms. **h**. U-shaped 5R bivalent with both centromeres orientated to the same pole and the neocentromere in each chromosome to the opposite pole.

3.4. 5R neocentromere in diploid rye

In one metaphase I cell from a F1 IPKxIMP plant a U-shaped 5R bivalent was found (Fig. 14). This 5R bivalent resembled the 5R bivalent in the wheat-5R disomic addition line (Fig. 13h) with active neocentromere in both 5R chromosomes simultaneously with the centromeres. In addition, in some rye meiocytes sister chromatids were kept together at the 5R constriction during anaphase I (e.g. Fig. 3c).

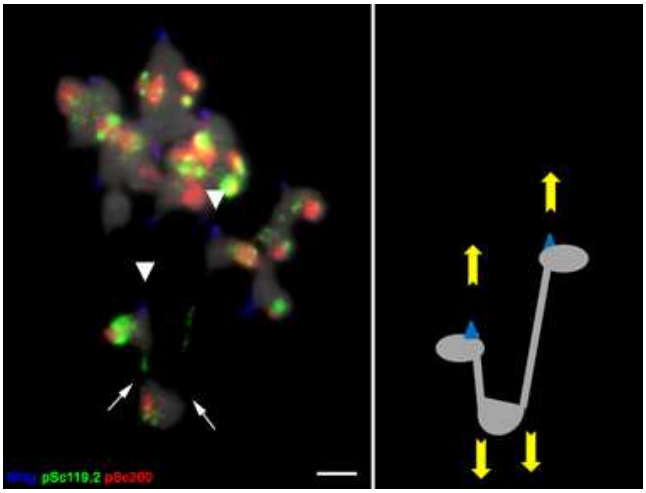

Fig. 14. Rye (F1 IPKxCI) 5R bivalent with the 5R neocentromere in both homologous active at metaphase I. **Right**. FISH. Probes are indicated in the picture. Arrowheads point to the 5R centromere, arrows point to the 5R neocentromere. **Left**. Scheme of the 5R bivalent. Blue triangles indicate the centromeres, yellow arrows indicate tension to the poles. Bar = 5 µm.

3.5. Terminal neocentromeres in diploid rye

Surprisingly, terminal neocentromeres were found in the rye cv. 'I89' (Fig. 15). The subtelomeric regions of one or two chromosomes were occasionally pulled to the poles at metaphase I and anaphase I. The FISH probes pSc200 and pSc119.2 were stretched at the terminal neocentromeres, either one (Fig. 15c, d, f) or both simultaneously (Fig. 15d, e). It seems that the amount of heterochromatin positively determines the stretching ability of the subtelomeric regions.

Terminal neocentromeres were found only in one I89 plant (I89-10) which did not show the 5RL constriction, whereas another I89-10 spike analyzed showed the 5RL constriction in 16.67% of cells but not terminal neocentromeres

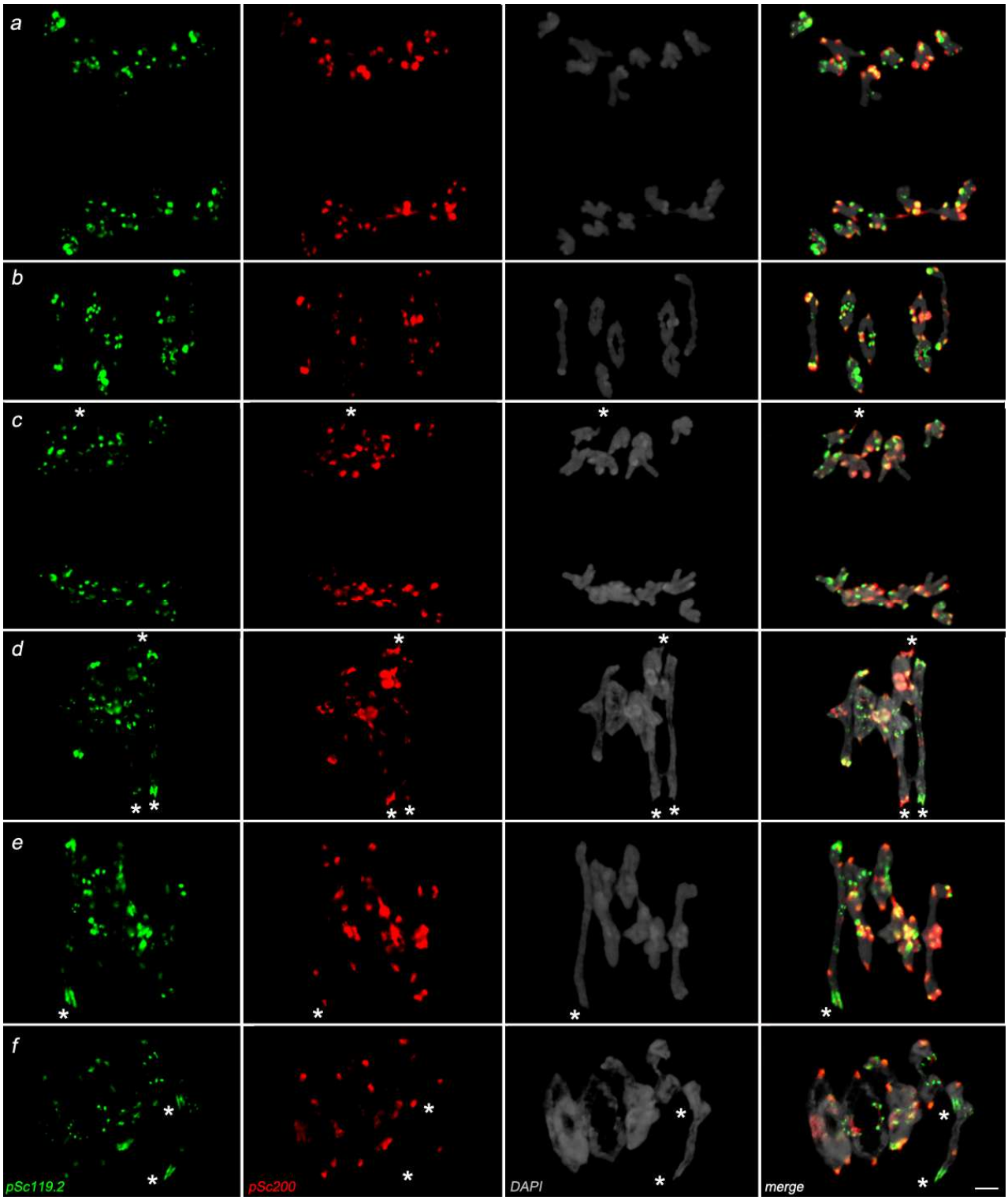

Fig. 15. Terminal neocentromeres in the rye cv. 'I89' after FISH. Probes are indicated in the figure. Asterisks indicate terminal neocentromeres. Bar = 5 µm.

a, **b**. No terminal neocentromeres. **a**. Anaphase I. **b**. Metaphase I.

c-**f**. Terminal neocentromeres. **c**. Anaphase I, only pSc200 is stretched to the pole. **d**. Metaphase I, only pSc200 is stretched to the pole in the left bivalent whereas both pSc119.2 and pSc200 are stretched in the right bivalent. **e**. Metaphase I, both pSc119.2 and pSc200 are stretched to the pole. **f**. Metaphase I, only pSc119.2 is stretched to the pole.

4. Characterization of the 5RL constriction with neocentromeric activity

4.1. DNA sequences

UCM600 and pSc119.2 are localized within the 5RL constriction. In some highly stretched constrictions UCM600 does not spread continuously but instead it leaves gaps that are partially labelled by pSc119.2 (Fig. 12b, c). Thus, both sequences occupy distinct regions within the constriction. Moreover, unlabelled regions indicate that other sequences are present in this region (Figs. 10e and 12b).

The hybridization pattern of pSc119.2 provided information about the stretching ability of the constriction and demonstrated that the kinetic activity is not fixed to a restricted region within the constriction. Thus, the location of pSc119.2 within the constriction varied in different cells from proximal to the centromere (Figs. 10e and 12f) to distal (Figs. 10d and 12d) or intermediate (Figs. 10c and 12a, c). In some cases the pSc119.2 signal co-localized with the site of kinetic activity (Figs. 10d and 12e), but not other times (Figs. 10c and 12c). In addition, this probe appeared sometimes stretched in consonance with the constriction (Figs. 10e and 12c) or not stretched, in contrast to the elongated constriction (Figs. 10c, d and 12d).

4.2. Centromeric proteins and pericentromere-associated histone modifications

Based on silver staining, Manzanero et al. (2002) reported that in wheat-5RL addition lines proteins of unknown nature are accumulated at the 5RL constriction during meiosis. In order to reveal which proteins are found at the 5RL constriction, immunolocalizations of known centromeric proteins and pericentromere-associated histone modifications were performed.

Antibodies against centromeric/kinetochore proteins of rye are not available. Therefore, antibodies specific for centromeric proteins of other plant species, particularly maize and rice, were tested for cross-reactivity in rye and wheat. Figure 16 shows the centromeric localization of cenH3, CENP-C and MIS12 in ‘Imperial’ rye.

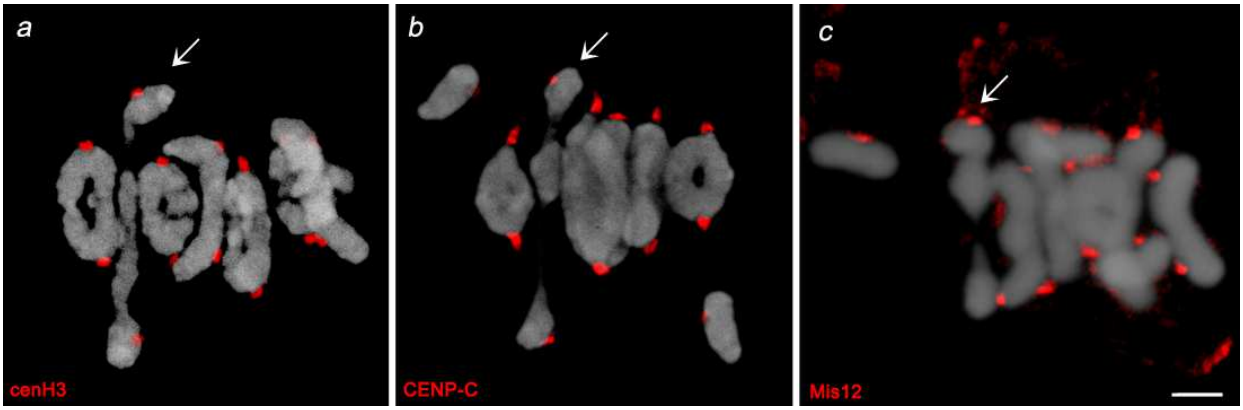

Fig. 16. Immunolocalization of centromeric proteins in rye metaphase I cells. Arrow points to the 5R bivalent, identified by the 5RL constriction. Bar = 5 µm.
a. anti- rice cenH3. **b**. anti- maize CENP-C. **c**. anti- maize MIS12.

cenH3

The antibody raised against rice cenH3 cross-reacted with rye and wheat centromeres (Figs. 16a and 17). cenH3 was not detected at the 5RL constriction neither when the neocentromere was inactive (Fig. 17a) nor active (Fig. 17b). To test whether the amount of cenH3 was under the resolution level of fluorescence Wide Field Microscopy (WFM), cenH3-immunolabelled cells were analyzed by Structure Illumination Microscopy (SIM) (Fig. 18), but no cenH3 signals were detected at the 5RL constriction.

CENP-C

The antibody against maize CENP-C cross-reacted with rye and wheat centromeres but it was not detected at the 5RL constriction (Figs. 16b and 19a).

H2AT133ph

The antibody against histone H2AT133ph from maize labelled wheat and rye 5R centromeres in wheat-5R and 5RL addition lines but not the 5RL constriction (Fig. 19b).

H3S28ph

An antibody against histone H3S28ph localized to wheat and rye centromeres in wheat-5R and 5RL addition lines, but not to the 5RL constriction (Fig. 19c).

H3K27me3

In wheat-5RL addition lines, wheat bivalents showed an enrichment of this heterochromatic mark at subtelomeric regions. In chromosome 5RL H3K27me3 labelling was found at a large terminal heterochromatic block and also in an interstitial band, but it was absent in the 5RL constriction as well as in the pericentromeric region (Fig. 19d).

Several other antibodies against proteins and histone modifications were tested but the antibodies were not informative; i.e. not cross-reacting (such as anti-rice Shugoshin), labelling the complete chromosomes at metaphase I (such as anti-H3S10ph) or not yielding a specific pattern (NDC80, H3K4me2, H3K9me1, H3K9me2, H3K27me1, H3T3ph and H3T11ph). The anti-MIS12 antibody cross-reacted with rye centromeres (Fig. 16c) but produced intense background in wheat-5R addition lines and therefore the presence or absence of this protein at the 5R neocentromere remains uncertain.

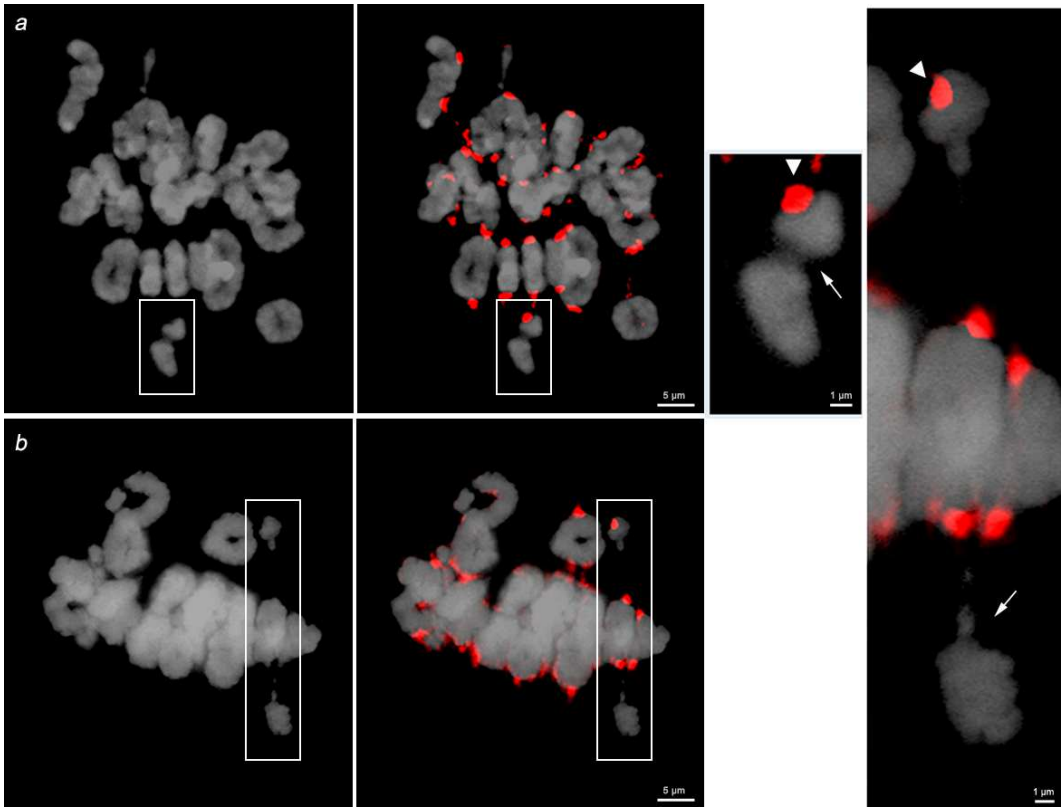

Fig. 17. Wheat-5RL metaphase I cells showing cenH3-positive wheat and rye centromeres and cenH3-negative 5RL constriction after immunostaining with an anti-rice cenH3 antibody (red). The 5RL chromosome is framed and enlarged to the right in both cells. Arrowheads point to the 5R centromeres, arrows point to the 5RL constriction (**a**) or to the 5R neocentromere (**b**).

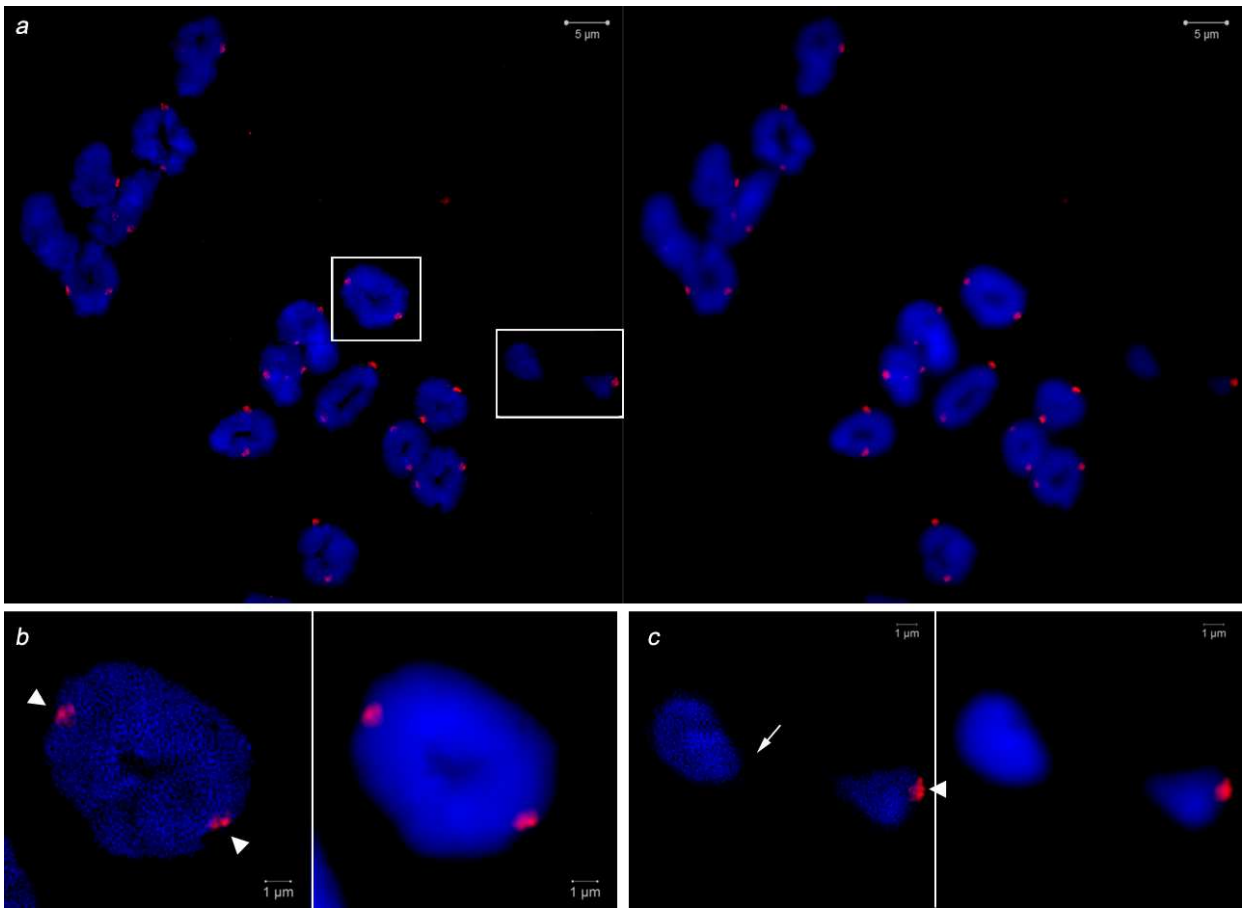

Fig. 18. Inset of wheat-5RL diakinesis cell showing cenH3-positive wheat and rye centromeres and cenH3-negative 5RL constriction after immunostaining with an anti-rice cenH3 antibody (red). High resolution imaging by Structure Illumination microscopy (SIM; **left**) compared to normal fluorescence Wide Field Microscopy (WFM; **right**). Enlargements of the white boxes in **a** are shown in **b** and **c**, respectively.

b. cenH3-positive wheat ring bivalent; note the distinguishable cenH3 signals corresponding to the two sister chromatids at the mono-orientated sister-centromeres (arrowheads). **c**. 5RL univalent with highly stretched cenH3-negative constriction (arrow) and cenH3-positive centromere (arrowhead).

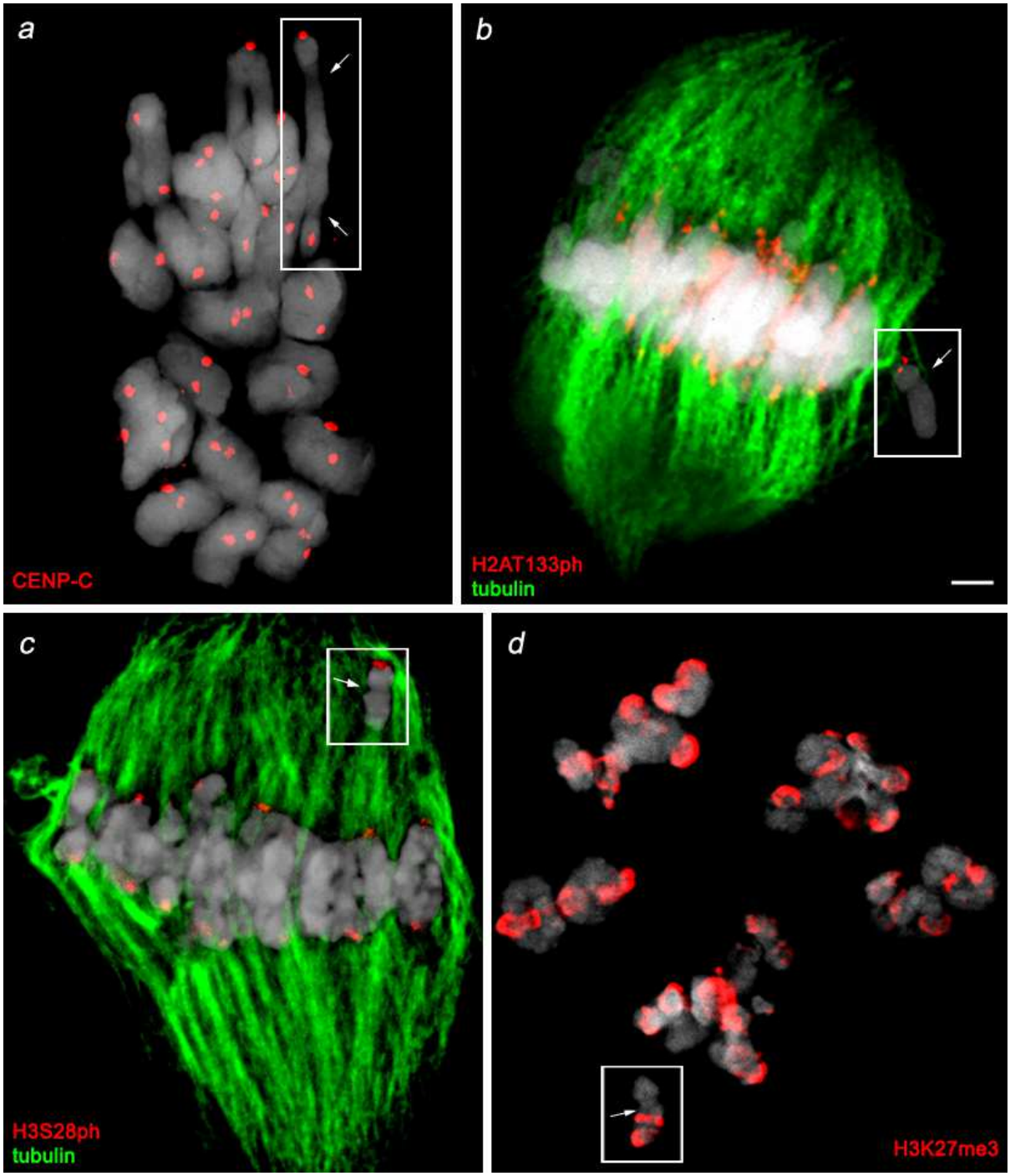

Fig. 19. Immunolocalization of kinetochore proteins and histone modifications in wheat-5RL meiocytes. 5RL chromosome or bivalent is framed. Arrows point to the 5RL constriction. Bar = 5 µm. **a**. Metaphase I cell showing CENP-C-positive wheat and rye centromeres and CENP-C-negative 5RL constriction. **b**. Metaphase I cell showing H2AT133ph-positive wheat and rye centromeres and H2AT133ph-negative 5RL constriction. Tubulin is attached to the 5RL constriction. **c**. Metaphase I cell showing H3S28ph-positive wheat and rye centromeres and H3S28ph-negative 5RL constriction. **d**. Diakinesis cell showing subtelomeric enrichment of the heterochromatic mark H3K27me3 in wheat and rye chromosomes but it is absent in the 5RL constriction as well as in the centromeres.

4.3. Antibodies atypically labelling the 5RL constriction in rye

In few rye meiocytes an antibody pattern at the 5RL constriction not comparable with the majority of cells was found. This might indicate features of the 5RL constriction under specific circumstances. This was the case for anti-MIS12 and anti-histone H2AT133ph antibodies.

In case of MIS12, one cell showed small signals at the 5RL constriction of both homologous in addition to strong signals at centromeres (Fig. 20a). In one of the 5R homologous two signals are distinguishable, possibly corresponding to both sister chromatids. In these cells, microtubules seem to be directed to the 5RL constriction.

The anti-histone H2AT133ph antibody cross-reacted with wheat and rye centromeres in meiocytes from the addition lines (Fig. 19b). The same occurred occasionally in rye meiocytes but frequently the signals appeared surrounding the bivalents, likely due to chromosomal penetration troubles of the antibody. However, in three cells the antibody labelled entirely 5RL constriction in both homologous whereas not the 5R centromeres (Fig. 20b).

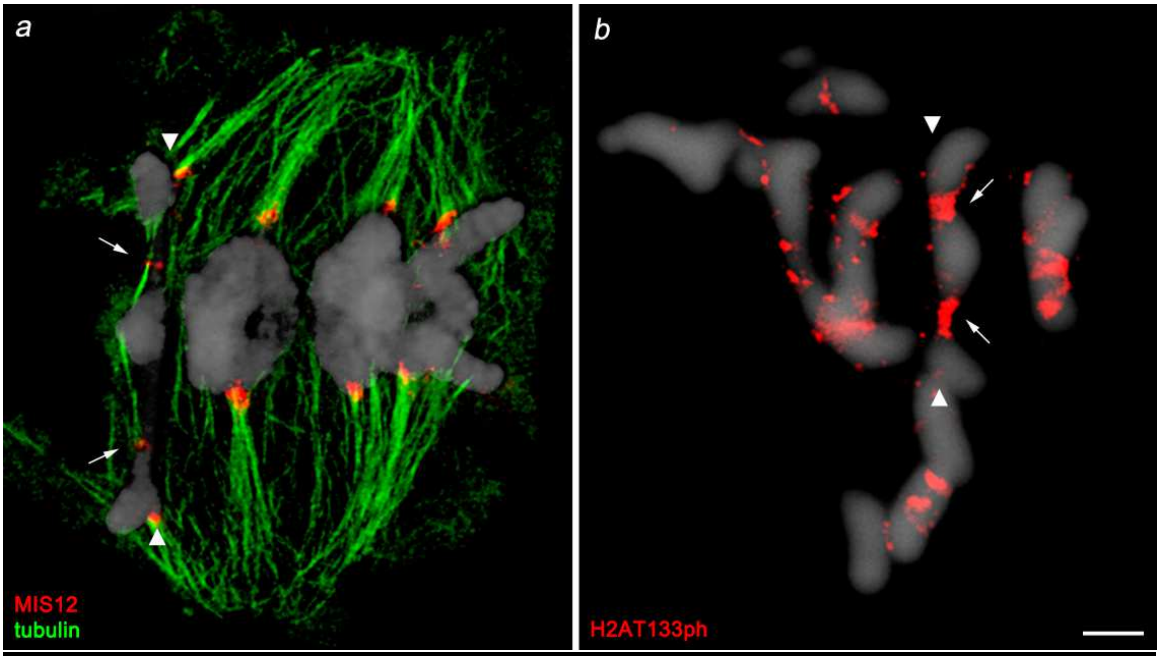

Fig. 20. Rye meiocytes with atypical immunolabelling of the 5RL constriction. Arrowheads point to the 5R centromere, arrows point to the 5RL constriction. Bar = 5 µm.

5. Influence of Diazinon on the frequency of the 5R neocentromere

Surprisingly, a commercial pesticide turned out to increase the frequency of the 5R interstitial neocentromere. When this product was applied to prevent insects harming the plants, these plants showed high frequency of neocentromeric activity in contrast to the low frequency observed in untreated plants.

Based on this finding, the neocentromeric activity was evaluated as the percentage of cells per plant showing the 5R neocentromere. Plants from all the addition lines were analyzed with and without pesticide treatment (referred to as 'treated and 'untreated', respectively). The observed morphologies were classified according to the previously established categories. When possible, anthers from the same plants were studied before the treatment as control.

5.1. Neocentromeric frequencies in a wheat-5RL monotelosomic addition line

Frequencies of monotelo-5RL univalent types at metaphase I in untreated and treated plants are shown in Table 7. The most frequent configuration of the 5RL univalent was type I (either IS or IA). Type II univalents (centromere and neocentromere active) were observed in a low frequency in untreated plants (6.38%) and type III univalents (only the 5RL neocentromere active, with two tension points within the constriction) were never found. In treated plants, the mean frequency of the 5R neocentromere increased from 6.38% to 22.35%, primarily due to a decrease in the frequency of univalents type IA. A contingency χ^2 test showed significant differences in the number of cells with neocentromeric activity between treated and untreated plants ($\chi^2 = 45.23$, $p = 0.000$).

Frequencies of cells with the 5R neocentromere before and after treatment varied between spikes from the same plant and even between anthers from the same spike. For example, anthers from the plant MT5RL-4 showed the 5RL neocentromere in 11.76 and 26.92% of the cells after treatment, but this value was higher than the frequency registered before the treatment (3.08%).

The frequency of occurrence of the 5R neocentromere did not show a significant correlation with the number of treatments ($r = 0.4517$, $p = 0.0910$) or with the number of days

after the treatment (r = –0.3048, p = 0.2693). The plant MT5RL-17 showed different percentages of cells with active neocentromere (from 13.79 to 27.38%) in a dosage- and time-independent way, but all frequencies were higher than in the untreated control spike (1.82%).

Table 7. Frequencies of cells with neocentromeric activity in wheat-5RL monotelosomic plants untreated and treated with the pesticide. Types I-IV are defined in figure 9.
T= Number of treatments; D= number of days from the last treatment to spike fixation.

Plant	Treatment	Cells without neocentromeric activity (n)			Cells with neocentromeric activity (n)		Frequency of cells with neocentromeric activity (%)	Total no. of cells
		IA	IS	IV	II	III		
MT5RL-6	Untreated	37	18	3	1	0	1.69	59
MT5RL-17	Untreated	26	20	8	1	0	1.82	55
MT5RL-4	Untreated	35	25	3	2	0	3.08	65
MT5RL-1	Untreated	66	44	4	11	0	8.80	125
MT5RL-14	Untreated	17	18	4	4	0	9.30	43
MT5RL-15	Untreated	14	18	7	6	0	13.33	45
Total untreated (%)		49.74	36.48	7.40	6.38	0.00	6.38	392
MT5RL-4	1T5D	26	29	5	8	0	11.76	68
MT5RL-17	3T1D	30	14	6	7	1	13.79	58
MT5RL-1	3T1D	3	6	3	2	0	14.29	14
MT5RL-16	1T10D	37	29	4	13	0	15.66	83
MT5RL-17	1T3D	18	16	10	9	2	20.00	55
MT5RL-5	1T7D	20	13	3	11	0	23.40	47
MT5RL-17	6T2D	17	12	3	8	2	23.81	42
MT5RL-4	1T5D	15	22	1	14	0	26.92	52
MT5RL-17	Treated	46	56	20	46	0	27.38	168
MT5RL-1	2T1D	9	7	3	15	1	45.71	35
Total treated (%)		35.53	32.80	9.32	21.38	0.96	22.35	622

5.2. Neocentromeric frequencies in a wheat-5RL ditelosomic addition line

Frequencies of the ditelo-5RL bivalent types in untreated and treated plants are shown in Table 8. In 4.5% of all metaphase I cells the two 5RL chromosomes were unpaired and occasionally the 5R neocentromere was active in the univalents. However, these cells are neither included in Table 8, nor have they been considered in the following calculations as they could not be classified in some of the established categories.

Also in the case of the 5RL ditelosomic line, the pesticide increased significantly the frequency of occurrence of the neocentromere, from 4.09% in untreated to 22.11% in treated plants (χ^2 = 42.95, p = 0.000). Type VI (centromere and neocentromere of both homologous inactive) in the ditelosomic line was reduced in treated plants (9.07%) compared to control plants (15.61%). Indeed, the number of cells with orientated bivalents (types I–V) versus non-orientated bivalents (type VI) significantly differed in untreated and treated plants (χ^2 = 9.56, p = 0.002).

Similarly to the 5RL monotelosomic line, plants from the 5RL ditelosomic line showed variable frequencies of neocentromeric activity. This is the case for DT5RL-1 with 0.00, 4.00 and 6.67% of cells with active neocentromere in different untreated anthers and 16.46 and 25.42% in treated anthers. Despite this variability, the neocentromeric frequency in treated plants was always higher. Unfortunately, data from the same plant before and after the treatment is not available in all cases.

In the ditelosomic line, the effect of consecutive pesticide treatments and the number of days from the last treatment to spike fixation were also analyzed. The data showed that none of these variables affected the frequency of occurrence of the 5R neocentromere.

Table 8. Frequencies of cells with neocentromeric activity in wheat-5RL ditelosomic plants untreated and treated with the pesticide. Types I-VI are defined in figure 11.
T= Number of treatments; D= number of days from the last treatment to spike fixation.

Plant	Treatment	Cells without neocentromeric activity (n)		Cells with neocentromeric activity (n)				Frequency of cells with neocentromeric activity (%)	Total no. of cells
		I	VI	II	III	IV	V		
DT5RL-1	Untreated	30	14	0	0	0	0	0.00	44
DT5RL-3	Untreated	40	4	0	1	0	0	2.22	45
DT5RL-1	Untreated	43	5	1	1	0	0	4.00	50
DT5RL-2	Untreated	36	8	0	2	0	0	4.35	46
DT5RL-1	Untreated	38	4	0	3	0	0	6.67	45
DT5RL-19	Untreated	29	7	0	3	0	0	7.69	39
Total untreated (%)		80.30	15.61	0.37	3.72	0.00	0.00	4.09	269
DT5RL-12	5T2D	28	3	0	1	2	0	8.82	34
DT5RL-15	1T1D	152	19	10	10	3	1	12.31	195
DT5RL-12	1T3D	11	2	0	1	1	0	13.33	15
DT5RL-19	1TXD	13	3	0	2	1	0	15.79	19
DT5RL-15	1T1D	53	5	4	6	1	0	15.94	69
DT5RL-1	1T1D	63	3	4	7	1	1	16.46	79
DT5RL-18	1T9D	23	16	1	8	0	0	18.75	48
DT5RL-16	3T1D	17	3	0	5	0	0	20.00	25
DT5RL-3	1T1D	49	5	6	8	2	0	22.86	70
DT5RL-1	1T5D	41	3	7	6	1	1	25.42	59
DT5RL-15	1T9D	126	16	14	35	7	1	28.64	199
DT5RL-15	1T9D	84	9	13	40	1	0	36.73	147
Total treated (%)		68.82	9.07	6.15	13.45	2.09	0.42	22.11	959

Plants from the wheat-5RL monotelo- and ditelosomic addition lines were treated with the pesticide during three consecutive years. The high frequency of occurrence of the 5R neocentromere following pesticide treatments was not inherited to the progeny; instead, the frequency of this activity rose every year to about the same frequency only in treated plants.

5.3. Neocentromeric frequencies in wheat-5R mono- and disomic addition lines

Three 5R monosomic and one 5R disomic plants of the respective addition lines were evaluated to determine the effect of the pesticide treatment. Frequencies of cells with neocentromeric activity in untreated and treated plants are shown in Table 9.

Treated spikes showed higher frequencies of cells with neocentromeric activity than corresponding untreated spikes, with similar frequencies as monotelo- and ditelosomic 5RL plants. Combining data from both monosomic and disomic 5R plants, significant differences between the number of cells with active neocentromere in treated and untreated plants was revealed by a contingency χ^2 test ($\chi^2 = 26.40$, $p = 0.000$).

As in the previous cases, a remarkable variability of neocentromere frequency was observed even between sister anthers, for example in M5R-5 (7.94 and 13.95% in two sister anthers from an untreated plant), M5R-17 (19.7 and 30.51% in two sister anthers from a treated plant) and M5R-2 (18.87 and 27.47% in two sister anthers from a treated plant). Besides this variability, in all cases the neocentromeric frequency in treated plants was higher than in the respective control plants.

Table 9. Frequencies of cells with neocentromeric activity in wheat-5R monosomic (M) and disomic (D) plants untreated and treated with the pesticide.
T= Number of treatments; D= number of days from the last treatment to spike fixation.

Plant	Treatment	Cells without neocentromeric activity (n)	Cells with neocentromeric activity (n)	Frequency of cells with neocentromeric activity (%)
M5R-17	Untreated	56	0	0.00
M5R-2	Untreated	50	1	1.96
M5R-5	Untreated	58	5	7.94
M5R-5	Untreated	74	12	13.95
Total untreated M (%)		92.97	7.03	
D5R-5	Untreated	54	3	5.26
Total untreated D (%)		94.74	5.26	
M5R-2	2T2D	45	6	11.76
M5R-2	1T5D	44	6	12.00
M5R-2	1T9D	43	10	18.87
M5R-17	2T2D	53	13	19.70
M5R-2	1T9D	37	14	27.45
M5R-17	2T2D	41	18	30.51
Total treated M (%)		79.70	20.30	
D5R-5	2T5D	46	12	20.69
Total treated D (%)		79.31	20.69	

6. Effect of Diazinon on the spindle at metaphase I

In order to test whether Diazinon alters the meiotic spindle, treated plants from the addition lines were studied by anti-α-tubulin immunostaining.

In four untreated plants (32 cells analyzed) nearly 100% of metaphase I cells showed normal spindles, with conspicuous bundles of microtubules between the poles and the centromeres (Fig. 21a). In eight treated plants (63 cells analyzed) about half of metaphase I cells showed abnormal spindles. In 11% of cells the spindle was open or split at the poles (Fig. 21b-d). In 38% of cells the spindle was strongly affected because either the poles or the microtubule bundles were undefined (Fig. 21e, f).

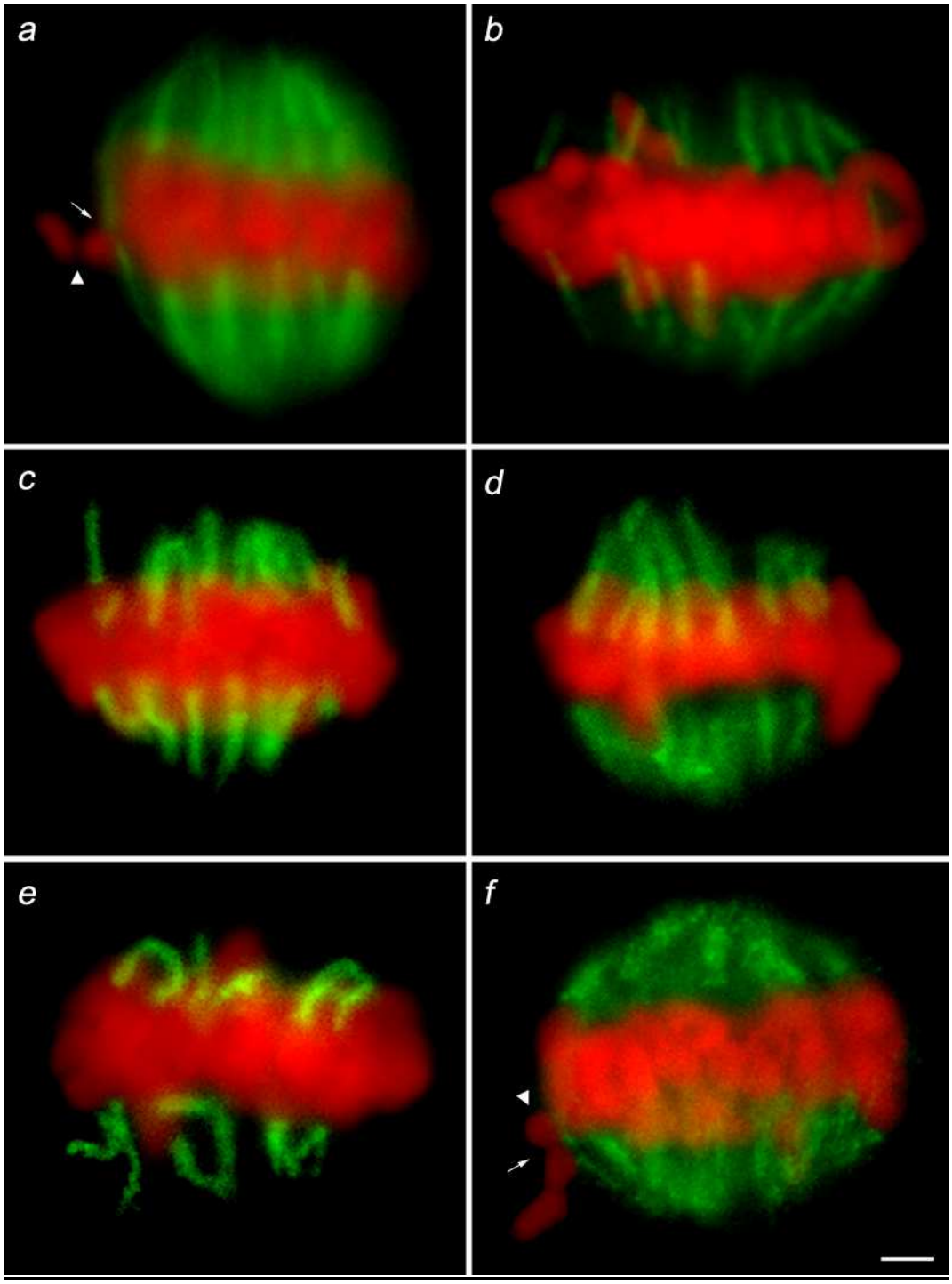

Fig. 21. Immunolocalization of α-tubulin (green) in wheat-5R and wheat-5RL addition lines at metaphase I (chromosomes in red). Bar = 5 µm.

a. Untreated monosomic 5R addition plant with normally shaped spindle. The 5R univalent is amphitelically orientated. A bundle of microtubules is joined to the 5RL constriction (arrow), but microtubules are not joined to the centromere (arrowhead).

b-f. Plants treated with Diazinon. **b**. Ditelosomic 5RL, split spindle. **c**, **d**. Monosomic 5R, split spindle. **e**. Monosomic 5R, the bundles of microtubules are not properly organized from the poles. **f**. Ditelosomic 5RL, the microtubules are altered. Microtubules are joined to the 5RL constriction (arrow) and not to the centromere (arrowhead).

Discussion

DISCUSSION

1. Occurrence of a secondary constriction in chromosome 5R of rye

Chromosome 5R of rye shows a secondary constriction in the long arm (5RL) due to an unusual organization of the heterochromatin of a C-band. The 5RL constriction is visible in different genotypes, including haploid rye, inbred lines and addition lines containing the chromosome 5R (Lamm 1936; Müntzing & Akdik 1948; Schlegel 1987; Manzanero et al. 2000b, 2002; Cuacos et al. 2011). In the present work, the 5RL constriction was found in wheat-5R and wheat-5RL monosomic, disomic, monotelosomic and ditelosomic addition lines as well as in diploid rye. Thus, the formation of the constriction is independent of the ploidy level, the presence of a complete or a misdivided centromere and it may occur in wheat and rye background. However, it is not meiosis-specific as previously reported, as mitotic metaphase cells with the 5RL constriction were observed for the first time. The frequency of the 5RL constriction in mitosis was much lower than in meiosis; likely the constriction is only visible in the highest condensed mitotic chromosomes following cold pretreatments.

The presence of the 5RL constriction in diploid rye was extensively analyzed. In *Secale cereale* cv. Imperial the constriction was consistently found, whereas other varieties showed it with variable frequencies and others never. The occurrence of the 5RL constriction was initially related to inbred lines (Lamm 1936). None of the lines analyzed were maintained by inbreeding and rye is naturally a cross-pollinating species. However, 'Imperial' is a cultivar commonly used in genetic and cytogenetic analyses and frequently the experimental populations are set up from few seeds. Thus, to test if consanguinity was influencing the formation of the constriction, all lines were analyzed recording the mean chiasma frequency (based on bound arms per bivalent) and the presence of univalents. Chiasma frequency at metaphase I decreases during inbreeding generations (studies in rye from Lamm 1936; Müntzing & Akdik 1948; Rees 1955) and univalents are a consequence of the absence of chiasmata.

Growing conditions of the plants, particularly temperature, can also influence the chiasma frequency (e.g. Higgins et al. 2012). In the present work, plants were grown under similar conditions; thus, temperature similarly influenced all rye varieties. Certainly spikes

were collected in different years from April to June, but several spikes from each genotype were collected simultaneously and therefore the effect of the temperature on the chiasma frequency was minimized.

A significant negative correlation between the presence of the 5RL constriction and the mean chiasma frequency was observed, in such a way that 'Imperial' showed the lowest chiasma frequency whereas varieties which never showed the constriction (*S. cereale* cv. 'Merced' and 'Transbaikal' and *S. ancestrale*) presented the highest chiasma frequency. Analysis of 'Imperial' demonstrates that self-pollinated plants (increasing consanguinity) show a higher frequency of the constriction than cross-pollinated plants. Even when a plant produced by cross-pollination was self-pollinated, the frequency of the constriction increased in the progeny. This indicates that consanguinity promotes the occurrence of the constriction, and in all probability, homozygosity for certain loci has an influence on the formation of the constriction.

To confirm this result, plants with ('Imperial') and without (*S. cereale* cv. IPK and Paldang and *S. ancestrale*) the 5RL constriction were reciprocally crossed to analyze the F1 and the F2. After crossing different lines, heterozygosity is increased in the progeny and less occurrence of the constriction would be expected. F1 plants showed increased chiasma frequency and accordingly occurrence of the constriction was reduced. In the F2 obtained by self-pollination the frequency of cells showing the 5RL constriction was increased, demonstrating an important role of homozygosity in the appearance of the constriction. Frequencies of the 5RL constriction in the F1 and F2 progeny do not adjust to the expected segregation of one or two loci, so there could be a possible control of the constriction by a polygenic system. This idea is supported by the variable degree of stretching of the constriction in different cells from conspicuous to subtle, resembling a quantitative trait.

It is noteworthy that the formation of the 5RL constriction in the hybrids was depending on the direction of the cross. In the cross between 'Imperial' and *S. ancestrale*, a vast majority of the progeny showed the 5RL constriction when 'Imperial' was the female parent but not when it was *S. ancestrale*. Such differences were not found in the crosses between 'Imperial' and 'IPK' or 'Paldang', where the 5R constriction was found in both directions. As the cytoplasm in the hybrids comes from the female plant, it seems that in *S. cereale* cytoplasm the constriction can be formed, whereas in *S. ancestrale* cytoplasm it is only rarely formed.

This influence of the cytoplasm might be based on extranuclear inheritance or imprinting. Both mitochondria and chloroplasts are commonly transmitted by uniparental inheritance (Birky 2008) but it is unlikely that organelles DNA influences the organization of the heterochromatin. Rather imprinting, which is the differential expression of alleles depending on the maternal or paternal origin, might influence the organization of heterochromatin. Most imprinted genes in plants are associated with the structure and function of the endosperm (Pignatta & Gehring 2012), but in *A. thaliana* some paternally expressed imprinted genes include transcription factors and genes that encode chromatin remodelers (Gehring et al. 2011). The 5RL constriction arises as a consequence of a different chromatin organization in a C-band. Thus, a possible differential expression of imprinted genes could explain the dissimilar behaviour of the constriction depending on the cross direction. Moreover, the imprinting of most of these genes in *Arabidopsis* is partial (Gehring et al. 2011) and a similar system in rye could explain the variable occurrence of the constriction in the progeny.

Strikingly, heteromorphic bivalents were not found in the hybrids, i.e. when one 5R chromosome showed the 5RL constriction so did the homologous. In addition, in some cases the same 'Imperial' or *S. ancestrale* plant was used as female parent in one cross and as male parent in a different cross, and the presence or absence of the 5RL constriction was only depending on which species was used as female parent. The 5R chromosome from *S. ancestrale* (never showing the 5RL constriction) could show it in the hybrids, and reciprocally the 5R chromosome from 'Imperial' (usually showing the 5RL constriction) could 'lose' it in the hybrids. This indicates that the capacity to form the constriction is equal in the 5R chromosome of both species.

The 5RL constriction was also visible in wheat-5R and 5RL addition lines, where the 5R chromosome from 'Imperial' is in wheat cytoplasm. In this situation, it is possible that the chromatin remodeling factors in the cell come from and are regulated by the wheat genome, and this could alter the normal chromatin organization of the C-band which is predisposed to form a constriction. Alternatively, the 'Imperial' 5R chromosome could have been added to wheat 'with' the constriction and neither the wheat genome nor the cytoplasm has any effect on the chromatin condensation of that region.

Taken into account these results, it can be concluded that the 5RL constriction is genetically and epigenetically regulated, as consanguinity in the plants as well as the cytoplasm influences the occurrence of the constriction (Fig. 22).

2. The 5RL constriction shows neocentromeric activity

The secondary constriction of rye 5R chromosome may behave as a neocentromere in haploid rye, in polyhaploid wheat-rye and wheat-Triticale hybrids and in wheat-5R and 5RL monosomic, disomic, monotelosomic and ditelosomic addition lines (Schlegel 1987; Manzanero et al. 2000b, 2002; Cuacos et al. 2011). In the present work, the activation of the neocentromere in the addition lines was analyzed.

The 5R neocentromere is active in meiosis only. The behaviour of centromeres is different during mitosis and meiosis; for example, a meiosis-specific cenH3 loading mechanism has been described in *A. thaliana* (Ravi et al. 2011; Lermontova et al. 2011) and *Drosophila* (Dunleavy et al. 2012); also, in the holokinetic chromosomes of *Caenorhabditis elegans,* depletion of cenH3 results in severe chromosome alterations during mitosis but not during meiosis. Similarly, a meiosis-specific regulation of the 5R neocentromere might be possible.

In addition, the 5R neocentromere may be active together with the endogenous centromere. The co-existence in one chromosome of a neocentromere together with the canonical centromere is one of the major differences between ‘de novo’ and ‘terminal’ neocentromeres: rye terminal neocentromeres are active in intact chromosomes only (Puertas et al. 2005), whereas most ‘de novo’ neocentromeres arise after loss or depletion of the endogenous centromere (Burrack & Berman 2012) (Table 10). In case of the 5R chromosome, truncation experiments could demonstrate whether this neocentromere may be active in acentric fragments.

The orientation centromere-neocentromere to opposite poles caused the stretching of the constriction until several times the chromosome length. This elasticity is in agreement with observations in amphitelically orientated human mitotic and plant meiotic chromosomes. There, centromeric DNA can stretch remarkably in response to the tension exerted by spindle microtubules (Shelby et al. 1996; Lukaszewski 2010). The elasticity of the constriction could explain why broken chromosomes were not found.

In spite of the atypical orientations displayed by the 5R chromosome when the centromere and the neocentromere were active simultaneously at metaphase I, segregation errors were rarely found. In lines with two copies of 5R or 5RL chromosomes, corresponding rod bivalents could show a U- or V-shape due to the centromere-neocentromere orientation to opposite poles. Occasionally, the two rye chromosomes were incorporated into the same

pole, but this frequency was much lower than the frequency of U- and V-shaped bivalents. In addition, plants with more than two 5R or 5RL chromosomes were not found. This indicates that tension is released before the end of anaphase I and the chromosomes migrate usually to opposite poles. When the centromere and the neocentromere were active simultaneously in the 5R or 5RL univalent, the chromosome was finally incorporated in one of the cell poles, what implies that the tension was released either from the centromere or the neocentromere. Indeed, both situations were observed, indicating that the centromere is not always 'stronger' and that the neocentromere may also lead the chromosome movement to the cell poles.

The kinetic activity of the constriction is based on the interaction with spindle microtubules (Manzanero et al. 2002). However, microtubules were also attached to the constriction in the absence of neocentromeric activity. This interaction occurred in the addition lines and in diploid rye (Manzanero et al. 2002; present work). Similarly, microtubules were associated to heterochromatic regions in maize and rye terminal neocentromeres (Yu et al. 1997; Puertas et al. 2005). This demonstrates that: i) microtubules have affinity for heterochromatic regions in specific situations and ii) the spindle attachment is necessary but not sufficient to promote the 5R neocentromere. In maize, microtubules are accumulated around chromosomes at prometaphase I as an initial step for spindle formation (Chan & Cande 1998). If this mechanism is true also for rye, this would create a favourable environment to allow the interaction of microtubules with the 5RL constriction. It is possible that these ectopic associations of microtubules occur often but they are released in most situations. In addition, Rieder et al. (1993) showed that microtubules have a higher affinity for meiotic than for mitotic chromosomes, and this might explain why the 5R neocentromere has not been observed during mitosis.

The ability to form a neocentromere is not restricted to a specific locus within the constriction, as the kinetic point varied in different cells. In the monotelosomic line, univalents 'type III' have two locations within the constriction showing simultaneously tension to the poles. Other evidence comes from the variable location of the FISH probe pSc119.2 relative to the kinetic point: either co-localizing with the neocentromere, or proximal or distal to the centromere within the constriction.

Remarkably, the 5R neocentromere can keep sister chromatids together at the 5RL constriction or between the centromere and the constriction at anaphase I (Manzanero et al. 2002; present work). Immunostaining with an antibody against rice Shugoshin (Wang et al. 2011) failed to detect the wheat and rye homologous at centromeres. Thus, the involvement of this protein in the chromatid cohesion observed at the 5RL constriction is unclear.

However, the heterochromatic composition of the constriction could determine this property, as heterochromatin is required for sister-chromatid cohesion in fission yeast (Bernard et al. 2001) and vertebrates (Fukagawa et al. 2004) and the lack of heterochromatin was associated with a subtle defect of cohesion at human neocentromeres (Alonso et al. 2010).

A similar morphology to that observed in the 5R chromosome, with a stretched constriction during meiosis, was found in one chromosome of *Aegilops markgrafii* (Schubert 2011). However, in this case the stretching was not due to an active neocentromere: cenH3 was found exclusively at the centromere and microtubules were not attached to the constriction (Schubert 2011). The 5R neocentromere does not possess cenH3 (discussed in section 4), but its neocentromeric activity is supported by its interaction with the spindle, the capacity to lead the chromosome to the pole and to maintain sister chromatids together at anaphase I.

3. The properties of the constriction could determine the neocentromeric activity

The 5R neocentromere arises at a heterochromatic C-band composed of repetitive sequences that forms a constriction. These features seem to be decisive for the neocentromeric activity.

Canonical centromeres are typically found at heterochromatic regions (Henikoff et al. 2000). Similarly, neocentromeres are frequently associated with heterochromatic regions, e.g. in fission yeast (Ishii et al. 2008), in *Drosophila* (Platero et al. 1999; Olszak et al. 2011), and in plant terminal neocentromeres (Dawe & Hiatt 2004). Human neocentromeres occur preferentially within euchromatic domains (Alonso et al. 2010), but Saffery et al. (2000) reported that Heterochromatin Protein 1 (HP1) was recruited in the otherwise euchromatic region of the neocentromeres.

The occurrence of the neocentromere within a constriction is in agreement with canonical centromeres of monocentric chromosomes and with human neocentromeres, both forming a constriction. In dicentric chromosomes, only the active centromere forms a constriction (Warburton 2004; Stimpson et al. 2012).

In addition, although centromeric sequences seem not to be determinant for the centromeric activity (reviewed for example in Sekulic & Black 2012), a common feature of the centromeric DNA in most species is that it is located within large arrays of repetitive DNA, mainly satellites and in plants also retrotransposon-derived sequences. The two DNA sequences found at the 5RL constriction meet this standard, as pSc119.2 is a satellite and UCM600 is a retrotransposon-derived sequence. Moreover, it is likely that these two sequences are not the only ones in the constriction, because FISH with pSc119.2 and UCM600 simultaneously revealed unlabelled regions within the highly stretched constriction. Thus, accumulation of repetitive DNA in this heterochromatic region could generate a higher-order structure supporting the kinetic activity, possibly by recruiting proteins that mediate the interaction with the spindle.

The precise role of pSc119.2 in the neocentromeric activity is unclear. This sequence does not always co-localize with the kinetic point suggesting that it is not responsible for the neocentromeric activity. The monomer length of this satellite is 120 bp (Bedbrook et al. 1980; McIntyre et al. 1990) and it is not in the range of the typical centromeric satellites unit length (around 150-180 bp) (Henikoff et al. 2001). Furthermore, this sequence is also present in the subtelomeric region of chromosome 5R, and this region was never observed active as a neocentromere in the addition lines. However, in the present work, terminal neocentromeres where pSc119.2 was stretched to the poles were found in one rye cultivar. Previous results demonstrated that pSc200, and not pSc119.2, was involved in the activity of rye terminal neocentromeres (Manzanero & Puertas 2003). The novel finding of pSc119.2 participating in the activity of terminal neocentromeres shows the capacity of this sequence to interact with microtubules and thus it could also contribute to the kinetic activity at the 5RL constriction.

It has been proposed that terminal neocentromeres could be a vestige of a proto-centromeric activity located at telomeric regions (Puertas & Villasante 2013) in the context of the hypothesis from Villasante et al. (2007) which suggests that centromeres were derived from telomeres during the evolution of the eukaryotic chromosome. During the evolution of rye chromosomes several translocation events occurred, as demonstrated by cytogenetic (Naranjo & Fernández-Rueda 1991, 1996) and molecular (Devos et al. 1993) studies. Interestingly, a translocation of the subtelomeric region of chromosome 4R to chromosome 5R occurred (Naranjo et al. 1987). The interstitial C-band where the neocentromere arises could have occupied a terminal kinetically active domain before the translocation event and the constriction might have retained kinetic activity. Alternatively, other chromosome rearrangements explaining the neocentromeric activity of chromosome 5R could have occurred, but information about karyotype evolution in rye is limited. Recently, Milczarski et

al. (2011) have developed an extensive linkage map of rye which may contribute to extend evolutionary analyses on rye chromosomes.

Nevertheless, pSc119.2 was involved in the terminal neocentromeres in a plant that neither show the 5RL constriction nor the 5R interstitial neocentromere. Thus, even if the kinetic activity is comparable in both the interstitial and the subtelomeric region, the regulation of both types of neocentromere seems to be different. In maize, the two repetitive DNA sequences involved in terminal neocentromeres in this species (180 bp and 350 bp-TR1) are independently regulated by (at least) two genes (Hiatt et al. 2002; Mroczek et al. 2006). Crossing terminal-neocentromere plants with 5R-neocentromere plants could show if both kinetic activities can occur simultaneously or not.

Non-coding RNAs transcribed from (peri-)centromeric regions are likewise involved in proper centromere function (reviewed in Hall et al. 2012). Also, in a human neocentromere the requirement of a LINE (Long INterspersed Elements) retrotransposon-derived RNA for proper neocentromeric activity was shown (Chueh et al. 2009). Whether non-coding RNAs derived from sequences located in the 5R constriction exist is unknown, but elucidate this will help to understand the 5R neocentromere structure and regulation.

4. Typical centromeric proteins are not detected at the 5R neocentromere

The presence of proteins at the 5RL constriction was demonstrated by Manzanero et al. (2002) based on silver staining. In the present work, immunostaining experiments were performed to disclose whether known (peri-)centromere-associated proteins and histone modifications are present at the 5R neocentromere.

The histone variant cenH3 was detected at wheat and rye centromeres using an antibody against rice cenH3 (Nagaki et al. 2004) but not at the 5R neocentromere. Facing the possibility that the amount of cenH3 in the constriction was under the resolution level of fluorescence Wide-Field Microscopy (WFM), Structure Illumination Microscopy (SIM) was employed but cenH3 was not detected in the constriction either. Therefore, the 5R interstitial neocentromere apparently lacks cenH3.

cenH3 is typically found at active centromeres. However, in *C. elegans* the meiotic kinetochore is assembled in the telomeric region of the chromosomes in a cenH3-independent way and even cenH3 is not detected during male meiosis (Monen et al. 2005). In addition, the role of cenH3 in the centromeric function may vary in different organisms. In *Drosophila*, cenH3 is sufficient to recruit kinetochore proteins and drive kinetic activity (Heun et al. 2006). In contrast, in human cells ectopic cenH3 recruited some proteins but it was not sufficient to promote neocentromeric activity (Van Hooser et al. 2001; Gascoigne et al. 2011). It has been suggested that cenH3 could only indicate the locus where the kinetochore must assemble. In the case of *C. elegans*, this locus is chosen depending on the location of a single chiasma (Monen et al. 2005) not depending on cenH3. In case of the 5R neocentromere, the kinetic site is pre-determined to the secondary constriction and the lack of cenH3-containing chromatin could support the variable location of the neocentromere within the constriction. Evidence supporting the hypothesis of cenH3 as a mere indicator of the centromeric locus comes from a work in human cells where the ectopic localization of CENP-C and the complex CENP-T/W was sufficient to recruit all the other kinetochore proteins bypassing the presence of cenH3 (Gascoigne et al. 2011). Thus, the absence of cenH3 could not interfere with a possible recruitment of other kinetochore proteins in the 5RL constriction.

As CENP-C was able to recruit kinetochore proteins in humans (Gascoigne et al. 2011) and was detected in human and barley neocentromeres (Saffery et al. 2000; Nasuda et al. 2005), its presence at the 5R neocentromere was analyzed. An antibody against maize CENP-C (Dawe et al. 1999) recognized wheat and rye centromeres but it was not detected in the 5RL constriction. In maize, Dawe et al. (1999) reported the existence of three *CENP-C* genes. The antibody generated failed to recognize one of the CENP-C proteins. Whether in rye there are more than one copy of the *CENP-C* gene is unknown, but a different CENP-C variant could exist in the constriction being not recognized by this antibody.

An antibody against maize MIS12 (Li & Dawe 2009) was employed, cross-reacting with rye and wheat centromeres. Unfortunately, in the addition lines it produced a strong background. Thus, it is unclear whether it localizes at the 5RL constriction. In rye an exclusive centromeric pattern was observed in most of the cells except one cell (discussed in section 5). MIS12 is thought to interact directly with cenH3 and CENP-C (Li & Dawe 2009). Therefore, the absence of these two proteins at the neocentromere would also explain the absence of MIS12. However, as many kinetochore proteins have not been identified in plants yet, a different regulation and/or protein-interactions could exist in plant kinetochores.

Additionally, an antibody against maize NDC80 (Du & Dawe 2007) was tested. This protein links the kinetochore with the spindle in plants, as it interacts with MIS12 and microtubules. Unfortunately, the antibody against the maize protein failed to detect corresponding homologous at rye or wheat centromeres.

Typically active centromeres are marked by distinct epigenetic modifications of centromeric and pericentromeric histones, particularly H3. Antibodies against some of these modifications were tested to check their presence at the 5RL constriction.

The labelling of an antibody against histone H3S10ph was (peri-)centromeric during the second meiotic division but during the first division the chromosomes were labelled along their entire length, as reported before (Manzanero et al. 2000a). Contrary, wheat and rye centromeres were H3S28ph-hyper-phosphorylated at metaphase I, as previously described (Gernand et al. 2003), but not the 5RL constriction. Additionally, an anti-histone H2AT133ph antibody from maize (Dong & Han 2012) was tested. Similarly to H3S28ph, the maize antibody cross-reacted with wheat and rye pericentromeric regions but it was not commonly found at the constriction. An unexpected labelling of the 5RL constriction with this antibody in rye will be discussed in section 5.

In wheat and the 5R chromosomes, H3K27me3 appeared mainly in terminal heterochromatic domains in agreement with previous observations (Carchilan et al. 2007). The antibody did not detect this modified histone at the centromeres and, interestingly, it neither labelled the 5RL constriction. As this modification is associated with a silenced chromatin state (repressive transcription) (Fuchs et al. 2006), the absence of this mark in the 5R centromere and the 5RL constriction could suggest transcriptional activity of sequences located within these regions. In future, it will be interesting to check if histone modifications associated with ‘open’ chromatin (permissive transcription), such as H3K4me3 (Carchilan et al. 2007), localize at the 5RL constriction.

Several additional histone modifications were checked but the results obtained were not informative. Thus, it was not possible to determine further similarities or differences between the centromere and the 5RL constriction with regards to epigenetic modifications.

It can be concluded that the 5R neocentromere does not bind typical centromere-associated proteins. The unavailability of antibodies against additional kinetochore proteins hampered their identification. Moreover, histone modifications detected at the 5R centromere were absent at the 5RL constriction. Only H3K27me3 was absent in both regions.

5. Active 5R neocentromere in diploid rye

A 5R bivalent in one rye meiocyte showed a morphology resembling that of 5R and 5RL bivalents from the addition lines with an active neocentromere in each 5R chromosome simultaneously with the centromeres. This is the first time that the 5R neocentromere is found in diploid rye.

In addition, immunostaining revealed an atypical hybridization pattern of MIS12 and H2AT133ph in diploid rye. After immunostaining with an anti-MIS12 antibody, one metaphase I cell was found with intense labelling in the centromeres and also two minor marks at the constriction in each 5R chromosome. These symmetric marks could be explained by the presence of the protein at both sister chromatids. Moreover, spindle microtubules seem to be attached there. Besides that, three cells were found where an anti-histone H2AT133ph antibody labelled the constriction and was absent at the centromeres. Despite the centromeric localization of H2AT133ph in most cells, this result opened the possibility that under specific circumstances the heterochromatin from the 5RL constriction could acquire epigenetic changes permissive for a kinetic activity.

Due to the low frequency of these patterns, it is unclear if a transient presence of MIS12 and H2AT133ph at the constriction occurs, allowing the activation of the 5R neocentromere.

6. A pesticide promotes the neocentromeric activity due to alterations of the spindle

Manzanero et al. (2000b, 2002) reported a significant variation in the frequency of the 5R neocentromere in two successive generations. Therefore, the authors proposed the influence of an environmental factor on the neocentromeric activity. Later on, it was discovered in our lab that the commercial pesticide Diazinon, containing organophosphate derivatives, was responsible for the high occurrence of the 5R neocentromere (Cuacos et al. 2011).

In all the addition lines the frequency of cells with the 5R neocentromere increased significantly from untreated to treated plants up to the frequencies reported by Manzanero et al. (2000b), demonstrating that this was the environmental factor promoting the

neocentromeric activity. The low frequencies observed in untreated plants represent a basal level of neocentromeric activity which might be explained by the properties of the 5RL constriction.

Frequencies were highly variable, but comparing untreated with treated spikes from the same plant confirmed that the frequency was always significantly increased by the pesticide treatment. In general, frequencies were slightly higher in monotelo- and monosomic than in ditelo- and disomic lines. Possibly, biorientation of bivalents disfavours the interaction of microtubules with the constriction. This interaction could occur easier when the univalent lies at the metaphase plate with syntelic and particularly with amphitelic orientation.

The effect of Diazinon was dosage- and time-independent. Additional treatments did not increase the frequency of the 5R neocentromere, likely because the amount of pesticide used in each treatment (recommended by the manufacturer) represented the saturation level for the plants to incorporate it. Supporting this idea, some of the highest frequencies of neocentromeric activity were registered with only one treatment. On the other hand, plants showed the maximum frequency of cells with the 5R neocentromere one day after the treatment and this effect did not decrease until ten days later; possibly because the product remained in the soil and the plant continued absorbing it.

The high frequency of neocentromeric activity is not a heritable feature; on the contrary, it arose newly every year only after pesticide treatment. When the pesticide was not used, the frequencies recorded were low (basal level). In addition, the self-pollinated progeny from treated plants showed the same frequency of cells with the 5R neocentromere than the self-pollinated progeny from untreated plants. This means that the pesticide is not inducing permanent changes in the chromosomes that are transmitted to the next generation.

An interesting hypothesis was that the pesticide promotes the 5R neocentromere by inducing changes in the spindle. It is known that herbicides containing carbamates disrupt mitosis as they affect microtubule organizing centers (MTOCs) splitting the spindle poles (Hepler & Jackson 1969; Yemets et al. 2008) and disrupting the orientation of microtubules (Coss & Pickett-Heaps 1974). In spite of the different composition of organophosphate- and carbamate-derivatives and the lack of reports of the effect of carbamates during meiosis, a similar effect on the microtubule system could occur following Diazinon treatments in the addition lines.

To test this hypothesis, immunostaining with an anti-α-tubulin antibody was performed in meiocytes from untreated and treated plants. In treated plants about half of the spindles were altered in different ways: in several cells spindles were divided and microtubules coming out of the MTOC were found; in other cells the microtubules were diffused or not well organized. It is possible that these spindle alterations promote the interaction of microtubules with the constriction and consequently the neocentromeric activity is increased. However, in spite of the spindle disturbances, wheat bivalents appeared well located at the metaphase plate and segregated correctly, and micronuclei (indicative of segregation errors) were not observed. Therefore the effect of Diazinon on the meiotic spindle was not as strong as that of carbamates during mitosis.

Alternatively, the pesticide could increase the frequency of the 5R neocentromere by several other mechanisms, e.g. it could interact directly with the constriction facilitating the binding of chromatin remodelers or promoting epigenetic changes.

The analysis of neocentromeric frequencies in untreated and treated plants revealed interesting differences in the behaviour of the chromosomes when the neocentromere was promoted. Thus, in the ditelosomic addition line the frequency of non-oriented bivalents (type VI) was strongly reduced from untreated to treated plants, indicating that the neocentromere could help to orientate the bivalent at metaphase I. In the monotelosomic addition line the frequency of type IA univalents (amphitelically orientated at metaphase I) experimented the highest frequency decrease from untreated to treated plants. Lukaszewski (2010) reported that separating chromatids was the most common feature of wheat univalents at anaphase I. In untreated plants around 50% of the cells showed a biorientated 5RL univalent parallel to the metaphase plate, but this frequency was reduced to around 35% in treated plants. Therefore, the neocentromere might help to orientate properly the univalent so that it migrates complete to one pole at anaphase I. This resembles the situation found in maize plants carrying one additional B chromosome, where the repetitive subtelomeric sequence ZmBs could co-orientate with the centromere to opposite poles and orientate the B univalent syntelically at metaphase I (González-Sánchez et al. 2007).

7. Concluding remarks

It has been shown in the present work that the 5RL constriction may behave as a neocentromere representing a third neocentromere type, which shows features of both 'terminal' and 'de novo' neocentromeres (Table 10).

Table 10. Comparison between the two types of neocentromeres previously described and the 5R neocentromere.

Feature	'De novo' neocentromere	'Terminal' neocentromere	5R neocentromere
Arise in centric or acentric chromosomes	Acentric	Centric	Centric (Acentric?)*
Substitute centromeric function	Yes	No	Yes (sometimes)
Genomic location	Eu- and heterochromatin	Subtelomeric heterochromatin	Interstitial heterochromatin
Visible constriction	Yes	No	Yes
Presence of cenH3	Yes	No	No
Centromeric proteins and histone modifications	Yes	No	No
Interaction with spindle	Yes (end-on)	Yes (lateral in maize, end-on in rye)	Yes (end-on)
Active in mitosis / meiosis	Yes / Yes	No / Yes	No / Yes
Maintain sister-chromatid cohesion	Yes	No in maize, Yes in rye	Yes
Species where they are described	Human, *Drosophila*, yeasts, maize, barley	Plants (e.g. maize, rye)	Rye, wheat-rye addition lines

*It has not been tested whether the 5R neocentromere may be active in an acentric fragment.

The heterochromatic composition and organization of the constriction seem decisive for the neocentromeric activity. However, the neocentromere is not always active in the situations where the constriction is visible, and the constriction only arises in specific situations. Figure 22 summarizes the parameters that may influence the occurrence of the 5RL constriction and the activation of the neocentromere.

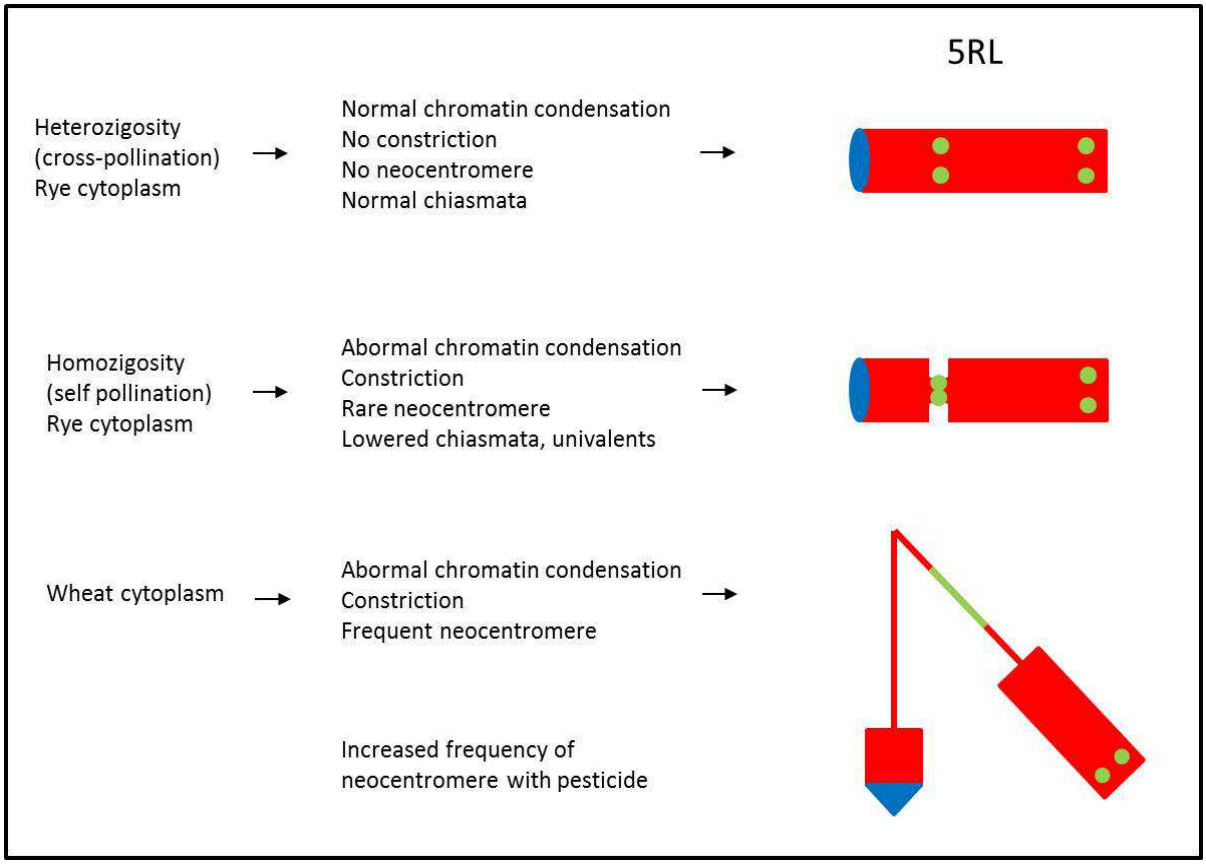

Fig. 22. Requirements for the occurrence of the 5RL constriction and the activation of the 5R neocentromere. It is represented the long arm of chromosome 5R (red), the centromere (blue) and the localization of the repetitive sequence pSc119.2 (green).

- In standard genotypes, such as cross-pollinated diploid rye, the centromere is active, the constriction is not visible and the neocentromere is inactive.
- In inbred lines of diploid rye the constriction is visible due to an abnormal chromatin condensation which is under genetic and epigenetic control. Rarely the neocentromere becomes active.
- In addition lines (where the 5R chromosome is in an alien cytoplasm) the constriction is always visible and the neocentromere is frequently active. The use of pesticides increases the frequency of the 5R neocentromere due to (at least) alterations in the spindle.

An ultimate hypothesis is that chromosome 5R could possess a permanent dicentric structure, in such a way that in diploid rye the neocentromere is silenced by a mechanism which is absent in the wheat background. The endogenous centromere would be ‘dominant’ on the neocentromere, but the neocentromere could occasionally be the leading force. Indeed, in the addition lines cells were found where the active neocentromere was substituting an inactive centromere; where none of them were active; and where both centromere and neocentromere were active simultaneously.

Conclusions

CONCLUSIONS

- The formation of the 5RL constriction is genetically and epigenetically regulated. It is influenced by consanguinity, it appears exclusively in certain genomic backgrounds and imprinting could be one of the reasons for the 'maternal effect' observed.

- The 5RL constriction behaves as a neocentromere in meiosis I in wheat-5R and 5RL addition lines, interacting with spindle microtubules, leading the chromosome movement to the poles and keeping sister chromatids together at anaphase I. This neocentromere is also active in diploid rye but with lower frequency.

- The basal frequency of the 5R neocentromere in the addition lines is significantly increased when plants are treated with a commercial pesticide. The pesticide treatment induces spindle alterations that could facilitate the interaction between microtubules and the neocentromere.

- The 5R neocentromere neither forms a kinetochore with typical centromeric proteins nor typical pericentromere-associated histone modifications are found. The results suggest a cenH3 independent neocentromeric activity during meiosis. The heterochromatic nature of the constriction likely enables kinetic activity.

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Appendix

Original Article

Cytogenetic and Genome Research

Cytogenet Genome Res 2011;134:151–162
DOI: 10.1159/000325744

Accepted: December 14, 2010
by B. Friebe
Published online: May 5, 2011

Activation of Rye 5RL Neocentromere by an Organophosphate Pesticide

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Key Words
Centromere · Chromosome 5R · Diazinon · Neocentromere · Rye · Wheat

Abstract
An interstitial constriction located on the long arm of rye chromosome 5R (5RL) shows neocentromeric activity at meiosis. In some meiocytes this region is strongly stretched orienting with the true centromere to opposite poles at metaphase I, and keeping sister chromatid cohesion at anaphase I. We found previously that the frequency of neocentric activity varied dramatically in different generations suggesting the effect of environmental factors. Here we studied the behavior of the 5RL neocentromere in mono- and ditelosomic 5RL, and mono-, and disomic 5R wheat-rye addition lines, untreated and treated with an organophosphate pesticide. The treated plants form neocentromeres with an about 4.5-fold increased frequency compared to untreated ones, demonstrating that the pesticide promotes neocentric activity. The neocentromere was activated irrespectively of the pairing configuration or the presence of a complete or truncated 5R centromere. Fluorescence in situ hybridization (FISH) with 2 repetitive sequences (UCM600 and pSc119.2) present at the constriction showed kinetic activity at several locations within this region. Immunostaining with anti-α-tubulin showed that treated plants have abnormal spindles in 46% of the metaphase I cells, indicating that disturbances in spindle formation might promote neocentromere activation.

A neocentromere is a chromosomal locus with kinetic activity outside the proper centromere. They have been described in animals and plants, with different characteristics. In animals they appear in somatic cells in chromosomes lacking a functional centromere. Human neocentromeres [Voullaire et al., 1993; du Sart et al., 1997] are the best characterized ones to date [Choo, 1998]. They arise in mitotically stable marker chromosomes and lack detectable quantities of α-satellite [Choo, 1997]. However, they assemble a functional kinetochore with the centromeric histone CENP-A, and other kinetochore proteins including CENP-C and -E [Choo, 1997; Saffery et al., 2000]. *Drosophila melanogaster* shows neocentromeres in acentric mini-chromosomes in regions adjacent to the centromere, with kinetochore proteins as well [Williams et al., 1998; Maggert and Karpen, 2001].

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On the other hand, classic plant neocentromeres are those found in intact chromosomes in addition to a standard centromere, they are active during meiosis, and may orient with the true centromere to opposite poles [reviewed in Guerra et al., 2010]. Recently, 2 neocentromeres have been described which constitute an exception of this general rule as they are de novo formed centromeres and fully substitute regular centromeres. One was reported in a barley chromosome added to common wheat after breakage [Nasuda et al., 2005]. Centromeric proteins were present at this site, unlike already known centromeric sequences. The fragments were stable at mitosis (comparable to animal neocentromeres) and meiosis. The second one was reported in a maize chromosome included in oat background [Topp et al., 2009]. It was similarly stable during mitosis, with the centromeric histone CENH3 (corresponding to CENP-A in humans) present at the neocentric site but without centromeric sequences. In contrast to the neocentromeres formed in *Drosophila* and barley, the maize neocentromere appeared at a locus distal from the centromere, which makes unlikely an extent of the centromere determinants to adjacent regions.

Classic plant neocentromeres are documented in 12 spermatophyte species and a moss [Dawe and Hiatt, 2004], but the best characterized are the terminal neocentromeres of maize and rye. In maize, neocentric activity arises at terminal heterochromatic domains (knobs) in the presence of the abnormal chromosome 10 [Rhoades and Vilkomerson, 1942; Peacock et al., 1981]. In that situation, chromosome arms are strongly directed polewards and the chromosome behaves as di- or polycentric. Two tandemly repetitive sequences may be present at knobs: a 180-bp and a 350-bp (TR-1) repeat [Peacock et al., 1981; Ananiev et al., 1998]. Kinetochore protein CENH3 [Zhong et al., 2002] does not localize at knobs [Dawe and Hiatt, 2004]. They interact with spindle microtubules, although in a lateral manner instead of in the end-on manner, typical of maize centromeres [Yu et al., 1997].

Katterman [1939] was the first to describe neocentromeres in rye. This species has 2 kinds of neocentromeres with distinct features. The 'terminal neocentromeres' were initially described in inbred lines [Katterman, 1939; Prakken and Müntzing, 1942; Rees, 1955], but later research identified neocentric activity in open pollinated varieties [Kavander and Viinikka, 1987; Manzanero and Puertas, 2003]. Analysis of segregation revealed that this neocentric activity may be controlled by 2 transacting genes [Puertas et al., 2005]. Neocentromeres may occur in all chromosomes of the normal set, but they are more frequent when terminal C-banded heterochromatic blocks are present. The repetitive subtelomeric sequences pSc34 and pSc74 were found to hybridize at the neocentromeres. Immunolocalization of α-tubulin revealed an end-on interaction with the spindle microtubules [Manzanero and Puertas, 2003].

The second type of rye neocentromeres is located in a proximal constriction present in the long arm of chromosome 5R (5RL) and was first described by Schlegel [1987] in haploid 'Petka' rye. This interstitial neocentromere was described in different plant materials: haploid rye, wheat-rye hybrids, *Triticale*-wheat hybrids, the monosomic 5R wheat-rye addition line [Schlegel, 1987] and in the 5RL monotelosomic and ditelosomic wheat-rye addition lines [Manzanero et al., 2000a, 2002]. The constriction is located at the interstitial heterochromatic C-band 5RL1–3 [Mukai et al., 1992; Cuadrado et al., 1995]. This constriction was observed in other materials where the neocentromere was not reported, as in inbred lines [Heneen, 1962] and some varieties of diploid rye [Levan, 1942].

Manzanero et al. [2000a, 2002] detected the neocentric activity by the orientation of this region with the centromere to opposite poles. They also reported that sister chromatid cohesion was kept in that region at anaphase I, which is another of the necessary functions of centromeres. The analysis by fluorescence in situ hybridization (FISH) showed that neither centromeric nor telomeric sequences were constituent of the constriction, but the subtelomeric repetitive sequence pSc119.2 was present in it [Bedbrook et al., 1980; McIntyre et al., 1990]. Immunolocalization with anti-α-tubulin and silver staining showed that centromeric and neocentromeric sites had a similar behavior, because microtubules were bound to the constriction in an end-on fashion and proteins were accumulated from metaphase I to anaphase II [Manzanero et al., 2002].

The frequency of neocentric activity varied dramatically in different generations [Manzanero et al., 2002], suggesting that an environmental factor could be promoting neocentric activity. In the present study we report that an organophosphate pesticide acts as neocentromere inductor.

Materials and Methods

Wheat-5RL monotelosomic and ditelosomic addition lines, and wheat-5R monosomic and disomic addition lines were used. Monotelo- and ditelosomic lines involve the addition of 1 and 2 copies, respectively, of the long arm of chromosome 5R of *Secale cereale* (2n = 2x = 14) var. Imperial to *Triticum aestivum* (2n =

6x = 42) cv. Chinese Spring. Mono- and disomic lines carry 1 or 2 copies, respectively, of the whole chromosome 5R. Plants of these genotypes were selected in the offspring of stocks produced by E.R. Sears [Driscoll and Sears, 1971].

Plants were grown in a greenhouse and treated before meiosis with the commercial pesticide diazinon (COMPO), adding 75 mg of diazinon per pot, as the dosage recommended by the manufacturer (30 g/m^2). Plants were treated 1–6 consecutive times to check the possible increase in the activation of the neocentromere. Other plants growing in different pots in the same greenhouse were untreated for control. Spikes were collected at meiosis 1–10 days after the last treatment, to test if the effect of the pesticide varied with time. When possible, before the first pesticide treatment, 1 spike was collected at meiosis to be used as untreated control of the same treated plants.

Spikes for FISH were fixed in ethanol:chloroform:acetic acid 3:2:1, in vacuum at 400 mm Hg during 10 min and stored at 4°C during 48 h. Then they were transferred to ethanol:acetic acid 3:1 and stored at 4°C. The appropriate meiotic stage was determined by anther squash in 1% acetocarmine, and anthers of the same flower were stored in ethanol:acetic acid 3:1 at 4°C. FISH was carried out as described in González-García et al. [2006].

The following DNA probes were used: (i) Bilby [Francki, 2001], specific to the rye centromeric region, kindly provided by Dr. P. Langridge (Univ. of Adelaide); (ii) the subtelomeric probe pSc119.2, derived from *S. cereale* containing a 120-bp family subclone from pSc119 [McIntyre et al., 1990]; (iii) the rye-specific UCM600, dispersed throughout the rye chromosomes, isolated in our laboratory as a 592-bp fragment from the rye-specific dispersed repetitive family R173 [Rogowsky et al., 1992].

UCM600 was biotin-labeled and detected with streptavidin-conjugated Cy3 (Sigma). Bilby and pSc119.2 were labeled with digoxigenin and detected with mouse anti-digoxigenin and anti-mouse fluorescein isothiocyanate (Sigma) as primary and secondary antibodies, respectively. Slides were counterstained with 4′,6-diamidino-2-phenylindole and mounted in anti-fade Vectashield.

For immunostaining, anthers were fixed during 45 min in freshly prepared 4% (w/v) paraformaldehyde solution containing the microtubule-stabilizing buffer MTSB (50 mM PIPES, 5 mM $MgSO_4$, 5 mM EGTA, pH 6.9), washed for 4 × 10 min in MTSB and digested at 37°C for 25 min in a mixture of 2.5% pectinase, 2.5% cellulase Onozuka R-10 and 2.5% pectolyase Y-23 (w/v) dissolved in MTSB. Anthers were then washed 3 × 5 min in MTSB. For slide preparations we adapted the technique of López-Fernández et al. [2009]. Anthers were gently disaggregated on pre-treated slides provided in the kit Halomax Proto-Tinca (ChromaCell SL, Madrid, Spain), with 20 μl of MTSB and 40 μl of low melting point agarose (1% agarose provided in the kit), and then covered with a coverslip. The slide was then placed on a cold metal plate at 4°C for 5 min to allow the agarose to set into a thin microgel. In this way all pollen mother cells are kept in the microgel. The coverslip was then gently removed and slides were immersed in MTSB until immunostaining treatment. Immunostaining with anti-α-tubulin was made following Manzanero et al. [2000b].

FISH and immunostained slides were examined using an Olympus BX60 fluorescence microscope and photographed with a CCD digital camera. Images were optimized for best contrast and brightness with Adobe photoshop 8.0.1.

Results

In wheat-rye addition lines, the rye chromosome was unambiguously identified with the rye-specific probes UCM600 (red in FISH figures) and Bilby (green in FISH figures). UCM600 labels the whole 5R rye chromosome, with the exception of the centromeric region, whereas Bilby is specific to the rye centromere.

The 5R chromosome shows a constriction in the long arm located at about one third of the arm length from the centromere. The constriction is conspicuous in all metaphase I cells, and appears more stretched in a variable number of cases behaving as a neocentromere that orients with the true centromere to opposite poles. The neocentric activity was evaluated by the morphology of the uni- or bivalent configuration at metaphase I and the frequency of cells showing neocentromere.

5RL Monotelosomic Line

The 5RL telochromosome showed 5 types of configurations at metaphase I (fig. 1). Type I represents the most common situation, when the neocentromere is not active. The 5RL univalent may show syntelic (fig. 1a, type IS) or amphitelic (fig. 1b, type IA) orientation at metaphase I. The neocentric activity is observed when the constriction is stretched due to the tension produced by the orientations of the centromere and the neocentromere to opposite poles (fig. 1c, d, type II). In very few cells the neocentric activity appears at both ends of the constriction (fig. 1e, type III) whereas the centromere seems inactive. In other cases the 5RL is neither oriented by the centromere nor the neocentromere (fig. 1, type IV).

The frequencies of these cell types in plants untreated and treated with the pesticide are shown in table 1. The most frequent configuration of 5RL univalent is type I (either IS or IA). Type II neocentromeres are observed in untreated plants but in a low frequency (6.38%). In treated plants the mean frequency of cells showing neocentromere activity increases to 22.35%. A contingency χ^2 test showed significant differences in the number of cells with neocentromeres between the treated and untreated plants ($\chi^2 = 45.23$, $p = 0.000$). However, the increase in the frequency of neocentromeres is variable between plants, and even in different anthers of the same plant. For example, neocentromere frequencies of 11.76 and 26.92% were found in 2 anthers of the treated plant MT5RL-4, although both frequencies are higher than the 3.08% found in the same plant before treatment.

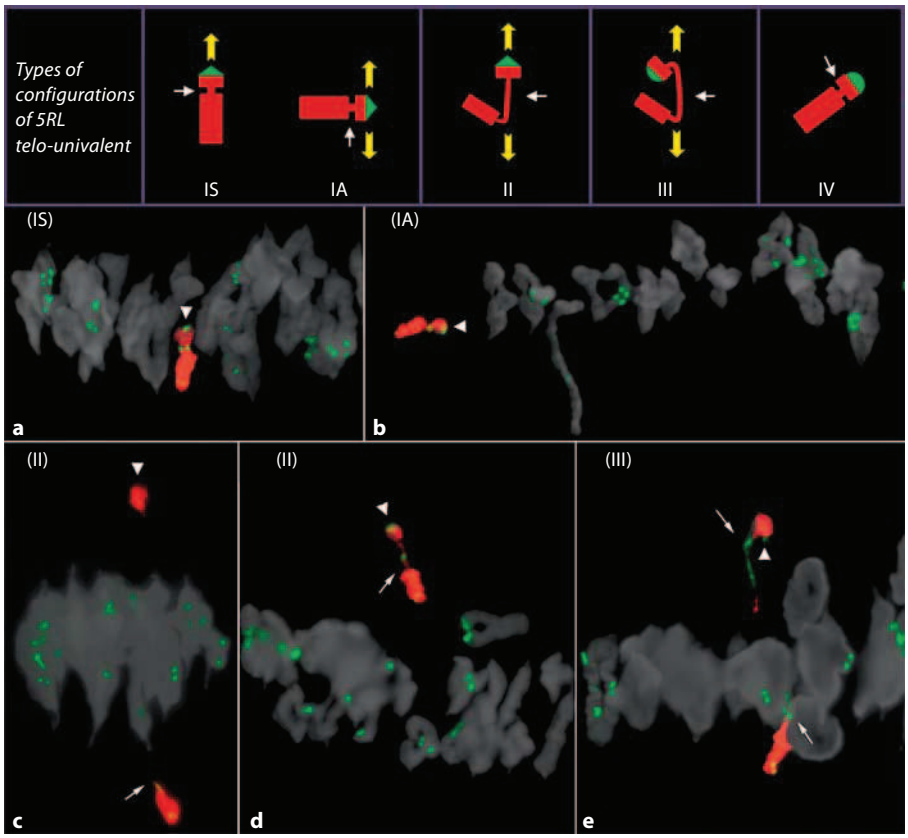

Fig. 1. Upper row. Scheme of the 5RL univalent configurations at metaphase I in the monotelosomic addition line. The green triangles represent centromeres with tension to the poles, the semicircles centromeres without tension to the poles. Yellow arrows indicate tension to the poles. Type I: visible constriction (white arrows) but inactive neocentromere; the univalent may show syntelic (IS) or amphitelic (IA) orientation. Type II: constriction stretched by the tension between the centromere and the neocentromere. Type III: neocentromere activity at both ends of the constriction; the centromere does not show tension to the poles. Type IV: univalent without orientation. Second and third rows. FISH. The 5RL is labeled with UCM600 (red) and the rye centromere with Bilby (green). The pSc119.2 probe partially labels the 5RL constriction and 10 wheat bivalents at subtelomeric positions (green). Arrowheads and arrows point to the centromeres and the neocentromeres, respectively. **a** Metaphase I, 5RL in syntelic orientation (IS). **b** 5RL in amphitelic orientation (IA). **c**, **d** 5RL showing the constriction stretched due to neocentric activity: centromere and neocentromere show tension to opposite poles (type II). **e** 5RL oriented to the opposite poles by 2 sites within the constriction (type III), centromere without tension. In **d** the pSc119.2 signal occupies a central position in the constriction, whereas in **e** it is proximal and in **c** it is distal to the centromere.

We did not find significant correlation between the number of treatments and the frequency of neocentromeres ($r = 0.4517$, $p = 0.0910$), or the number of days after the treatment and the frequency of neocentromeres ($r = -0.2635$, $p = 0.4079$).

The number of cells with univalents showing amphitelic or syntelic orientation did not differ in treated or untreated plants ($\chi^2 = 2.79$, $p = 0.095$). Similarly, the number of cells with univalents showing oriented centromere (types I, II, III) versus univalents showing nonoriented centromere (type IV) did not differ in treated or untreated plants ($\chi^2 = 1.14$, $p = 0.28$).

5RL Ditelosomic Line

The 2 copies of the 5RL chromosome pair in a bivalent with different morphologies depending on the activation of the neocentromeres. The types established for this line are shown in figure 2, following those reported by Manzanero et al. [2000a].

Type I is the most common situation forming a rod bivalent (fig. 2a). The ditelobivalent may be V-shaped with both centromeres oriented to the same pole and the neocentromere in one of the 5RL chromosomes to the opposite pole (fig. 2b, type II). The ditelobivalent may be U-shaped with 1 neocentromere in each 5RL chromosome,

Table 1. Cell types and frequencies of cells showing neocentric activity observed in the monotelosomic 5RL wheat addition line treated and untreated with the pesticide

Plant	Treatment	Cells without neocentric activity, n			Cells with neocentric activity, n		Frequency of cells with neocentromeres, %	Cells, n
		IA	IS	IV	II	III		
MT5RL-6	Control	37	18	3	1	0	1.7	59
MT5RL-17	Control	26	20	8	1	0	1.8	55
MT5RL-4	Control	35	25	3	2	0	3.1	65
MT5RL-1	Control	66	44	4	11	0	8.8	125
MT5RL-14	Control	17	18	4	4	0	9.3	43
MT5RL-15	Control	14	18	7	6	0	13.3	45
Total control (%)		49.7	36.5	7.4	6.4	0.0	6.4	392
MT5RL-4	1T5D	26	29	5	8	0	11.8	68
MT5RL-17	3T1D	30	14	6	7	1	13.8	58
MT5RL-1	3T1D	3	6	3	2	0	14.3	14
MT5RL-16	1T10D	37	29	4	13	0	15.7	83
MT5RL-17	1T3D	18	16	10	9	2	20.0	55
MT5RL-5	1T7D	20	13	3	11	0	23.4	47
MT5RL-17	6T2D	17	12	3	8	2	23.8	42
MT5RL-4	1T5D	15	22	1	14	0	26.9	52
MT5RL-17	Treated	46	56	20	46	0	27.4	168
MT5RL-1	2T1D	9	7	3	15	1	45.7	35
Total treated (%)		35.5	32.8	9.3	21.4	0.9	22.4	622

T = Number of treatments; D = number of days from the last treatment to spike fixation. Types I–IV are defined in figure 1.

orienting to the opposite pole of the centromeres (fig. 2c, type III). Types II and III show the constriction remarkably stretched. However, the chromatin fiber is never broken at metaphase I. In type IV only 1 centromere is active, orienting to the opposite pole of the neocentromere in the homologous chromosome (fig. 2d, type IV). In type V the bivalent is oriented by the neocentromeres in both chromosomes, whereas centromeres seem to be inactive (fig. 2e, type V). In type VI the bivalent is not oriented and does not show any tension from centromeres or neocentromeres (fig. 2, type VI). The frequencies of these cell types in plants treated and untreated with the pesticide are shown in table 2.

In 4.5% of the metaphase I cells the two 5RL chromosomes were unpaired. These cells are neither included in table 2, nor have they been considered in the following calculations.

Also in the case of the 5RL ditelosomic line, the pesticide increased the frequency of neocentromeres, from 4.09% in control to 22.11% in treated plants. A contingency χ^2 test showed significant differences between the number of cells with neocentromeres in the treated and untreated plants ($\chi^2 = 42.95$, $p = 0.000$).

Type VI (none of the centromeres or neocentromeres oriented) in the ditelosomic line was reduced in treated plants (9.07%) compared to control plants (15.61%). Thus, the number of cells with bivalents showing 1 or 2 oriented centromeres (types I–V) versus bivalents showing nonoriented centromeres (type VI) significantly differ in treated and untreated plants ($\chi^2 = 9.56$, $p = 0.002$).

In the ditelosomic line we also studied the effect of several doses and different days of collection after pesticide treatments. The data showed that none of these variables strongly affected the frequency of neocentromeres.

The treatment with the pesticide was performed during 3 years in monotelo- and ditelosomic plants. It was observed that the high frequency of neocentromeres was not inherited from treated plants to their selfed progeny, but the frequency of neocentromeres rises every year to about the same frequency in treated plants only.

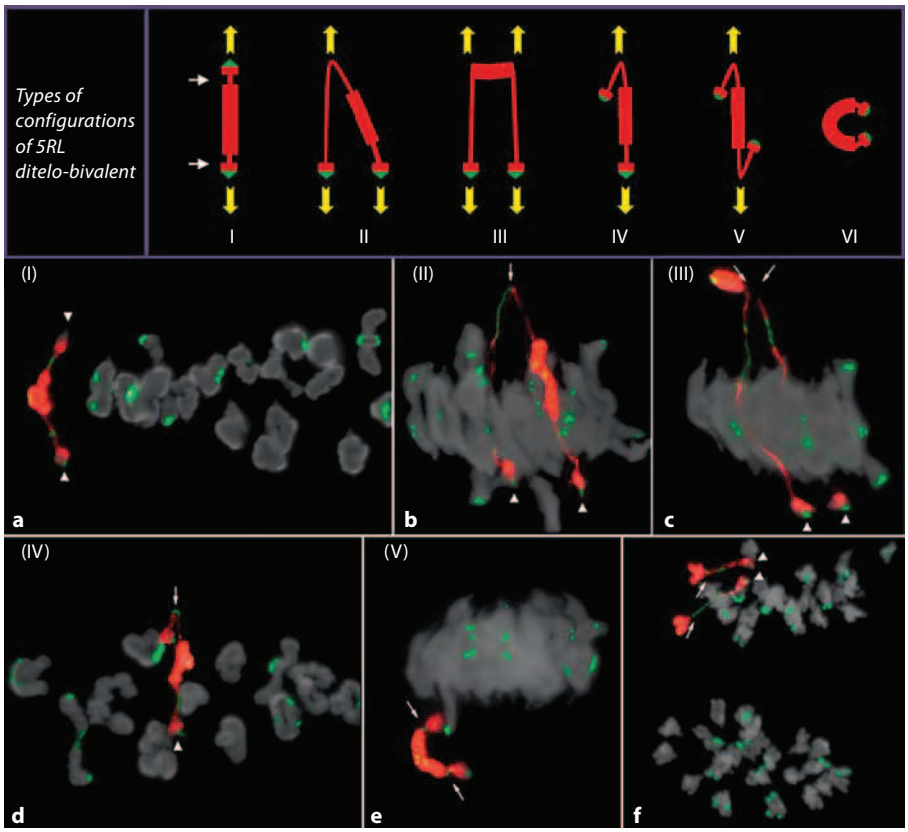

Fig. 2. Upper row. Scheme of the 5RL bivalent configurations at metaphase I in the ditelosomic addition line. The green triangles represent centromeres with tension to the poles, the semicircles centromeres without tension to the poles. Yellow arrows indicate tension to the poles. Type I: the bivalent is oriented by the centromeres, the stretched constrictions (white arrows) are visible, but the neocentromeres are not active. Type II: the constriction of one of the chromosomes is strongly stretched due to the tension between the centromeres and the neocentromere; the bivalent is V-shaped. Type III: the neocentromere appears in the constriction of both chromosomes (bivalent U-shaped). Type IV: one of the chromosomes is oriented by the centromere and the homologous by the neocentromere. Type V: the centromeres are not active and the bivalent is oriented by the neocentromeres. Type VI: nonoriented bivalent. Second and third rows. FISH with the same probes as in figure 1. Arrowheads and arrows point to the centromere and the neocentromere, respectively. **a** Metaphase I. The 5RL rod bivalent shows orientation to the poles by the centromeres, the constriction is conspicuous (type I). **b** V-shaped ditelobivalent where both centromeres are oriented to the same pole and the neocentromere to the opposite pole (type II). **c** U-shaped ditelobivalent where both centromeres are oriented to the same pole, and 2 neocentromeres to the other pole (type III). **d** Ditelobivalent oriented by 1 centromere and 1 neocentromere, the upper centromere is apparently inactive (type IV). **e** Ditelobivalent oriented by 2 neocentromeres. Both centromeres are apparently inactive (type V). **f** Anaphase I with two 5RL chromosomes migrating to the same pole. The constriction keeps the cohesion of sister chromatids.

In the type II of the monotelosomic line, and II and III of the ditelosomic line the constriction was very stretched, but in spite of the strong tension the chromatin fiber was rarely broken. In the case of the univalent, the tension stops before the end of anaphase I and thus the chromosome migrates to the centromere or the neocentromere pole. In the ditelosomic line the segregation of the homologs was correct in most cases. This suggests that the bivalent obtains a proper orientation before anaphase I.

5R Monosomic and Disomic Addition Lines

We studied 1 disomic and three 5R monosomic plants of the respective addition lines. The 5R univalent or bivalent showed the constriction with neocentric activity in the long arm (fig. 3) as in 5RL mono- and ditelosomic addition lines.

In the monosomic addition line, the 5R univalent behavior is similar to the 5RL telochromosome. It may be amphitelically or syntelically oriented (fig. 3a), and the constriction may be very stretched due to the orientations

Table 2. Cell types and frequencies of cells showing neocentric activity observed in the ditelosomic 5RL wheat addition line treated and untreated with the pesticide

Plant	Treatment	Cells without neocentric activity, n		Cells with neocentric activity, n				Frequency of cells with neocentromeres, %	Cells, n
		I	VI	II	III	IV	V		
DT5RL-1	Control	30	14	0	0	0	0	0.0	44
DT5RL-3	Control	40	4	0	1	0	0	2.2	45
DT5RL-1	Control	43	5	1	1	0	0	4.0	50
DT5RL-2	Control	36	8	0	2	0	0	4.4	46
DT5RL-1	Control	38	4	0	3	0	0	6.7	45
DT5RL-19	Control	29	7	0	3	0	0	7.7	39
Total control (%)		80.3	15.6	0.4	3.7	0.0	0.0	4.1	269
DT5RL-12	5T2D	28	3	0	1	2	0	8.8	34
DT5RL-15	1T1D	152	19	10	10	3	1	12.3	195
DT5RL-12	1T3D	11	2	0	1	1	0	13.3	15
DT5RL-19	1TXD	13	3	0	2	1	0	15.8	19
DT5RL-15	1T1D	53	5	4	6	1	0	15.9	69
DT5RL-1	1T1D	63	3	4	7	1	1	16.5	79
DT5RL-18	1T9D	23	16	1	8	0	0	18.8	48
DT5RL-16	3T1D	17	3	0	5	0	0	20.0	25
DT5RL-3	1T1D	49	5	6	8	2	0	22.9	70
DT5RL-1	1T5D	41	3	7	6	1	1	25.4	59
DT5RL-15	1T9D	126	16	14	35	7	1	28.6	199
DT5RL-15	1T9D	84	9	13	40	1	0	36.7	147
Total treated (%)		68.8	9.1	6.2	13.5	2.1	0.4	22.1	959

T = Number of treatments; D = number of days from the last treatment to spike fixation. Types I–VI are defined in figure 2.

of the centromere and the neocentromere to opposite poles (fig. 3b). Interestingly, the tension can be stronger in the neocentromere, pulling the chromosome to the pole (fig. 3c).

In the case of the disomic line, both 5R chromosomes pair in a ring or rod bivalent (fig. 3d1, d2). We found configurations of the bivalent resembling the type III of the ditelobivalent with both centromeres oriented towards the same pole and the neocentromeres from each 5R chromosome to the opposite pole (fig. 3f).

Treated plants always showed higher frequencies of neocentromeres than their corresponding control plants, with about the same range as in the monotelo- or ditelosomic plants (table 3). A contingency χ^2 test showed significant differences between the number of cells with neocentromeres in the treated and untreated plants ($\chi^2 = 26.40$, $p = 0.000$).

As in the previous cases, a remarkable variability of neocentromere frequency was observed between plants, or between spikes from the same plant.

In all types of plants studied, it was commonly observed that the sister chromatid cohesion was maintained in the neocentromere at anaphase I, as in figures 2f and 3e.

Effect of Diazinon on the Spindle at Metaphase I

One monotelosomic, 4 ditelosomic, 1 monosomic and 1 disomic 5R plants were studied with the immunostaining technique, using anti-α-tubulin to observe the effect of the pesticide on the spindle (fig. 4). In 20 metaphase I cells of each of 4 untreated plants, nearly 100% showed normal spindle, with conspicuous bundles of microtubuli between the poles and the centromeres (fig. 4a) and only 1 case of split spindle. However in treated plants, about half of metaphase I cells showed abnormal spindle. In 11% of the cells, the spindle was split at the poles (fig. 4b–d). In 38% of the cases the spindle was strongly affected because either the poles or the microtubule bundles were undefined (fig. 4e, f).

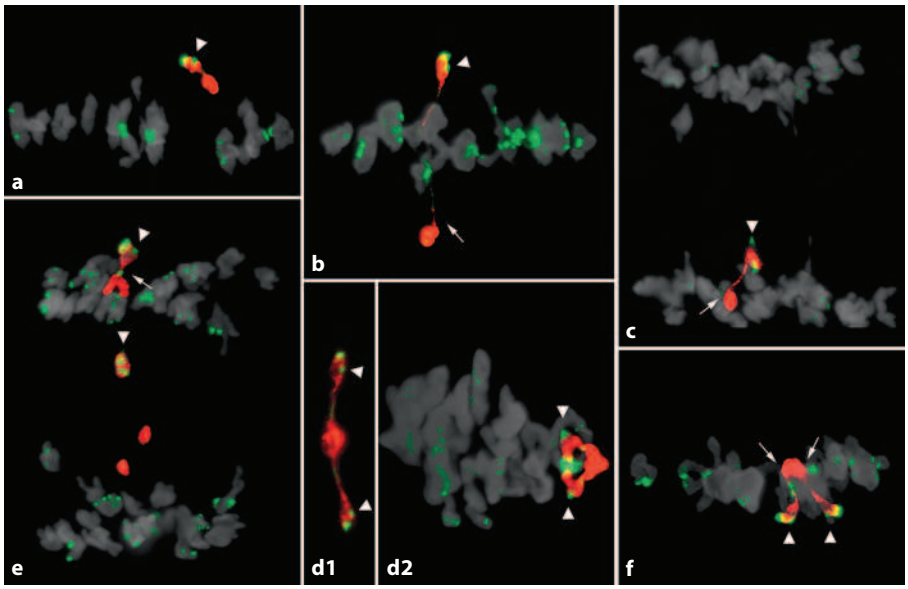

Fig. 3. Monosomic (upper row) and disomic (lower row) 5R rye-wheat addition lines. FISH with the same probes as in figure 1; pSc119.2 also labels a large terminal band in the short arm of 5R. Arrowheads point to the centromeres and arrows to the neocentromeres. **a**, **b** 5R univalent at metaphase I. In **b** the 5R univalent is pulled to the opposite poles by the centromere and the neocentromere. **c** The 5R chromosome has reached the pole of the neocentromere. **d1** Rod 5R bivalent with conspicuous constrictions. **d2** Ring 5R bivalent showing the constriction in the long arm. **e** Anaphase I. The 5R chromosome at the upper pole shows chromatid cohesion at the constriction. The lagging 5R is broken at the constriction resulting in 3 fragments, one including the 5RS, the centromere and the proximal part of the 5RL, and the other 2 fragments correspond to the chromatids of the distal part of the 5RL. **f** U-shaped 5R bivalent with both centromeres oriented to the same pole and neocentromeres to the other pole.

Table 3. Cell types and frequencies of cells showing neocentric activity observed in the monosomic (M) and disomic (D) 5R wheat addition lines treated and untreated with the pesticide

Plant	Treatment	Cells without neocentric activity, n	Cells with neocentric activity, n	Frequency of cells with neocentromeres, %
M5R-17	Control	56	0	0.0
M5R-2	Control	50	1	1.9
M5R-5	Control	132	17	11.4
Total control M (%)		92.9	7.0	
D5R-5	Control	54	3	5.3
Total control D (%)		94.7	5.3	
M5R-2	2T2D	45	6	11.8
M5R-2	1T5D	44	6	12.0
M5R-2	1T9D	80	24	23.1
M5R-17	2T2D	94	31	24.8
Total treated M (%)		79.7	20.3	
D5R-5	2T5D	46	12	20.7
Total treated D (%)		79.3	20.7	

T = Number of treatments; D = number of days from the last treatment to spike fixation.

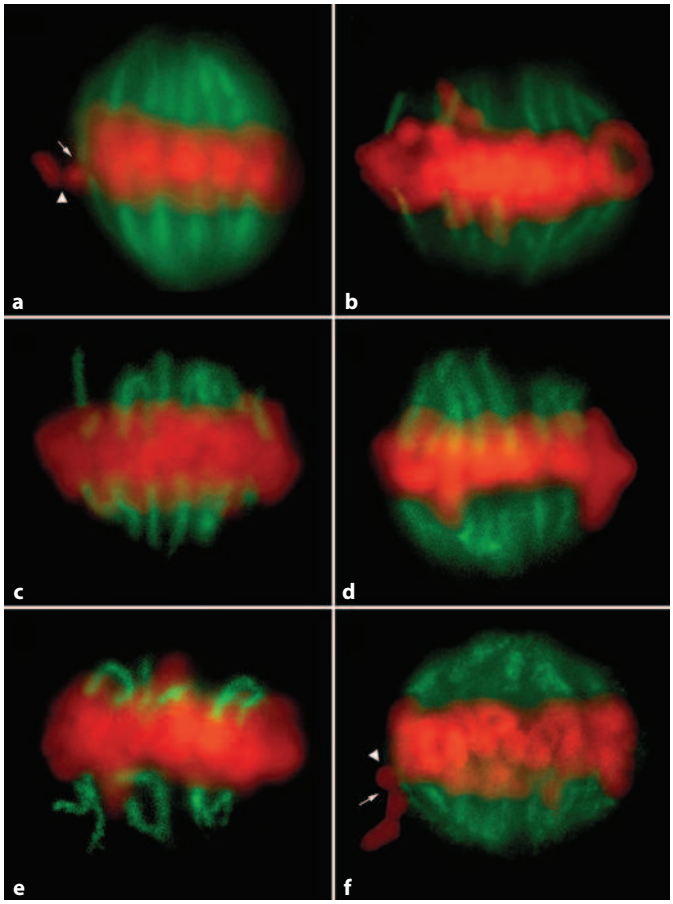

Fig. 4. Immunostaining with anti-α-tubulin. **a** Untreated monosomic 5R addition plant with normally shaped spindle. The 5R univalent is in amphitelic orientation. A bundle of microtubuli is joined to the constriction (arrow), but microtubuli are not joined to the centromere (arrowhead). **b–f** Plants treated with diazinon. **b** Ditelosomic 5RL; **c** and **d** monosomic 5R show split spindle. In **e** (monosomic 5R) the bundles of microtubuli are not properly directed to the poles. In **f** (ditelosomic 5RL) the microtubuli are altered. Microtubuli are joined to the constriction (arrow), and not to the centromere (arrowhead).

In most cases the 5RL chromosome or bivalent was undistinguishable amongst wheat bivalents, but in a few cases it was possible to observe microtubuli joined to the 5RL constriction (fig. 4f).

Discussion

Neocentromere activity was observed in the heterochromatic constriction of the 5RL chromosome in the 5RL monotelo- and ditelosomic wheat-rye addition lines by Manzanero et al. [2000a, 2002]. The frequencies of neocentromeres were very variable and not conserved in 2 successive generations, thus suggesting the influence of an environmental effect on neocentric activation. In the present work we show that the organophosphate pesticide diazinon promotes the neocentric activity in the 5RL chromosome, up to the frequencies reported by Manzanero et al. [2000a], and was actually used in the greenhouse in that occasion against an ant plague. Besides the monotelo- and ditelosomic addition lines, we have studied mono- and disomic 5R addition lines with complete centromeres. In all cases the pesticide raised the frequency of neocentromeres about 4.5-fold. However, there was a basal frequency of neocentromeres in most untreated plants, and the types of chromosome configurations showing neocentromeres were the same in untreated and treated plants. Moreover, the increase in frequency of neocentromeres in treated plants was not heritable, because in selfed progeny from treated plants the frequency of neocentromeres was the same as that of the progeny of selfed untreated plants. Therefore, this chromosome region itself has the ability of acting as a neocentromere and the pesticide raises its frequency.

The frequency of neocentromeres was similar in the monotelo- and ditelosomic lines, in spite of the different configuration of the univalent and the bivalent at metaphase I. On the other hand, neocentromeres appeared with similar frequency in the mono- and disomic addition lines, where the whole centromere is present. These results indicate that the neocentromere can be activated irrespective of the pairing condition or the presence of the complete centromere.

In a low percentage of cells, the 5RL did not show any orientation to the poles (types IV and VI in the monotelo- and ditelosomic addition lines, respectively) indicating that the truncated centromere may not be as functional as the complete one. This never happened in the monosomic and disomic addition lines, where the complete centromeres were always active, together or not with the neocentromeres.

In the monotelosomic line the number of cells without neocentromeres (types IA, IS and IV) did not differ between treated or untreated plants, indicating that the treatment did not affect the behavior of the single centromere in the univalent. However, the frequency of nonoriented bivalents (type VI) in the ditelosomic line was strongly reduced in treated plants compared to control plants. Thus, the 5RL neocentromere could help to orientate the bivalent at metaphase I. Moreover, in some cells neocentric activity could entirely substitute the centro-

meric function as in types III (monotelosomic line) and IV and V (ditelosomic line), pulling the chromosome to the pole at anaphase I, while the centromere remained apparently inactive because of the lack of tension to the pole.

In a previous work we observed microtubuli joined to the 5RL constriction [Manzanero et al., 2002]. In the present work, immunostaining with anti-α-tubulin in treated plants revealed that the pesticide diazinon disturbs the spindle. Spindle disturbances and mitosis disruption by pesticides containing carbamates have been reported at plant mitosis [Hepler and Jackson, 1969; Clayton and Lloyd, 1984; Hoffman and Vaughn, 1994; Giménez-Abián et al., 1997; Yemets et al., 2008]. Diazinon is a synthetic organophosphate insecticide commonly used in agriculture and gardens to control plagues of ants, flies, cockroaches and fleas [Eto, 1974]. The active compound of the pesticide is (O,O-diethyl-O-(2-isopropyl-6-methyl-pyrimidine-4-yl)phosphorothioate). Diazinon causes insect death by inhibition of acetylcholinesterase, an enzyme which hydrolyzes the neurotransmitter acetylcholine. This leads to muscular paralysis and asphyxia. Carbamate insecticides are derivatives of carbamic acid, $HOC(O)NH_2$. Carbamate and organophosphate insecticides have different chemical nature, although the effect exerted on cholinesterase on the insect nervous system is similar.

In all probability, disturbances in spindle formation prompt microtubule joining between the constriction and the poles, raising the frequency of neocentromere activation in treated plants. Diazinon might increase the decondensation in the chromatin at metaphase I exposing determined DNA sequences to microtubules and/or the splitting effect of diazinon on the spindle, changing its normal shape, might help the interaction between the constriction and the microtubuli.

In spite of spindle disturbances, wheat bivalents appeared well located at the plate, the anaphase poles appeared normal at first and second division, and micronuclei were hardly observed at second division. It seems that the pesticide affects the 5RL constriction at metaphase I by its special features and does not affect other chromosomes or meiotic stages. Therefore, the binding with the spindle and neocentric activation seems to be consequences of the chromatin features at the constriction.

The frequency of the neocentric activity does not depend on the number of treatments, or the number of days between the treatment and the spike collection. We used for each treatment the dosage recommended by the manufacturer to control the insect plagues. With one dosage we found high frequencies of neocentromeres, thus it could be sufficient for neocentromere activation. The pesticide has a half-life from 2–6 weeks; therefore, the product surely remained in the soil after 10 days of treatment.

The constriction of the 5RL chromosome appears stretched in the univalents when the neocentromere is active, and in the bivalents (both 5R and 5RL) with and without neocentric activity. This elongation of the constriction reveals special features of the chromatin, which is unusually decondensed in this material. Plant neocentromeres are reported within heterochromatic domains as terminal neocentromeres in maize and rye [Dawe and Hiatt, 2004; Guerra et al., 2010]. It indicates that heterochromatin is a necessary requirement for the neocentromeric as well as centromeric function [Allshire, 1997]. Furthermore, chromatin at the 5RL neocentromere is unusually decondensed. It has been suggested that the decondensed state of the heterochromatin is necessary for binding centromeric determinants in *Drosophila* and human chromosomes [Ahmad and Henikoff, 2001]. This state may provide the necessary characteristics to assemble a functional kinetochore [Manzanero et al., 2002]. However, the 5RL constriction has appeared in situations where neocentromeres were not active, being observed from diakinesis to telophase I. This demonstrates that the decondensed state of the heterochromatin is necessary but not sufficient for the neocentromeric activity.

An interstitial constriction morphologically similar to that formed in the 5RL was described in the chromosome E of *Aegilops markgrafii* at metaphase and anaphase I in the monosomic wheat-*Ae. markgrafii* addition line. However, it has been shown that the constriction formed in this case does not behave as a true neocentromere because it does not contain CENH3 and does not join microtubuli [Schubert, 2011]. In contrast, 5RL constriction fulfilled 3 main centromeric features: orienting the chromosome to the pole, joining spindle microtubuli, and keeping sister chromatid cohesion at anaphase I. In normal chromosomes, chromatid cohesion at anaphase I is essential to ensure the migration of n chromosomes to each pole, ensuring the reduction of chromosome number at first meiotic division.

The repetitive sequences UCM600 and pSc119.2 were found within the constriction of the 5RL chromosome in this work. UCM600 was a key tool to distinguish the rye chromosome among the wheat bivalents without performing genomic in situ hybridization on the wheat-rye addition lines. Interestingly, when the constriction is extremely stretched, there is 1 gap in the labeling of the

chromatin fiber by this probe, which is partially covered by pSc119.2. In some cells the pSc119.2 label was observed within the constriction at the neocentromere. However, in other cells the pSc119.2 label appeared at different positions, in a proximal, central or distal location with respect to the site of neocentric activity, and differently elongated. Therefore, the pSc119.2 sequence does not seem to be determinant for the neocentromere activity. The neocentromere may appear at any location within the whole constriction. This is reinforced with the observation of type III configuration in the monotelosomic line. Although its frequency was low, it strongly supports that the neocentric activity is not restricted to 1 locus within the constriction.

Centromere primary sequence is not determinant of centromeric function, but repetitive sequences are necessary [Allshire, 1997]. Plant neocentromeres are usually related with tandemly repetitive sequences as well [Houben and Schubert, 2003; Nasuda et al., 2005; Guerra et al., 2010]. The sequence pSc119.2 is organized as tandem arrays of a 118-bp monomeric unit [Bedbrook et al., 1980; McIntyre et al., 1990; Vershinin et al., 1995; Vershinin and Heslop-Harrison, 1998]. This sequence size does not fit with the typical unit length from the centromeric satellite arrays [Henikoff, 2001], but the presence of other repetitive sequences within the constriction is not excluded. These sequences could provide the necessary environment for the neocentric activity, presumably facilitating a higher-order structure [Choo, 2000] supporting kinetochore formation.

Finally, the interstitial neocentromere described here has unique characteristics which provide an excellent opportunity to study the neocentromere occurrence in plants. Future studies are necessary to prove whether it needs a *cis*-acting centromere to operate or it can replace the centromeric function. The organophosphate pesticide promotes its appearance and thus constitutes an excellent tool to study the neocentromere.

Acknowledgements

This work was funded by the projects AGL2008-04255 and BFU 2006-10921 from the Ministry of Science and Innovation of Spain. M. González-García is a grant holder of the Ministry of Education of Spain. M. Cuacos is a grant holder of the University Complutense. We thank Dr. E. Benavente (Universidad Politécnica, Madrid) for providing greenhouse space.

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