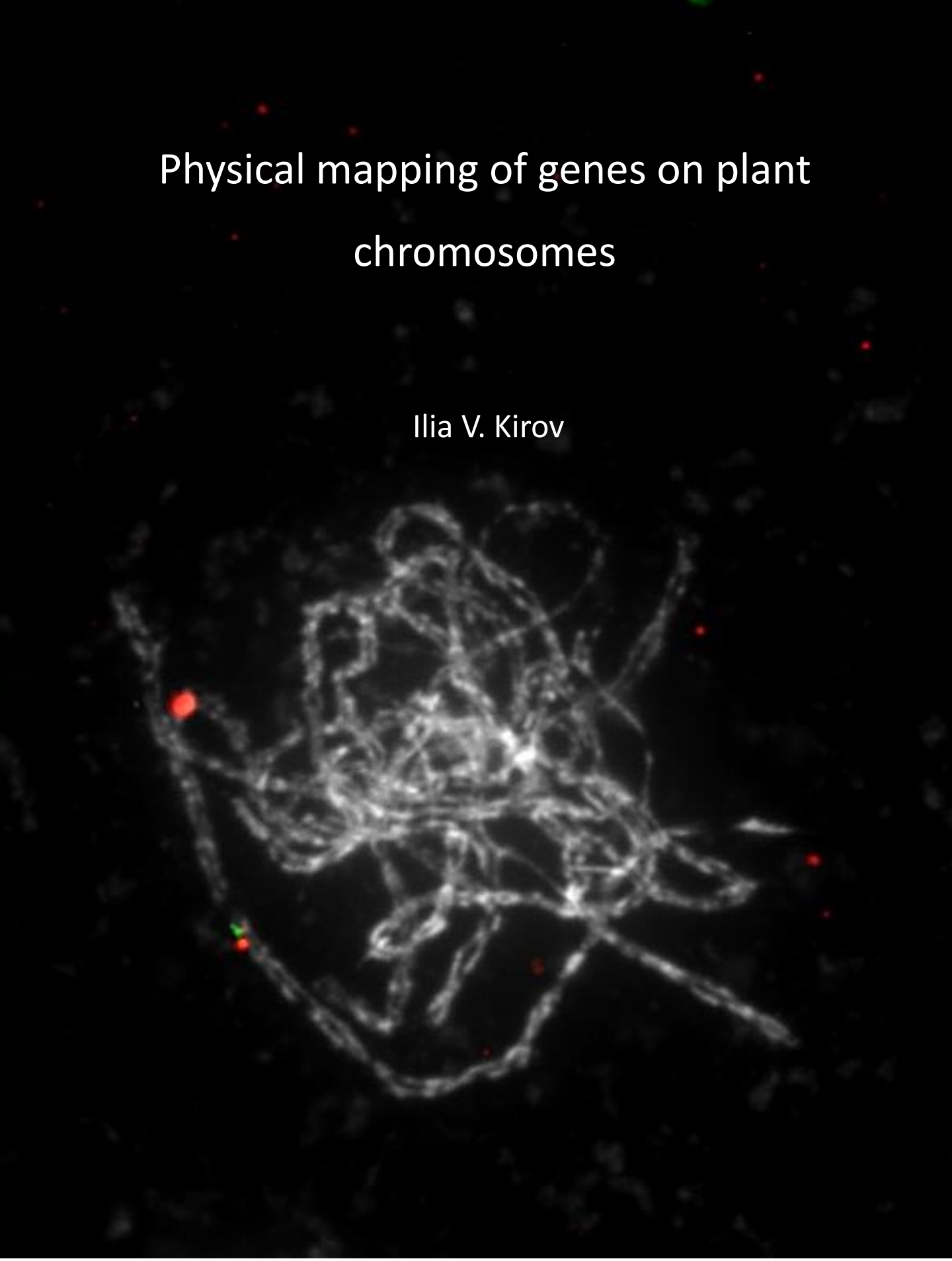
Physical mapping of genes on plant chromosomes

Ilia V. Kirov



GHENT UNIVERSITY

FACULTEIT GENEESKUNDE EN GEZONDHEIDSWETENSCHAPPEN

This PhD thesis is dedicated to my wife

Nadya, daughter Angelika and son Luka

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TABLE OF CONTENT

ABBREVIATIONS

BSA	Bovine Serum Albumin
CY3	Cyanine-3
EST	Expressed Sequence Tag
FISH	Fluorescence In Situ Hybridization
FITC	Fluoresceine Isothiocyanate
HRM	High Resolution Melting
HRP	Horse Radish Peroxidase
NGS	Next generation sequencing
OOMT	Orcinol O-MethylTransferase
PAL	Phenylalanine Ammonia Lyase
P5CS	Pyrroline-5-Carboxylate Synthase
SA	Streptavidin
SNP	Single Nucleotide Polymorphism
SSR	Single Sequence Repeat
TSA	Tyramide Signal Amplification
Tyr-Bio	Biotinylated tyramides

SUMMARY

Physical maps provide unique information about genome structure and organization. They show the real position of DNA sequences in the genome and have been widely applied in comparative genomics, genome research and gene isolation. Due to the genome complexity, development of a physical map is a challenging task. Physical mapping by Fluorescence In Situ Hybridisation (FISH) analysis allows direct visualization of DNA sequences on the chromosomes, but the length of the chromosomal fragment that can be routinely visualized by FISH is 5-10 kb, which is higher than the average plant gene length of 2500 – 4000 bp. To visualize individual genes on plant chromosomes, a very sensitive method is required, such as Tyramide-FISH. Tyramide-FISH was originally developed for application on human and animal chromosomes (Raap et al., 1995). The first Tyramide-FISH applied for plant cytogenetics was on onion chromosomes (Khrustaleva and Kik 2001). Up till now, Tyramide-FISH methods are only well established for model plants (e.g. *Allium*, wheat).

The aim of this thesis was to optimise Tyramide-FISH for physical mapping of unique short DNA probes in non-model plants. *Allium*, as a model plant, and *Rosa*, as a non-model plant, were used for the technology development followed by application of Tyramide-FISH for physical mapping of single-copy genes and physical map construction. *Alllium* is a herbaceous monocotyledon with a basic chromosome number of 8 (x=8), while roses are woody dicotyledons with a basic chromosome number of 7 (x=7). Moreover, *Allium* has very large chromosomes (mean total length - 69.7 µm) (Khrustaleva and Kik 2001), while the mean total length and compaction rate of *Rosa* chromosomes are eight and seven times less than in *Allium cepa*. Because of the small genome, small-sized chromosomes, low mitotic index in shoot and root tips, weak root development and thin roots, the application of any cytogenetic technique on *Rosa* chromosomes is very challenging. In contrast to *Rosa*, *Allium* is the favorite histological and cytological object for chromosome and mitotic cell observation (Patau et al. 1953; Gardner and Mertens 1975; Johnson 1973). Because of the advantages of *Allium* as a model plant for cytological studies, the development and optimizations of physical mapping in this thesis were first performed on this genus and the obtained knowledge was then transferred to rose chromosomes.

Knowing that the quality of chromosome preparation plays an essential role in the success of short probe physical mapping, a new protocol for chromosome preparation, named 'SteamDrop', was developed. This "SteamDrop" method was successfully applied on 28 plant species with large and small chromosomes (Kiseleva et al. 2014; Kriov et al. 2014; Kirov et al., 2015; Laskowska et al. 2015; Romanov

et al. 2015; Alexandrov and Karlov, 2016). The obtained chromosome slides are suitable for molecular cytogenetic methods and short DNA probe mapping.

Secondly, the Tyramide-FISH detection technique was optimized. Direct and indirect detection systems were tested for *Rosa wichurana* and *Allium* chromosomes. The results indicated that the indirect detection system was superior to the direct detection system. In the indirect system, the signal frequency was between 25% and 40% and background signals were only observed when the direct detection system was applied. HRM markers for 3 gene fragments, which were positioned on the chromosomes by Tyramide-FISH, were developed and made it possible to include these gene fragments into the existing genetic linkage map of *Rosa wichurana* (Moghaddam et al. 2012). This allowed us to anchor three linkage groups to the physical chromosomes. To further improve the Tyramide-FISH mapping procedure, multicolor and high-resolution Tyramide-FISH were established for *R. wichurana* which allowed a 10-20 times higher resolution for gene mapping.

Identification of individual chromosomes is required for effective physical mapping. FISH-based chromosome markers were therefore developed for the chromosomes of *Rosa* and *Allium* using conserved repetitive DNA sequences (45S and 5S rDNA, *Arabidopsis*-type telomeric repeat) and a bioinformatic search for new tandem repeats. By combining our cytogenetic markers with the chromosome morphology measurements, all 7 mitotic chromosomes of *R. wichurana* and all 8 chromosomes of *A. fistulosum* could be identified.

Using our optimised methods seven genes were successfully mapped on pachytene chromosomes 4 and 7 of *Rosa wichurana* and a detailed pachytene map was constructed for these chromosomes. The developed protocols for multi-colour Tyramide-FISH allowed us to simultaneously visualize three genes and revealed the physical order of closely linked genes. An integration of the physical and genetic map was performed (Moghaddam et al. 2012).

SAMENVATTING

Fysische kaarten zijn zeer waardevol in de studie naar genoomstructuren en organisatie. Deze fysische kaarten tonen de reële positie van DNA sequenties in het genoom. Daarom worden ze veel gebruikt in comparatieve genomische studies, genomisch onderzoek en gen isolatie. Echter, door de grote complexiteit van het genoom is het maken van fysische kaarten geen eenvoudige taak. Fluorescentie In Situ Hybridisatie (FISH) maakt het mogelijk om DNA sequenties direct te visualiseren op de chromosomen. De lengte van het chromosomaal fragment dat routinematig kan gevisualiseerd worden door FISH is 5-10Kb, wat echter veel groter is dan de gemiddelde lengte van een plantengen, nl. 2500 – 4000 bp. Om individuele genen te visualiseren op de chromosomen zijn dus zeer gevoelige FISH methoden nodig, zoals Tyramide-FISH. Tyraminde-FISH werd oorspronkelijk ontwikkeld voor toepassingen in diergeneeskundig en geneeskundig onderzoek (Raap et al. 1995). De eerste Tyramide-FISH experimenten bij planten dateren van 2001 bij ajuin (Khrustaleva and Kik 2001). Tot op vandaag is Tyramide-FISH enkel succesvol gebruikt bij model planten, vb. *Allium* and tarwe.

Het doel van deze thesis was om de Tyramide-FISH technologie ook te optimaliseren voor het fysisch mappen van unieke korte DNA probes in niet-model planten. Voor deze optimalisatie werd *Allium* gebruikt als model plant en *Rosa* als niet-model plant. Het op punt gestelde protocol werd dan toegepast bij *Allium* en *Rosa* voor het mappen van single-copy genen en het opstellen van een fysische kaart. *Allium* is een kruidachtige monocotyl met een basis chromosoomaantal van 8 (x=8), terwijl rozen houtachtige dicotylen zijn met een basis chromosoomgetal van 7 (x=7). Verder heeft *Allium* zeer grote chromosomen (gemiddelde totale lengte = 69.7 µm) met een zeer hoge compactheid (249.6 MB/µm) (Khrustaleva and Kik 2001). Dit in tegenstelling tot *Rosa* waar de chromosoomlengte en -compactheid 8 en 7 keer kleiner zijn. Het kleine genoom, de kleine chromosomen, de lage mitotische index in de worteltoppen, en de zwakke ontwikkeling van de fijne wortels, maken dat het toepassen van cytogenetische technieken bij *Rosa* zeer moeilijk is. *Allium* daarentegen is een populair histologisch en cytologisch gewas voor chromosoomanalyse (Patau et al. 1953; Gardner and Mertens 1975; Johnson 1973). Door de voordelen van *Allium* als model gewas voor cytogenetische studies, zal in deze thesis de ontwikkeling en optimalisatie van Tyramide-FISH en fysisch mappen van genen in eerste instantie gebeuren bij dit genus. De opgedane kennis zal dan vertaald worden naar een optimaal protocol voor de roos chromosomen.

De kwaliteit van de chromosoom preparaten speelt een cruciale rol in het succes van fysisch mappen van korte probes. Daarom was de eerste focus het ontwikkelen van een nieuw protocol voor het maken van chromosoom preparaten. Het “steamdrop” protocol dat op punt werd gesteld, werd met succes

toegepast op 28 planten genera, waaronder zowel planten met grote chromosomen als planten met kleine chromosomen. De verkregen preparaten zijn goed bruikbaar in verdere moleculaire cytogenetische experimenten en mappen van korte DNA probes.

Vervolgens werd de tyramide-FISH detectie op punt gesteld. Zowel directe als indirecte detectiemethodes werden getest bij *Rosa wichurana* en *Allium* chromosomen. De resultaten toonden aan dat het indirecte detectiesysteem te verkiezen was boven het directe detectiesysteem. Bij het indirecte systeem, was de signaal frequentie tussen 25% en 40%. Achtergrond signalen werden vooral geobserveerd wanneer het directe systeem gebruikt werd. HRM merkers voor 3 gen fragmenten, die via Tyramide-FISH op de chromosomen gevisualiseerd werden, werden ontwikkeld en maakten het mogelijk om deze gen fragmenten te integreren in de bestaande (Moghaddam et al. 2012) genetische koppelingskaart van *Rosa wichurana*. Op die manier werden 3 linkage groepen gekoppeld aan de overeenkomstige fysische chromosomen. Verdere optimalisatie van Tyramide-FISH mapping bestond erin multikleur-FISH en hoge-resolutie FISH op punt te stellen voor *Rosa wichurana*. Hierdoor werd de resolutie voor gen visualisatie 10 tot 20 keer hoger.

Om op een efficiënte manier genen fysisch te mappen, is het nodig om de chromosomen individueel te kunnen herkennen. Hiervoor werden FISH-gebaseerde chromosoommerkers ontwikkeld voor *Rosa* en *Allium* gebruik makend van conservatieve repetitieve DNA sequenties (45S, 5S rDNA, *Arabidopsis*-type telomeric repeat) en van via bioinformatica tools opgespoorde tandem repeats. Door een combinatie van de ontwikkelde cytogenetische merkers en chromosoom morfologische kenmerken, konden alle 7 chromosomen van *Rosa wichurana* en alle 8 chromosomen van *Allium fistulosum* geïdentificeerd worden.

Door toepassen van de geoptimaliseerde methodes konden in totaal 7 genen gelokaliseerd worden op pachyteen chromosomen 4 en 7 van *Rosa wichurana* en kon een gedetailleerde fysische kaart gemaakt worden van deze chromosomen. Verder werden 3 genen gelijktijdig gevisualiseerd door gebruik van multi-kleur Tyramide-FISH. Op die manier kon de fysische plaats van dichtbij elkaar gelegen genen toch bepaald worden. Als laatste stap hierbij werd een integratie gedaan van de fysische en genetische kaarten (Moghaddam et al. 2012).

PART I - INTRODUCTION AND RESEARCH OBJECTIVES

1. PHYSICAL MAPPING OF PLANT GENOMES

1.1 RELEVANCE

The organization and structure of a genome is a central topic in modern molecular biology and genomics. Evidence is accumulating that the function of genes and their physical location are strongly linked (Talbert and Henikoff, 2006; Mandakova et al. 2015). Changes in the physical order of genes on a chromosome have significant consequences for cells and whole organisms. To reveal the physical organization of DNA sequences in genomes, a physical map is constructed, showing the real position of sequences (e.g. genes, repetitive DNA, regulatory sequences, epigenetic modifications etc.) on the chromosomes (cytogenetic maps) or scaffolds (sequenced genomes). Therefore, physical maps are indispensable in modern biology and are widely used in comparative genomic and evolutionary studies to reveal synteny and collinearity between genomes and to shed light on genome organization and structure (O'neill and Bancroft, 2000). Knowledge about genome structure and chromosome rearragements has shown importance in many applications from human diseases to gene cloning in plants (Miller et al. 2010; Jander et al. 2002; D'Orso et al., 2015; Himi and Taketa, 2015). Development and application of physical maps also support molecular plant breeding by allowing a significant shortening of time for breeding and selecting new crop varieties (Dohm et al. 2014). Identification of plant genes involved in pathways of economic important traits, such as disease resistance and tolerance to abiotic stresses, is enhancing efficient plant breeding towards continuously changing climate conditions. Thereby physical maps provide a platform for the efficient cloning of genes (Philippe et al. 2013).

However, despite the great importance and need of physical maps, their construction is very challenging especially in plants. The genome complexity (allo- and autopolyploidy) and high number of repetitive DNA sequences significantly slow down the efficiency of physical mapping. Development of new advanced methods for physical mapping that deal with these challenges is an important task in this (post)genomic era.

1.2. PHYSICAL MAPS VERSUS GENETIC MAPS

An important milestone in genome mapping was the publication of the first genetic map in 1913 (Sturtevant, 1913). A genetic map shows the relative position of genetic markers along the chromosomes based on recombination frequency (Collard et al. 2005). The distance between markers is expressed in centi-Morgans (cM) and 1cM is equal to 1% of recombination. Since then, genetic maps have been published for many plant genera. In plant breeding programs, genetic maps can be used for marker-assisted selection (Ibitoye and Akin-Idowu, 2015), genetic study of qualitative and quantitative traits (Xiao et al. 1996; Mohan et al. 1997; Doerge , 2002; Yim et al. 2002), introgressive breeding (Dufey et al. 2015; Yohannes T. et al. 2015), map-based cloning of genes (Mohan et al. 1997; Mickelson-Young et al. 1995; Shimizu et al. 2015) and genome assembly (Ariyadasa et al. 2014; Deokar A. A. et al. 2014; Dohm J. C. et al. 2014; Argyris et al. 2015; Wu P. et al. 2015). However, while genetic maps are important in biology, they do not show the real physical distance between genes/markers due to unequal distribution of recombination frequencies along the chromosomes. One cM on a genetic map can be equivalent to a few kilobases as well as to millions of base pairs of physical distance (Kunzel et al. 2000; Blenda A. et al. 2012; Sun et al. 2013; Ariyadasa et al. 2014; Si et al. 2015).

The development of the next generation sequencing techniques (NGS) exponentially increased the sequencing capacity and made whole-genome sequencing fast and robust (Varshney et al. 2009). Using NGS technologies, tens of plant genomes have been sequenced and genomes were assembled into pseudochromosomes or scaffolds, providing valuable information about the physical organization of genomes. But the genome complexity (allo- and autopolyploidy) and high number of repetitive DNA sequences make genome assembly and scaffold order arrangement very difficult. In spite of the huge progress in obtaining sequencing data, at present there are no good tools to solve the puzzle putting all short DNA reads obtained after genome sequencing together. The way from single base pair level to chromosome level is nowadays the main challenge. Using a genetic map as an intermediate between scaffolds and chromosomes, scaffolds can be accurately mapped in the regions with high (“hot spot”) recombination rates (Shearer et al. 2014). In contrast, genome assembly in centromeric, heterochromatic and other genomic regions with low recombination rates (“cold spot” of recombination) is problematic. Since recombination poor regions, such as centromeres and heterochromatin, occupy a significant portion of the genome (Kunzel et al. 2000; Mayer et al. 2011), it is impossible to unravel the organisation of the genome only by genetic mapping. Recent publications

suggest massive discrepancies between the in *silico* assembled version of the genome and nuclear genome. Most of these misassemblies occurred in the 'cold' spot recombination regions (Yang et al. 2012; Karafiátová et al. 2014; Shearer et al. 2014). Therefore, to achieve high-quality assembly of genome sequences, an integrated approach is needed including different sequencing strategies such as long-size insert libraries, long-read sequencing (e.g. PacBio sequencing), Hi-C data based scaffolding as well as instruments not relying on recombination frequency such as cytogenetic mapping and optical mapping (Korbel and Lee, 2013; Cao et al. 2016; Chaney et al. 2016).

Integration of cytogenetic and genetic maps have been carried out for many plant species and genera including *Brassica rapa* (Xiong et al. 2000), *Allium cepa* (Masamura et al. 2012; Romanov et al. 2015); *Cucumis sativus* (Han et al. 2011; Lou et al. 2013), *Zea mays* (Figueroa and Bass, 2012), *Solanum* species (De Jong et al. 1999; Szinay et al. 2008; Tang et al. 2009), *Gossypium* (Wang et al. 2010; Cui et al. 2015), *Phaseolus vulgaris* (Fonsêca et al. 2010), *Vicia faba* (Ruiz-Rodriguez et al. 2014), *Beta vulgaris* (Paesold S. et al. 2012), *Brachypodium distachyon* (Febrer et al. 2010), *Lotus japonicus* (Ohmido et al. 2010), *Carica papaya* (Zhang et al. 2010; Wai et al. 2012). Map integration in these studies significantly extended the understanding of plant genome organization, recombination frequency and epigenetic signature distribution along the chromosomes. Filling the gaps between genome sequencing data and the (sub)chromosomal level is one of the current goals in modern genome biology.

1.3 PHYSICAL MAPPING USING FLUORESCENCE IN SITU HYBRIDIZATION

(Molecular) cytogenetic methods allow to directly assign the position of DNA sequences to the physical chromosomes. This makes fluorescence in situ hybridization (FISH) a favorable tool for physical mapping of a wide range of plant genomes even without any prior knowledge about the genome sequence.

The principle of FISH is illustrated in Figure 1.

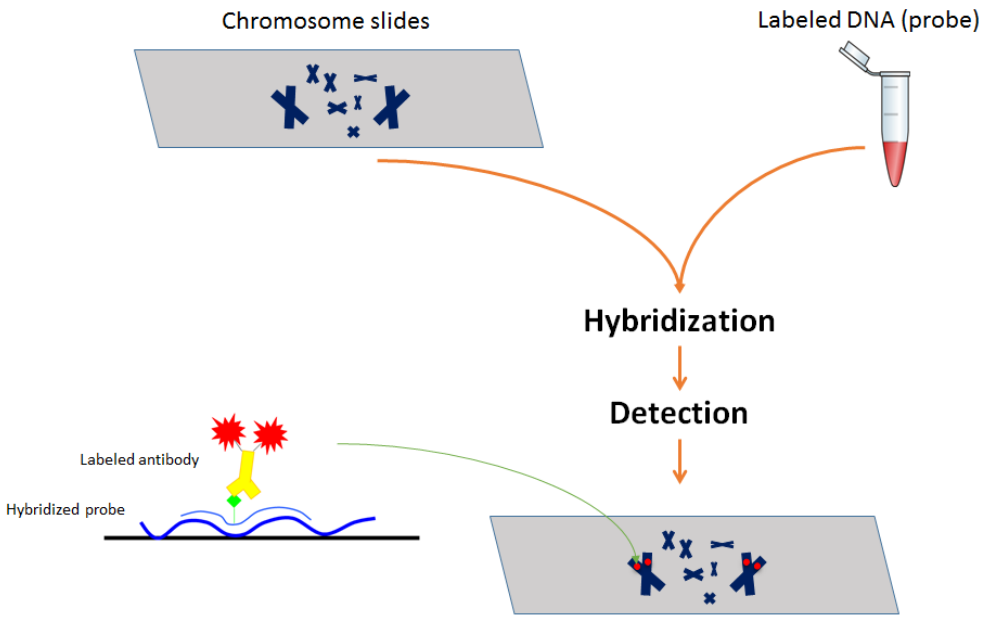

Figure 1. FISH workflow. Labeled DNA (probe) is added to the chromosome slide followed by hybridization (37°C, 2 – 28h). Detection of the hybridization sites (red dots on the chromosomes) is performed by fluorochrome-labeled antibodies.

The DNA of a probe is labeled by incorporation of a hapten (e.g. biotin, digoxigenin) or fluorochrome labeled nucleotides. This probe is hybridised to the chromosomes on the slide followed by fluorescent detection. Several layers of antibodies can be used to increase signal intensity but this often results in the simultaneous increase of background signals.

The signals from the hybridization sites are collected and the relative position (RP) on the chromosomes is determined by the formula:

$$RP = \frac{\text{Distance from the centromere to the signal} * 100}{\text{Length of the chromosome arm}}$$

Maps constructed using FISH techniques are called cytogenetic maps.

The FISH-based cytogenetic maps have a number of advantages:

1. the real position of sequences on the chromosome is shown,
2. the sequences are located in the context of major chromosomal landmarks (e.g. centromeres, telomeres, heterochromatin),
3. a mapping population is not required (Jiang and Gill, 2006),

4. a plant collection with different chromosomal rearrangements (e.g. deletions) is not needed,

5. all genome parts can be mapped regardless of their genome location (genome position independent mapping),

6. they can be integrated with genetic and physical maps

However, cytogenetic mapping is a laborious, time-consuming and low-throughput method. A high quality of the chromosome slides is crucial. The plant cell has a rigid cell wall making the chromosome preparation procedure quite time consuming. In addition, a high number of chromosomes and the difficulty to find a good source of dividing cells (e.g. pollen mother cells) further detain the chromosome preparation procedure. Between plant genera many differences in chromosome number and size as well as in mitotic index exist. Therefore, there is no standard chromosome preparation protocol directly suitable for all plant genera. Additional obstacles for cytogenetic mapping are the complexity of a plant genome containing high numbers of repetitive DNA and the frequent occurence of allo-/autopolyploidy.

BAC-FISH is the main tool used for cytogenetic mapping of plant genomes. The large genomic inserts of BAC clones make them suitable probes for FISH both on mitotic as well as on pachytene chromosomes (De Jong et al. 1999; Szinay et al. 2008). BAC-FISH has been recently applied for high-density cytogenetic mapping of tomato (Shearer et al. 2014) and *Spirodela polyrhiza* (Cao et al. 2016) to verify and guide genome assembly, respectively. BAC-FISH results provided anchors for scaffold arrangement. The repetitive DNA in BAC clones may significantly hamper the process of BAC-FISH physical mapping because the FISH signals from the repeats are scattered over many loci on the chromosomes as a result of extensive cross-hybridization of repetitive DNA (Szinay et al. 2008). The problem can be circumvented by the application of repetitive fractions of genomic DNA (e.g. Cot fraction) as blocking DNA (Szinay et al. 2008; Chang et al. 2007). BAC-FISH was mostly used for species with a small genome (*Arabidopsis*, tomato etc.) while its application for species with large genomes (grasses, onion etc.) is challenging. For species with a large genome and a high number of repeats, FISH with single copy genes is applied.

2. PHYSICAL MAPPING OF INDIVIDUAL GENES BY FISH

Single-copy FISH has been used to map genes and to create a cytogenetic map for maize, wheat, barley and oat (Wang et al. 2006; Lamb et al. 2007; Maron et al. 2013; Danilova et al. 2012, 2014; Poursarebani et al. 2014; Karafiátová et al. 2013). These species have large chromosomes and optimized protocols for chromosome preparation and FISH are widely described. The progress in application of FISH with single copy sequences in non-model species is slow. In addition, recent studies suggested that the FISH sensitivity is about 5 – 10 Kb (Jiang and Gill, 2006; Lamb et al. 2007; Danilova and Birchler, 2008; Karafiátová et al. 2013; Danilova et al. 2014). The average length of plant genes as is known from genome sequencing projects is about 2.5Kb (The *Arabidopsis* Information Resource website; Ming et al. 2008) which is lower than the sensitivity of FISH. To overcome this limitation, different approaches have been applied, including Tyramide-FISH (Raap et al. 1995; Khrustaleva and Kik, 2001), PRINS (primed *in situ* labeling) (Koch et al. 1989; Kubaláková et al. 2001) and modified probe labeling (Kato et al. 2006). PRINS has been used for single copy genes (Abbo et al. 1993; Zhu et al. 1995; Kaczmarek et al. 2007) as well as for repetitive DNA mapping (Kubalakova et al. 1997; Menke et al. 1998). However, the application of this method for physical mapping of short unique probes is limited due to high levels of background signals. To reduce background and increase signal-to-noise ratio (the number and intensity of the non-specific and background signals compared to intensity of the real signal), Kato et al. (2006) proposed a modified procedure for probe labeling. Using fluorochrome labeled nucleotides and higher concentration of DNA polymerase I, authors showed that the effectiveness of labeled nucleotide incorporation into DNA molecules is increased, resulting in a more sensitive FISH procedure. This method has been applied for mapping of genes with a length as small as 2.6Kb (Danilova and Birchler, 2008; Acevedo-Garcia et al. 2013; Danilova et al. 2012, 2014). To further increase the sensitivity of FISH mapping, Tyramide-FISH (Section 3 of this chapter) was proposed. Besides sensitivity, also the resolution of FISH, indicating the minimum distance at which two neighboring FISH signals can be distinguished, is important. In general, the resolution of FISH mapping is correlated to the resolution of the fluorescence microscopy, which is 200-250 nm, and the degree of chromatin compaction. The resolution of FISH on highly compact mitotic chromosomes is near 10 Mb, while on streched fiber DNA the resolution is 1 Kb (De Jong et al. 1999). However, the disadvantage of high-resolution fiber FISH is that the orientation of extended DNA fibers with respect to the centromere or telomere is undetermined. To increase the resolution of FISH but keep the chromosome structure, pachytene chromosomes can be used as they

have a compaction 7 – 100 times lower than mitotic chromosomes. Using pachytene chromosomes the resolution is increased to a few hundreds Kb (De Jong et al. 1999; Shearer et al. 2014). However, for many plant species pachytene chromosome preparation is very challenging (De Jong et al. 1999; Kulikova et al. 2001; Lacia and Pinto-Maglio, 2013; De Capdevill et al. 2009).

Also other cellular and experimental factors influence the effectiveness of FISH (Figure 2).

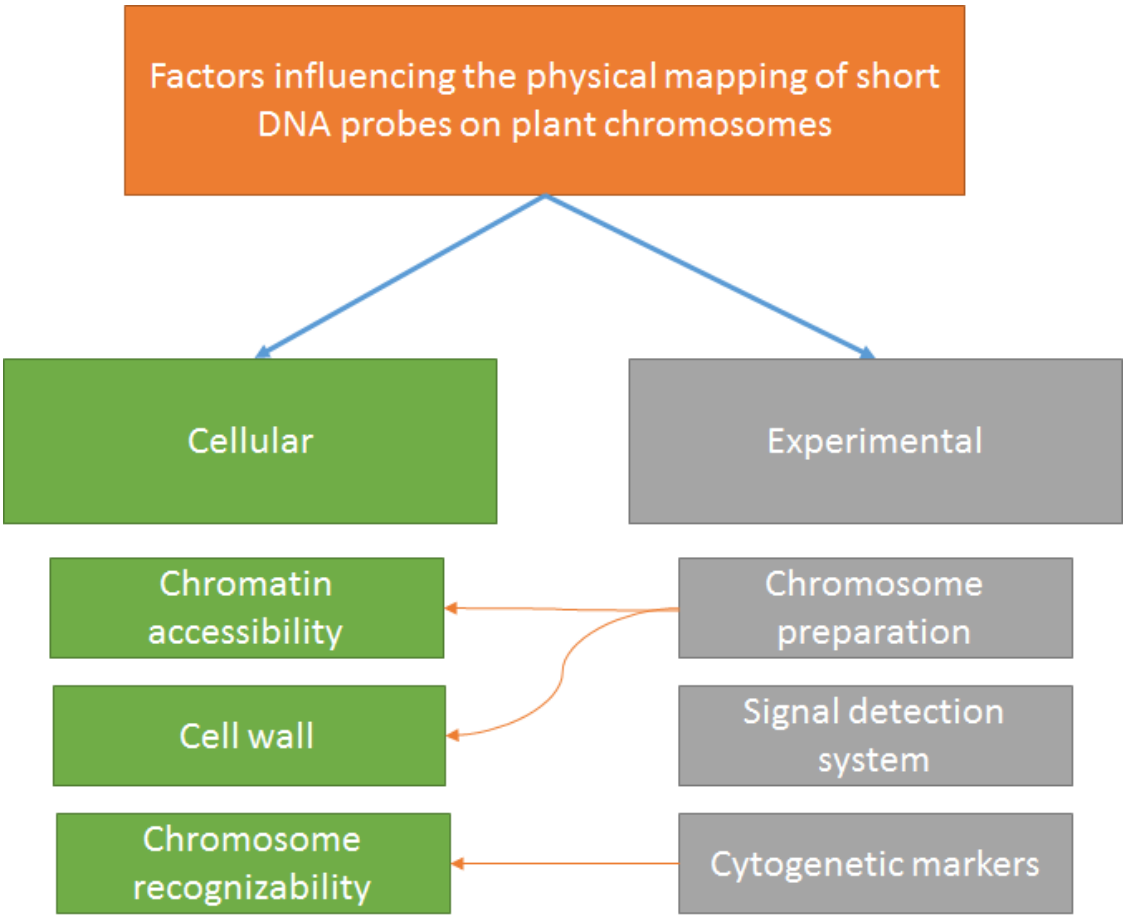

Figure 2. Experimental (gray) and cellular (green) factors that significantly influence the short DNA probe visualization. Orange arrows indicate cellular factors which can be compensated by experimental factors.

Low chromatin accessibility results in weak or no FISH signals. The chromatin accessibility itself depends on several parameters:

1. compaction of chromosomes
2. degree of chromatin damage after chromosome slide preparation
3. quality of chromosome slides

All these parameters cause changes in signal intensity and have an impact on the signal-to-noise ratio. To increase the chromatin accessibility, pachytene chromosomes (De Jong et al. 1999), DNA fibers (Jiang and Gill, 2006) or interphase nuclei (Jiang et al. 1995) can be used. Fiber-FISH and FISH on interphase nuclei provide superior FISH resolution but do not allow to assign the signals to specific chromosomal positions.

Experimental procedures can compensate for the influence of all these cellular factors. For example, by enzyme digestion the cell wall is removed and cannot influence the FISH results. If chromosome identification by morphology is difficult, molecular cytogenetic markers can solve the problem. Thus, optimization of the experimental procedures plays a key role in successful visualization of short DNA sequences on a physical chromosome circumventing cellular factors influencing FISH efficiency.

2.1 CHROMOSOME PREPARATION

The chromosome preparation procedure has a very strong impact on chromatin accessibility and short probe detection (Ambros et al. 1983). Chromosome slides are considered to be suitable for FISH application if:

1. they are free of cytoplasm and cell wall debris
2. they have nicely spread chromosomes
3. the chromosome structure is well preserved (Ambros et al. 1986; Schwarzacher et al. 1989).

There are currently three methods for plant chromosome preparation: the squash (Belling, 1921; Schwarzacher et al. 1980; Schwarzacher and Leitch, 1994), spread (Pijnacker and Ferwerda, 1984; Fukui and Ilijima, 1991) and drop (Mouras et al. 1978; Murata, 1983; Ambros et al. 1983; Andras et al. 1999; Anamthawat-Jonsson, 2003; Kato et al. 2004) method. Squash and spread methods have been commonly used during decades while the drop method was originally developed for human cells and is less common in plant science. In contrast to animal cells, plant cells have a rigid cell wall making the application of the drop technique challenging. The first drop method was applied to protoplasts having no or a weak cell wall (Murata, 1983). However, the described protocol had several disadvantages:

1. a low mitotic index due to cell loss during protoplast isolation and protoplast damage under hypotonic treatment
2. polyploidy caused by spontaneous fusion of protoplasts
3. a labor-intensive protocol
4. protoplast isolation from intact plant tissue is more difficult than from cultured cells.

Kato et al. (2004) modified the drop method and proposed a new air dry drop method. According to that protocol, the cell suspension was consecutively washed in water, 100% ethanol and acetic acid/ethanol (9:1), dropped onto a glass slide in a humid box and dried slowly. Kato et al. (2004) proposed two main modifications for improvement: application of a high concentration of acetic acid or even pure acetic acid for a better chromosome spreading and use of 'laughing gas' (nitrous oxide) as an anti-tubulin agent. The method of Kato et al. (2004) was intensively used for chromosome preparation of maize (Kato et al.2006; Danilova and Birchler, 2008), soybean (Gill et al. 2009) and wheat (Komuro et al. 2013; Yuan et al. 2015). However, the application of this method for species with large sized chromosomes remained problematic (Komuro et al. 2013). Studies carried out on animal cells demonstrated that the chromosome spreading during chromosome preparation is a complex and highly dynamic process that depends on many factors (Spurbeck J. L. et al. 1996; Hliscs et al. 1997; Henegariu et al. 1999; Claussen U. et al. 2002; Deng et al. 2003; Ami et al. 2014). In contrast to animals, the study of the dynamics of chromosome spreading and the influencing factors is not yet undertaken for plants. Filling this gap can improve the current protocols for plant chromosome preparation.

2.2 DETECTION SYSTEM

There are two types of detection systems: direct and indirect detection systems (Leitch et al. 1994). In the direct detection system, the probe is directly labeled by fluorochromes and sites of hybridization are visualised under the fluorescence microscope without making use of any further detection layers. During the last decade, application of FISH with fluorochrome labeled probes and direct detection has been frequently used on plant chromosomes. New protocols for probe labeling by fluorochromes (Kato et al. 2004, 2006) together with the modern digital imaging systems using CCD

(charge coupled device) cameras enable chromosome mapping by direct FISH detection much faster and efficient. This system is especially useful for repetitive DNA mapping (Kato et al. 2011; Komuro et al. 2013; Danilova et al. 2014) but also unique and short DNA probes can be detected with this system (Maron et al. 2013; Danilova et al. 2014; Tiwari et al. 2015). However, by direct detection the signal intensity for single copy sequences is relatively low compared to the indirect detection system, in which the probe is labeled by a hapten (e.g. biotin, digoxigenin) and the sites of hybridization are visualised after additional detection with antibodies conjugated with a fluorochrome or with enzymes (enzymatic detection). Indirect detection can also be performed without antibodies but with a 'click-chemistry' reaction (Hesse et al. 2016) to couple the fluorochromes to the alkyne-modified target DNA after probe hybridization. This approach was successfully used for repetitive DNA detection (Hesse et al. 2016). Antibody based indirect detection systems can be performed with different numbers and types of antibodies. Generally, indirect detection systems allow to amplify signal intensity by increasing the number of fluorochromes per labeled nucleotide. This can be achieved via sequential application of fluorochrome labeled antibodies or/and via an enzymatic reaction bringing the multiple fluorochrome molecules to the site of probe hybridization. However, each amplification step in the indirect detection procedure also increases the background signals. Therefore, optimization is required to increase the signal to noise ratio and to reach maximum sensitivity.

2.3 CHROMOSOME MARKERS

To assign the FISH signals to a specific chromosome or chromosome pair the chromosomes have to be distinguishable in the karyotype. A robust method for chromosome discrimination is important for successive cytogenetic mapping (Jiang and Gill, 2006). The morphology of chromosomes (centromere index (CI = length of short arm * 100 / length of entire chromosome) and relative chromosome length provides limited information for chromosome identification. Especially when small or/and high number of chromosomes are present in a karyotype, chromosome identification is very challenging. The application of cytogenetic banding techniques and molecular-cytogenetic (FISH-based) markers accelerate the process of chromosome identification (Fuchs et al. 1996; Kato et al. 2004; Koo et al. 2005; Divashuk et al. 2014; Lombello and Pinto-Maglio, 2004; Pedrosa et al. 2000; Muravenko et al. 2010). FISH-based markers are a versatile tool for plant chromosomes (Jiang and Gill, 2006). The sources of the DNA probes which can be used as cytogenetic markers include BAC and YAC clones, genes and tandemly

organized repeats. The latter source is more frequently used for chromosome identification because the location of tandem repeats on the chromosomes can be easily detected by FISH. Plant genomes are enriched by various types of repetitive DNA e.g. tandem repeats and FISH with tandem repeats often results in chromosome specific patterns (Snowdon et al. 2000; Hasterok and Maluszynska, 2000; Navratilova et al. 2003; Koo et al. 2005; Muravenko et al. 2009; She et al. 2015; Badaeva et la., 2016; Tran et al. 2016). Tandem repeats can be either conserved among different plant species (e.g. 45S, 5S rDNA, telomeric repeat) or highly variable (e.g. satellite DNA). The rDNA repeats, 45S and 5S, are often used for plant chromosome discrimination as they usually occupy distinct regions of a certain chromosome and they are easy to visualize. In addition, the probes can be generated even from clones belonging to distantly related species, enabling probe design without preliminary knowledge about the DNA sequence of the studied species. Compared to the rDNA sequences, satellite DNA sequences are highly variable in terms of chromosome location as well as DNA sequence similarity. But patterns of chromosomal location of the satellite DNA sequences can be unique for specific chromosomes, allowing them to be identified as was demonstrated for *Vicia faba* (Macas et al. 2003), *V. sativa* (Navratilova et al. 2003) and *Triticum* species (Cuadrado and Schwarzacher, 1998; Komuro et al. 2013; Badaeva et al. 2016). In order to isolate satellite DNA sequences from plant genomes without knowledge about their genomic sequence, several molecular methods were developed (reviewed by Hemleben et al. 2007). The breakthrough in satellite DNA isolation and characterization was the introduction of high-throughput next generation sequencing (NGS) and specific bioinformatic algorithms for identification of satellite DNA sequences. One of this algorithm is the RepeatExplorer (Novak et al. 2010, 2013). RepeatExplorer uses NGS data from low genome coverage sequencing and performs all-to-all read comparison followed by a graphical reconstruction (Novak et al. 2013). The RepeatExplorer output provides information about the repeatome composition and allows identification of tandemly organized repeats based on their cluster shape and contig sequences. This tool was successfully used for isolation of tandemly organized repeats from a number of plant species (http://www.repeatexplorer.org/).

3. TYRAMIDE-FISH – A HIGHLY SENSITIVE TECHNIQUE FOR CYTOGENETIC MAPPING OF SHORT DNA PROBES ON PLANT CHROMOSOMES

One of the methods used for the visualization of short DNA probes is Tyramide-FISH (also called TSA (tyramide signal amplification)-FISH, CARD (Catalised reported deposition)-FISH or Tyr-FISH). This method was first introduced by Raap et al. (1995) and applied in animal cells. In 2001, Tyramide-FISH was used on plant chromosomes for the first time to map a T-DNA insertion (710bp) in shallot (Khrustaleva and Kik, 2001). Tyramide-FISH uses an enzymatic detection of hapten-labeled probes. The principle of Tyramide-FISH is based on the capacity of horseradish peroxidase (HRP) to oxidise phenolic molecules (e.g. phenols, tyramine, tyrosine etc.) in the presence of hydrogen peroxide and to catalyse their polymerization (Figure 3).

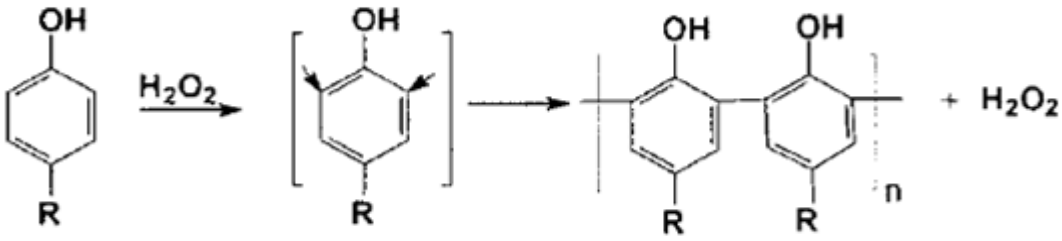

Figure 3. Polymerization of phenol derivatives by horseradish peroxidase (Gross et al. 2001).

In an aqueous solution, a single molecule of HRP can catalyse the oxidation of 10^7 substrate molecules per minute (Zhang et al. 2013). The oxidized substrate molecules can then bind to identical substrate molecules causing di-, tri- or polymerization or can couple to other phenolic molecules. To prevent polymerization, exogenic electron-rich molecules are used. Electron-rich molecules play a role as carrier to which the HRP oxidized substrate molecules can bind (Watabe et al. 2011; Bhattacharya et al. 1999). Proteins can be used for this, at least if they contain electron-rich amino acids such as tyrosine, tryptophan or phenylalanine (Figure 4).

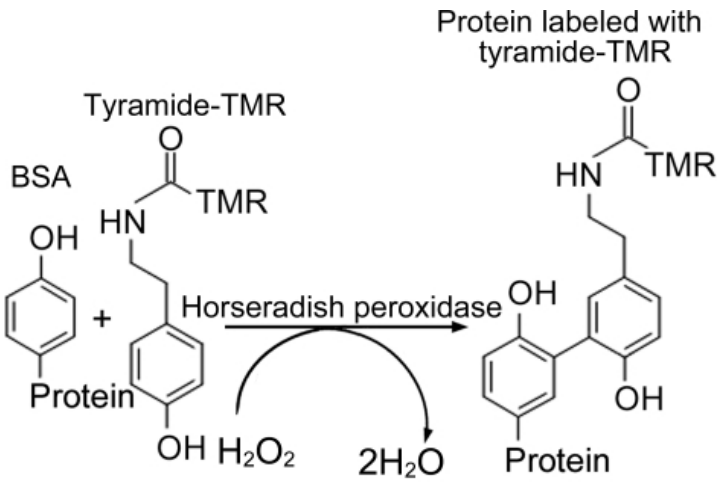

Figure 4. Binding between oxidized tyramides labeled with tetramethyl rhodamine (TMR) and proteins by HRP and H_2O_2 (Watabe et al. 2011).

To manipulate the HRP reaction, Bhattacharya et al. (1999) created an artificial electron-rich protein (based on Bovine Serum Albumin (BSA)) and applied it in Dot-ELISA. To increase the sensitivity of the Dot-ELISA method, electron-rich proteins were used instead of normal blocking proteins (conventional BSA) to cover the Dot-ELISA membrane before the HRP reaction with tyramide-HRP starts. Authors hypothesized that by increasing the number of "landing places" for HRP, oxidized tyramides will allow more tyramides to be left on the membrane after washing and, as a consequence, a higher sensitivity can be achieved up to approximately 10000 times (Bhattacharya et al. (1999)). The same principal was also used in some other studies (Pal and Dhar, 2004; Zhang et al. 2011; Huang et al. 2013).

To use this HRP reaction in a FISH protocol, the following modifications were made:

1. the HRP enzyme was conjugated with an antibody or streptavidin molecule
2. the tyramide molecules as substrate for HRP were conjugated with fluorochromes (tyramide-FLU, direct Tyramide-FISH) or haptens (tyramide-HAP, indirect Tyramide-FISH) (Figure 4).

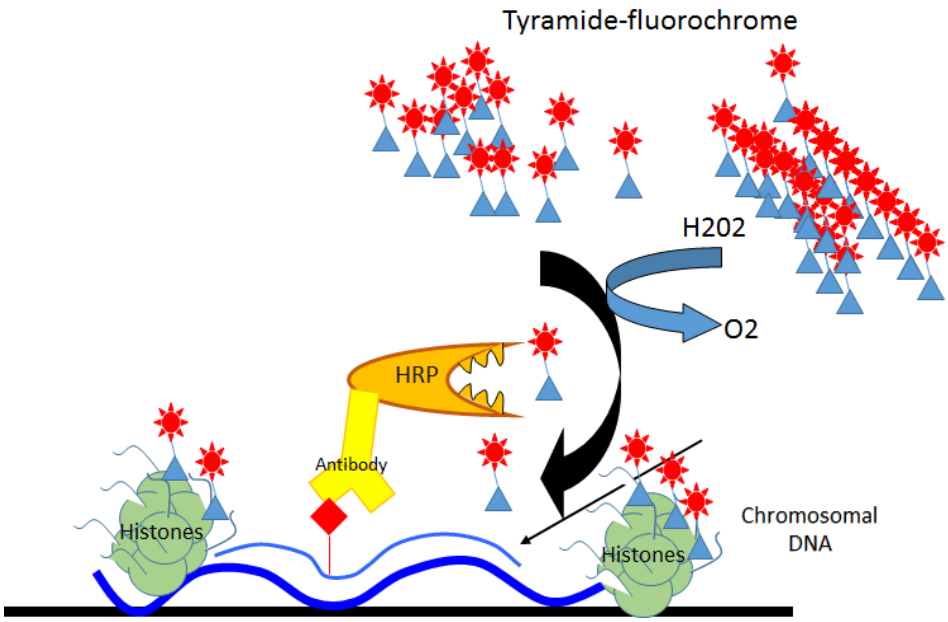

Figure 4. The principal of Tyramide-FISH (direct Tyramide-FISH is shown). Sites of hybridization are detected by antibodies conjugated with horseradish peroxidase (HRP). Adding tyramide-fluorochromes (or tyramide-haptens) molecules in the presence of hydrogen peroxide results in tyramide oxidation by HRP and a covalent coupling of the tyramide-fluorochromes to electron-rich molecules such as tyrosine, tryptophan and phenylalanine amino acids of histones.

Both direct and indirect Tyramide-FISH detection systems can be used for physical mapping of repetitive and unique DNA sequences on plant and animal chromosomes. Comparison of the effectiviness of these two systems for short DNA probe mapping was performed for human (Schriml et al. 1999). In the indirect detection system (Figure 5) tyramide-biotin/streptavidin-fluorochrome reagents mostly showed superior results.

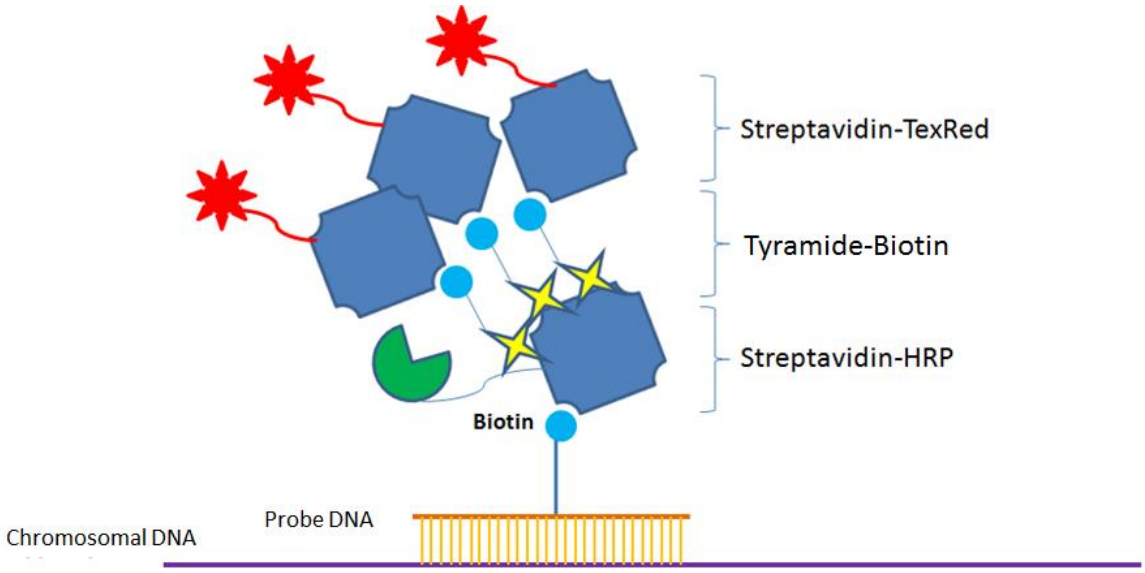

Figure 5. Indirect Tyramide-FISH detection system. Tyramides used for indirect detection system are conjugated with haptens (e.g. biotin). By reacting with HRP the tyramide-hapten molecules are detected by hapten-specific antibodies or streptavidin (if tyramide-biotin conjugates are used) conjugated with fluorochromes (e.g. TexRed).

4. APPLICATIONS OF FISH BASED PHYSICAL MAPS

4.1 ASSISTANCE IN GENOME ASSEMBLY

Cytogenetic mapping may be successfully implemented in the final steps of genome assembly to validate and establish the position of long genomic sequences (e.g. scaffolds and pseudochromosomes) (Cao et al. 2016). The number of examples of successful application of cytogenetic mapping in plant genome sequencing is growing. FISH verified genome assembly for example in *Amborella* (Chamala et al. 2013). In total, authors were able to cytogenetically locate 176 Mb, or 25% of the genome of this genus. Shearer et al. (2014) applied BAC-FISH in combination with optical mapping to verify the tomato genome sequence. Unexpectedly, they found that 45 of the 91 scaffolds of the tomato genome did not correspond to the order as established by linkage maps. The length of all inappropriately arranged scaffolds was equivalent to 34% of the genome consisting of thousands of genes. In addition, authors showed that nearly 5% of the tomato genome was not incorporated in the genome sequence, but was represented as gaps. Most of the discrepancies were located in the heterochromatic regions (Shearer

et al. 2014). Recently, 91% of the genome of duckweed (*Spirodela polyrhiza*) was assembled using FISH (Coa et al. 2016). After assembly of the genome based on NGS data, contigs were obtained. To anchor these contigs to the chromosomes, authors identified BAC clones corresponding to the contigs and used these in FISH experiments. A total of 110 BAC clones representing 6.4% of the *Spirodela* genome were localised on the chromosomes providing anchors for whole genome assembly.

FISH-based physical maps can be integrated with genetic maps and used for scaffold arrangement. As the genetic map resolution is very low in pericentromeric regions in which genetic recombination is suppressed and recombination events are not equally distributed along the chromosomes (Blenda A. et al. 2012; Sun et al. 2013; Ariyadasa et al. 2014), integrated maps are more useful for genome assembly verification as it was demonstrated, for example, in cucumber and tomato (Szinay et al. 2008; Sun et al. 2013). All these studies show that FISH based maps are indispensable tools for genome assembly to confirm the physical locations of markers on linkage groups, to identify mis-assembled clones and to evaluate the size of the remaining gaps in the assembly (Sun et al. 2013).

4.2 COMPARATIVE GENOMICS

A comparative analysis of the genome organization between different species allows shedding light on genome evolution and speciation events. Cytogenetic maps are valuable tools for a comparative genomic analysis as they allow comparing the location of DNA sequences on chromosomes of different species and by this reveal macrosynteny and collinearity between their genomes. A change in gene order and chromosomal location in different species may influence gene functionality by disrupting the link between gene and its regulatory elements or by placing the gene inclose proximity to the heterochromatin (Mandakova et al., 2015; Talbert and Henikoff, 2006). Therefore, understanding of interspecific chromosomal differences can help to unravel mechanisms of gene regulation and adaptation. Cytogenetic maps were used for comparative genome analysis in many plant genera including cotton (Cui et al. 2015), Solanaceae (Szinay et al. 2008; Iovene et al. 2008; Tang et al. 2008), *Cucumis* (Li et al. 2011; Yang et al. 2014), Brassicaceae (Fransz et al. 2000, Lysak et al. 2005), Graminae (Ma et al. 2010; Aliyeva-Schnorr et al. 2016; Danilova et al. 2014) among others. Iovene et al. (2008) reported FISH mapping of 30 genetic marker-anchored BACs on the pachytene chromosome 6 of potato. Using these markers for FISH on tomato pachytene chromosomes the authors demonstrated that short

arms of chromosome 6 of tomato and potato differ by inversion (Figure 6). Because many R genes have been found in the short arm of chromosome 6, Tang et al. (2008) speculated that this inversion may influence the trend of R gene evolution in this region of tomato and potato.

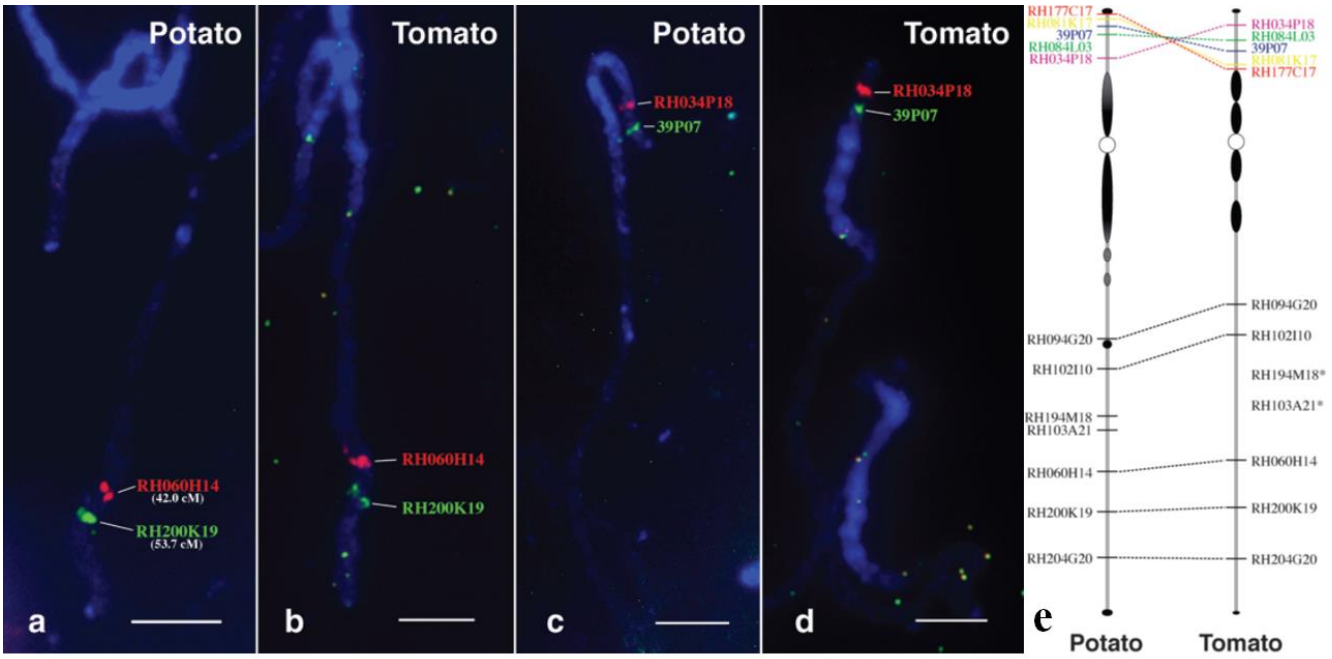

Figure 6. Comparative FISH analysis of pachytene chromosome 6 of tomato and potato (Iovene et al. 2008). BAC-FISH revealed an inversion differentiating the short arms of chromosome 6 of tomato and potato. Bars are 5 µm.

To reveal genomic differences between *Cucumis sativus*, *C. hystrix* and *C. melo,* Yang et al. (2014) performed large-scale comparative pachytene FISH mapping using more than 100 fosmid clones and demonstrated a number of structural chromosomal differences between species.

4.3 GENETIC AND PHYSICAL MAP INTEGRATION

Integration of genetic and physical maps can be useful for:

1) studying recombination event distribution along chromosomes (Aliyeva-Schnorr et al. 2015; Lou et al. 2013);

2) calculation of the physical distance between genetically mapped markers (Khrustaleva et al. 2013);

3) revealing the order of genes in the regions with low recombination frequency (Aliyeva-Schnorr et al. 2015; Karafiatova et al. 2013);

4) anchoring linkage groups to physical chromosomes (Ruiz-Rodriguez et al. 2014) and

5) assisting in genome assembly (Sun et al. 2013; Shearer et. al. 2014).

Understanding the relations between genetic and physical distance is important for map based gene cloning (Tanksley et al. 1992). To perform map integration the same DNA sequences are usually used for genetic and cytogenetic mapping. Map integration using this approach is efficient and cost-effective and has been conducted for a number of plant genera (Xiong et al. 2000; Masamura et al. 2012; Romanov et al. 2015; Han et al. 2011; Lou et al. 2013; Figueroa and Bass, 2012; Wang et al. 2010; Cui et al. 2015; Fonsêca et al. 2010; Ruiz-Rodriguez et al. 2014; Paesold S. et al. 2012; Febrer et al. 2010; Ohmido et al. 2010; Zhang et al. 2010).

5. CYTOGENETICS IN *ROSA* AND *ALLIUM*

5.1 *ROSA*

The genus *Rosa* belongs to the Rosaceae family which contains a number of economically important species such as cherry, apple, apricot, pear etc. Cytogenetic studies in the Rosaceae family are scarce. Until recently only 45S and 5S rDNA have been used in cytogenetic research on *Rosa* species. The main focus of these studies was to perform FISH mapping of these rDNA probes and evaluate the obtained results in the context of phylogenetic relationships between species (Ma and Chen, 1991, 1992; Ma et al. 1997a, b; Fernandez-Romero et al. 2001; Akasaka et al. 2002, 2003; Liu et al. 2008, Jian et al. 2013a, 2013b). In all these studies, it was shown that almost all diploid *Rosa* genomes have one chromosome pair that possesses 45S rDNA signals on the Nucleolus Organizing Region (NOR). However, exceptions were also found as in *R. foliolosa* which has 3 chromosome pairs with 45S rDNA loci (Akasaka et al. 2003). Fernandez-Romero et al. (2001) used FISH with 45S rDNA to elucidate the origin and chromosome complements in diploid, triploid and tetraploid ancestor species of the subgenus *Rosa*. By this, the authors proved the autotriploid and allotetraploid nature of *R. chinensis* and *R. gallica*, respectively (Fernandez-Romera et al. 2001). In contrast to 45S rDNA loci, 5S rDNA loci are located on two chromosome pairs of *Rosa* species belonging to "subset A" (Akasaka et al., 2002). One of the 5S rDNA loci is usually collocalized with 45S rDNA genes on the NOR bearing chromosome (Akasaka et al., 2002; Lim et al. 2005). Two color FISH with 45S and 5S rDNA probes was successfully used to study chromosome pairing in *R. canina* (2n=5x=35). FISH revealed that two chromosome sets of *R. canina* are involved in bivalent formation and no recombination occurred between the other three chromosome sets (Lim et al. 2005).

Application of molecular cytogenetic techniques on *Rosa* chromosomes is hampered by:

1) the small size of the genome (the diploid genome size is 0.83 to 1.30 pg/2C; Roberts et al. 2009) and chromosomes (basic chromosome number x=7) which are difficult to distinguish;

2) polyploidy in the genus (Vamosi and Dickinson, 2006), ranging from 2n=2x=14 to 2n=10x=70 (Jian et al. 2010);

3) the low mitotic index in roots and shoots and weak and tiny roots which are difficult to handle (Ma et al. 1996).

To overcome these challenges, optimization of the chromosome preparation method will be required as a first step towards advanced molecular cytogenetic techniques including physical mapping of short DNA probes. Cytogenetic markers for rose chromosomes are now limited to 45S and 5S rDNA Akasaka et al. 2003). So also, better cytogenetic markers will accelerate the process of physical mapping of rose genomes.

5.2 *ALLIUM*

Allium belongs to the subfamily Allioideae of the Alliaceae family (Asparagales) and consists of nearly 780 species (Friesen et al. 2006). Most of the *Allium* species are diploids and have a basic chromosome number of x=8 while some *Allium* species have a basic chromosome number of x=7 and x=9. Due to the large chromosomes and high mitotic index, some *Allium* species (*A. cepa* and *A. fistulosum*) have been used as model species for cytogenetic research. Moreover, *A. cepa* and *A. fistulosum* are important crops growing world-wide. Extensive breeding programs stimulate the use of molecular cytogenetic methods (FISH, GISH and Tyramide-FISH) for *Allium*. FISH was used to reveal the chromosomal location of major repetitive elements including 45S rDNA, 5S rDNA (Hizume 1994; Lee and Seo 1997; Shibata and Hizume, 2002), and the subtelomeric repeat (Fesenko et al. 2002). These studies suggested that 45S and 5S rDNA are useful cytogenetic markers for 2 (*A. fistulosum*) or 3 (*A. cepa*) chromosome pairs. Recent research performed on *A. fistulosum* shed light on its centromeric DNA (Nagaki et a., 2012). Fajkus et al. (2015) conducted a broad search on *Allium* telomere repeats and showed that the *Allium* telomere contains an unusual repeat, (CTCGGTTATGGG)n. This study also provides a new marker for *Allium* chromosomes assisting in chromosome end identification. *Allium* species have huge genomes varying from 7 pg/1C (*A. altyncolicum*) to 31.5 pg/1C (*A. ursinum*) with a high fraction of repeat sequences (Ricroch et al. 2005). Physical mapping of such a large genome is challenging because of various types of repetitive DNA. Application of BAC-FISH to *A. cepa* chromosomes resulted in signals distributed on all chromosomes for most of the BAC clones (Suzuki et al. 2001). Using Cot-100 fraction for blocking of non-specific hybridization signals allowed to perform BAC-FISH for physical mapping of LFS genes (Masamure et al. 2012). To overcome problems with non-specific hybridization of repetitive DNA, Khrustaleva and Kik

(2001) optimized the Tyramide-FISH technique and showed that DNA sequences as small as 710 bp can be visualized on onion chromosomes by this method. Recently, Tyramide-FISH was used for physical mapping of markers linked to the onion Male Sterility (Ms) locus (Khrustaleva et al. 2016). The Ms markers are physically located near the centromere of onion chromosome 2 providing evidence that the Ms controlling gene is located far away from the genetic markers. The pericentromeric location of the Ms locus suggested that map-based cloning of Ms controlling genes can be challenging (Khrustaleva et al. 2016). To extend the physical map of the onion genome, Romanov et al. (2015) used Tyramide-FISH to locate EST clones on chromosome 5. This onion chromosome is known to carry loci controlling a number of desirable traits including dry matter content, pungency, storability of bulbs, amounts and types of epicuticular waxes, and resistances to abiotic stresses (Romanov et al. 2015). Five unique probes were positioned on *A. cepa* chromosomes and an integration of the physical and genetic map for this chromosome was achieved.

Although members of the *Allium* genus have been widely used for cytological studies (e.g. *Allium* test) the physical map construction by FISH is significantly hampered by repetitive DNA and the huge genome. Ongoing sequencing of *Allium cepa* and extensive breeding programs require knowledge about the physical location of genes. Development of robust cytogenetic markers for chromosome identification is also an important step towards the understanding of the genome organization and evolution in *Allium*.

RESEARCH OBJECTIVES

Since the first application of Tyramide-FISH, no significant optimization of the method was carried out. Questions considering the HRP reaction with tyramide on the slide remain to be answered to achieve higher reproducibility of the results and improved signal-to-noise ratio. For example: How do the chromosome preparation and the pretreatment of slides influence Tyramide-FISH results? With what kind of molecules do tyramides oxidized by HRP bind in the site of HRP localization? Can Tyramide-FISH be applied to pachytene chromosomes to further increase spatial resolution of the method? Unraveling of the influencing factors and optimization of the technology would help to improve this method and would make physical mapping of short DNA probe by Tyramide-FISH more effective and provide higher throughput.

Chromosome preparation, FISH detection systems and cytogenetic markers play a key role in successfull physical mapping of short DNA probes by Tyramide-FISH. These factors are only optimized for a limited number of plant genera including the cytogenetic model plants maize (Wang et al. 2006), wheat (Danilova et al. 2014) and barley (Aliyeva-Schnorr et al. 2016). Development and optimization of new, easy-to-use, cytogenetic tools for other plant species is a challenge. Especially for plants of which genome sequencing is ongoing, it will be interesting to be able to integrate the genome sequence data and the cytogenetic data to accelerate genome assembly.

The general focus of this thesis is to improve short and unique DNA probe physical mapping for *Allium* and *Rosa* species. To achieve this aim, we choose plant species from the genera *Allium* and *Rosa*. *Allium* species are model plants for cytological studies having high mitotic index in root meristems and large chromosomes while *Rosa* species were rarely used for molecular cytogenetic studies because of their small chromosomes, tiny roots and challenging chromosome preparation and FISH application.

In addition, *R. wichurana* is one of the species involved in the origin of most of the modern rose cultivars and is a valuable source of resistance genes (Dugo et al. 2005; Moghaddam et al. 2012; Leus et al. 2009). It is diploid and has shoot meristems that are very suitable for chromosome preparation. Genome sequencing for *R. wichurana* is ongoing and good cytogenetic methods to verify the physical order of contigs and the genetic map will be of great importance for full genome assembly.

More detailed objectives of this thesis are:

1. to develop a highly efficient chromosome preparation method suitable for species with large and small chromosomes;
2. to optimize and evaluate direct and indirect Tyramide-FISH detection systems.
3. to develop a chromosome marker system for *Allium* and *Rosa* chromosomes;
4. to optimize high resolution and multicolor Tyramide-FISH using *R. wichurana* pachytene chromosomes;
5. to perform physical mapping of target genes (1.1 – 3.5 Kb) on chromosomes of *Allium* and *Rosa* species;
6. to carry out anchoring of linkage groups of *R. wichurana* to the physical chromosomes;

RESEARCH OUTLINE

Results in this thesis (Part II) are divided into four chapters addressing specific research questions and objectives. The first three chapters describe different technology optimisation processes and focus on the development of a chromosome preparation method (chapter 1), the set-up of the Tyramide-FISH detection system (chapter 2), and the use of a cytogenetic marker system (chapter 3). We aimed to answer different research and technical questions including: How can the chromosome preparation procedure be improved to be applicable for a wide range of plant species? (chapter 1); Will the chromosome preparation procedure influence the results of Tyramide-FISH? (chapters 2 and 4); Which detection system, direct or indirect, is optimal for the visualization of unique genes on *R. wichurana* chromosomes (chapter 2)? What DNA sequences are useful for chromosome identification in *R. wichurana* and *A. fistulosum* (chapter 3)? Can high resolution and multi-color Tyramide-FISH be applied for physical mapping on *R. wichurana* pachytene chromosomes (chapter 4)?

In chapter 4 the application of the optimized techniques for physical mapping of target genes (1.1 – 3.5 Kb) is described on chromosomes of *Rosa wichurana* and *Allium*. Based on the physical mapping results we looked for an answer on biological questions: What are the differences in gene distribution between *A. cepa* and *A. fistulosum* chromosomes? To what extent is the macro synteny level between *R. wichurana* and other *Rosaceae* genomes?

Chapter 1: Development of a highly efficient chromosome preparation method suitable for species with small and large chromosomes

The study of the dynamics of chromosome spreading occurring on the slide during chromosome preparation and the influencing factors is not yet undertaken for plants. Filling this gap can improve the current protocols for plant chromosome preparation. In Chapter 1 the study of the dynamics of plant chromosome spreading and the development of a chromosome preparation method named “SteamDrop” is described. It is shown that steam stimulates rapid chromosome spreading and chromosome stretching. Based on these observations, the “SteamDrop” protocol was designed for the preparation of well-spread mitotic and pachytene chromosomes and successfully applied to 28 plant species with large and small chromosomes. Both mitotic and meiotic chromosomes prepared by “SteamDrop” are suitable for FISH experiments with repetitive and short DNA probes.

Chapter 2: Evaluation of direct and indirect detection systems for efficient physical mapping of genes

Tyramide-FISH has previously been used for *Allium* (Khrustaleva and Kik, 2001) but never for *Rosa*. Because the effectiveness of the standard direct detection protocol was very low for *R. wichurana* chromosomes, we had to set up an alternative detection system. In this chapter, we describe the results of optimizing the indirect detection system and its successful application for physical mapping of 3 gene fragments on *Rosa wichurana* mitotic chromosomes.

Chapter 3: Development of cytogenetic markers for Rosa and Allium chromosome identification

Chapter 3 presents the results of the development of cytogenetic markers for *Rosa wichurana* and *A. fistulosum*. By the application of FISH with conservative DNA probes, it was shown that 45S rDNA and 5S rDNA can be used for distinguishing 5 out of 7 *R.wichurana* chromosome pairs. Another strategy for identification of FISH-based cytogenetic markers is to perform a bioinformatic search for tandemly organized repeats. This resulted in the isolation of 2 new tandem repeats in *Allium fistulosum*, CAT36 and HAT58, which were validated as useful cytogenetic markers.

Chapter 4: Physical mapping of target genes on chromosomes of *Allium* and *Rosa* species

In this chapter we demonstrate that the developed protocols are useful for individual gene mapping for plant chromosomes. By application of the newly developed "SteamDrop" protocol, the optimized indirect detection system and the cytogenetic markers, we were able to physically map 7 genes (1.7 – 3 kb) on pachytene chromosomes 4 and 7 of *Rosa wichurana*. Using multicolor Tyramide-FISH 3 closely located genes were simultaneously visualized on chromosome 7. In this chapter, we also present the results of Tyramide-FISH mapping of ESTs clones and of the alliinase gene on chromosomes of *A. cepa*.

Part III represents a general discussion on the results and gives some future perspectives for further research based on our findings.

PART II - RESULTS

CHAPTER 1

Development of a highly efficient chromosome preparation method suitable for species with small and large chromosomes

Chromosome preparation is the key step in all cytogenetic techniques including Tyramide-FISH physical mapping. Depending on the quality of the chromosome slides, the results of FISH and Tyramide-FISH can vary dramatically. Obtaining high quality chromosome slides is challenging for many plant genera including *Rosa* and other ornamental plants. Therefore, the aim of this part of the thesis was to develop a novel chromosome preparation protocol suitable for a wide range of plant genera with small as well as large chromosomes. The new chromosome preparation protocol, named "SteamDrop", allows efficient chromosome spreading with reduced cell loss. Chromosome slides prepared by the "SteamDrop" method are suitable for FISH and Tyramide-FISH.

RESULTS ARE DESCRIBED IN PAPER 1:

KIROV, I., DIVASHUK, M., VAN LAERE, K., SOLOVIEV, A., & KHRUSTALEVA, L. (2014). AN EASY "STEAMDROP" METHOD FOR HIGH QUALITY PLANT CHROMOSOME PREPARATION. *MOLECULAR CYTOGENETICS*, *7*(1), 1-10.

MOLECULAR CYTOGENETICS

METHODOLOGY Open Access

An easy "SteamDrop" method for high quality plant chromosome preparation

Ilya Kirov[1,2,3], Mikhail Divashuk[1,2], Katrijn Van Laere[3], Alexander Soloviev[1] and Ludmila Khrustaleva[1,2*]

Abstract

Background: The chromosome preparation is a crucial step for obtaining satisfactory results in molecular cytogenetic researches. The preparation of plant chromosomes for molecular cytogenetic purposes remains a challenge for some species. In contrast to human chromosome preparation, the processes occurring during plant chromosome preparation and causing chromosome spreading are still poorly understood.

Results: We studied the dynamics of plant chromosome spreading after dropping cell suspension on slides. We showed that steam stimulates cytoplasm hydrolysis and rapid chromosome spreading and that chromosomes stretch during this chromosome spreading. Based on these observations, we developed a novel method, named "SteamDrop", for the preparation of well-spread mitotic and pachytene chromosomes and successfully used it for 28 plant species with large and small chromosomes. We applied cell suspensions in ethanol instead of the commonly used ethanol/acetic acid fixative. Mitotic and meiotic chromosomes prepared via "SteamDrop" were used in fluorescent in situ hybridization (FISH) experiments with repetitive and unique DNA probes. Long storage of cell suspensions in ethanol did not impair the quality of chromosome preparations.

Conclusion: The SteamDrop procedure provides a robust and routine method for high quality plant chromosome preparations. The method can be applied for metaphase as well as pachytene chromosome preparation in wide range of species. The chromosomes prepared by SteamDrop are well suitable for repetitive and unique DNA visualization.

Keywords: Plant chromosome preparation, Fluorescence in situ hybridization, Steam application, New method

Background

Chromosome preparation is a key step in all cytogenetic techniques. Most of the modern molecular cytogenetic techniques such as FISH, GISH and Tyramide-FISH require well-spread and morphologically intact chromosomes. Several reports were dedicated to elucidating the chromosome spreading dynamic for improving human chromosome preparations [1-8], while equivalent studies are largely lacking for plants. Difficulties in obtaining well-spread plant chromosome preparation are due to the presence of a cell wall. Moreover, because the high diversity of species possessing small or large chromosomes, low or high chromosome number and different compounds in their cytoplasm, many researches on a modification of a good chromosome preparation method are conducted. There are three main methods of plant chromosome preparation: squashing [9-11], spreading [12,13] and dropping [14-22]. A squashing method has been the common procedure for chromosome counting in plant cytogenetics during decades. Another air dry/spreading method [12] involves a cell suspension preparation which is generated directly on a slide by maceration with the tip of a needle and scattered along a slide. This method is more suitable for plants with small chromosomes. A modification of the method of Pijnacker and Ferwerda [12] was made by Fukui and Ilijima [13] for rice chromosome preparation. The air dry/spreading method was developed also for studying maize somatic chromosomes [23,24]. The drop technique was developed for human cells more than a half century ago [25]. Since then, numerous improvements of the technique and comprehensive studies of all parameters influencing

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the human chromosome spreading dynamics have been done [3,5,7]. The first drop method on plant chromosomes was applied to isolate protoplasts [16]. That method had several disadvantages, however: (i) a low mitotic index due to cell loss during protoplast isolation and metaphase protoplast damage under hypotonic treatment; (ii) polyploidy caused by spontaneous fusion of isolated protoplasts; (iii) labor-intensive protocol; and (iv) protoplast generation from intact plant tissue is more difficult than from cultured cells. Kato et al. [20] developed a novel air dry drop method that was based on enzymatically digested root meristems and preparation of cell suspension in a tube. The cell suspension was consecutively washed in water, 100% ethanol and acetic acid/ethanol (9:1), dropped onto glass slides in a box lined with wet paper towels and dried slowly. The method was successfully used for FISH on mitotic metaphase and pachytene chromosomes of maize [20,21,26] and on mitotic metaphase chromosomes of soybean [27,28]. However, application of this method for species with large sized chromosomes remains problematic because of a low number of non-overlapped metaphases [29].

In spite of the many protocols available for plant chromosome preparation, no robust and generally applicable method has been developed. Therefore, by studying all steps of plant chromosome preparation in detail, we developed a simple protocol "SteamDrop" for reliable chromosome preparation of mitotic and meiotic plant chromosomes. A key step in the preparation of well-spread chromosomes is the application of steam at the moment of meniscus formation over cells during fixative evaporation. The applicability of "SteamDrop"-prepared chromosomes for FISH mapping of repetitive DNA as well as individual genes has been demonstrated. The chromosome preparation allowed physical mapping of small DNA fragments of onion genes using Tyramide-FISH. The "SteamDrop" method was applied for 28 species with different chromosome size and number, belonging to 13 monocot (20 species) and 7 dicot (8 species) genera.

Methods

Plant material

Root meristematic cells were obtained from seedlings: *Allium cepa* (2n = 2 × =16; chromosome size), *A. fistulosum* (2n = 2 × =16), *A. schoenoprasum* (2n = 2 × =16), *A. altaicum* (2n = 2 × =16), *Linum usitatissimum* (2n = 2 × =16), *Triticum aestivum* (2n = 6 × =42), *Cannabis sativa* (2n = 2 × =20).

Root meristematic cells were obtained from intensively grown plants in greenhouse: *Allium roylei* (2n = 2 × =16), *A. wakegi* (2n = 2 × =16), *Humulus japonicus* (2n = 2 × =17 for male or 2n = 2 × =16 for female plants), *H. lupulus* (2n = 2 × =20), *Rosa wichurana* (2n = 2 × =14), *Populus nigra* (2n = 2 × =38), *Brassica oleracea* (2n = 4 × =36), *Ricinus communis* (2n = 2 × =20), *Anthurium andreanum* (2n = 2 × =30), *Monstera deliciosa* (2n = 4 × =60), *Philodendron scandens* (2n = 2 × =32), *Spathiphyllum wallisii* (2n = 2 × =30), *Syngonium auritum* (2n = 2 × =24), *Zantedeschia elliotiana* (2n = 2 × =32), *Aloe vera* (2n = 2 × =14), *Hippophae rhamnoides* (2n = 2 × =24), *Festuca arundinacea* (2n = 6 × =42) and *Lolium perenne* (2n = 2 × =14), *Thinopyrum ponticum* (2n = 10 × =70), *Th. elongatum* (2n = 2 × =14).

Shoot meristems collected from seedlings for *Triticum aestivum* and Triticale (2n = 6 × =42) or from plants in the greenhouse for *R. wichurana* were also used as a source of divided cells for chromosome preparation.

Metaphase arresting, fixation and enzyme treatment

For pretreatment and metaphase arresting, see Table 1. After pretreatment, the roots, shoots or anthers with pollen mother cells (PMC) were fixed in 3:1 (ethanol: acetic acid) for 30–50 min at room temperature. Cell suspension were prepared strait away or cell sources were stored overnight in the freezer at −20°C preceeding enzyme treatment.

The stock enzyme mixture, containing (w/v) 6% Pectolyase Y-23 (Kikkoman, Tokyo, Japan), 6% Cellulase Onozuka R-10 (Yakult Co. Ltd., Tokyo, Japan) and 6% Cytohelicase (Sigma-Aldish Co.LLC, France), was prepared in 0.1 M citric buffer (pH4.8). Concentrations of the work enzyme mixture and incubation time for different species are listed in Table 1.

Protocol of chromosome preparation using the "SteamDrop" method

Enzyme treatment

1. Wash roots (anthers or shoots) in water for 10–30 min
2. Dissect meristems and transfer them into 0.1M citric buffer, pH 4.8
3. Transfer 1–5 meristems to 0.5 ml tubes with 20–30 μl of enzyme mixture (Table 1)
4. Incubate at 37°C for 1–2.5 h depending on species (Table 1)

Cell suspension preparation

1. Vortex the tubes with digested meristems to get cell suspension
2. Add 600 μl of distilled water and mix
3. Centrifuge at 10,000 rpm for 45 sec
4. Remove supernatant using a Pasteur pipette
5. Add 600 μl of 96% ethanol and mix (cell suspension can be stored at −20°C for at least 6 months)
6. Centrifuge at 11,000 rpm for 30 sec
7. Discard supernatant by inverting the tube

Table 1 Condition of metaphase arresting and enzyme treatment

Species	Reagents and conditions	Enzyme concentration	Incubation time in enzyme
Allium cepa, *Allium fistulosum*, *Allium schoenoprasum*, *Allium altaicum*	0.75 mM hydroxyurea for 20 h (RT), 0.05% colchicine for 3.5-4 h (RT)	0.6%	90–100 min
Humulus japonicus, *Humulus lupulus*, *Linum usitatissimum*, *Cannabis sativa*, *Ricinus communis*	2 mM 8-hydroxyquinoline, 4 h	0.6%	100–120 min
Triticale, *Triticum aestivum*, *Thinopyrum ponticum*, *Thinopyrum elongatum*	0.2% colchicine, 2 h[1]	1.2%	100–120 min
Brassica nigra, *Brassica oleracea*,	1-bromnaphtalene (1:1000 water solution) overnight 4°C	0.6%	90–100 min
Aloe vera,		1.2%	100–120 min
Rosa wichurana		0.3%	90–120 min
Anthurium andreanum, *Monstera deliciosa*, *Philodendron scandens*, *Spathiphyllum wallisii*, *Syngonium auritum*, *Zantedeschia elliotiana*, *Hippophae rhamnoides*, *Festuca arundinacea* and *Lolium perenne*		0.1%	60–90 min
Rosa wichurana[2]	Mix of 0.1% colchicine and 2 mM 8-hydroxyquinoline (4 h, RT)[3]	1.2%	120–150 min
Allium wakegi, *Allium roylei*	Nitrous oxide gas (1.0 MPa), 3 h	0.6%	90–100 min
Allium cepa PMC	-	1.5%	180–200 min

[1]the same treatment was used for shoot meristems of Triticale and *Triticum aestivum*.
[2]applied to shoot meristems.
[3]this procedure was carried out according to Ma et al., 1996 [30].

8. Resuspend the pellet in 20–100 μl of 96% ethanol, depending on cell concentration.

Chromosome preparation

1. Drop 10 μl of cell suspension onto a slide* and wait till the surface becomes granule-like, i.e. ethanol meniscus occurred on the top of the cells, (10–15 sec)
2. Drop 18–22 μl of fixative (1:1, 2:1, 3:1 or 5:1 ethanol:acetic acid)** and wait till the surface becomes granule-like and the layer of fixative becomes thin (25–35 sec)
3. Put the slides upside down under the steam from a water bath at 55°C (10–15 cm from water surface of the water bath) for 3–5 sec
4. For double "SteamDrop", repeat step 2 but with less volume (3–6 μl) of fixative and higher concentration of acetic acid. Perform Step 3 for 1 sec only.
5. Immediately dry slides with air flow (e.g. a tabletop fan).

Note

*-for preparation of large size chromosomes it is useful to coat slides with APES (3-aminopropyl-triethoxy-silane) to prevent a chromosome partial detachment. APES coating of slides: 1.5% APES in 100% acetone for 30 sec, twice wash in distilled water and dry for 1 h at 37°C.
**– the protocol allows an easy correction of enzyme treatment results - check the level of tissue enzymatic digestion in the first chromosome preparation slide under microscope, if tissue is underdigested use a high proportion of acetic acid in fixative (1:1 or 2:1); if tissue is overdigested use a low proportion of acetic acid in fixative (5:1 or 10:1).

Probe preparation

LFS and bulb alliinase gene fragment

The LFS and bulb alliinase gene fragment probes were obtained using specific primers (for LFS: LFSbeF: 5′-AAATGGAGCTAAATCCTGGTG-3′, LFSbeR: 5′-CATAATGCATCACAGCACTGAA-3′; for alliinase: Allbe1F: 5′-GGTCATCTCCCTTTCACCAA-3′, Allbe1R: 5′-TGATCAAACTCAAACGCAC-3′) designed by Primer 3.0 software (http://frodo.wi.mit.edu/) using LFS [GenBank: AB094593.1] and alliinase [GenBank: L48614] sequences from GenBank at the NCBI (http://www.ncbi.nlm.nih.gov/genbank/). The PCR conditions were 94°C – 1 min, 35 cycles; 94°C – 1 min; 58°C – 1 min; 72°C – 1 min; final elongation: 72°C – 3 min. The PCR products were cloned by pPCR-TOPO kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's descriptions. Plasmid DNA was isolated from white colonies, sequenced and sequences were analyzed by BLASTN. Plasmids with high similarity to LFS gene or to bulb alliinase gene fragment were selected for labeling with the Biotin Nick Translation Mix (Roche Diagnostics Gmbh, Mannheim, Germany).

5S rDNA

A plasmid carrying the 5S rRNA gene of rye (pScT7, [31] was labeled by Biotin-16-dUTP using Biotin- Nick

Translation Mix according to the manufacturer's protocol (Roche Diagnostics Gmbh, Mannheim, Germany).

HJSR KpnI

A plasmid carrying a HJSR *Kpn*I repeat of *Humulus japonicus* [32] was labeled by Digoxigenin-11-dUTP using Digoxigenin - Nick Translation Mix according to the manufacturer's protocol (Roche Diagnostics Gmbh, Mannheim, Germany).

$(AAC)_5$ oligonucleotide

$(AAC)_5$ oligonucleotide labeled with biotin in the 3′- and 5′ -ends was synthesized in ZAO 'Syntol' (Moscow, Russia).

DNA isolation

Genomic DNA was isolated according to Rogers and Bendich [33].

Tyramide-FISH

Probe hybridization and signal detection was performed according to Khrustaleva and Kik [34] with minor modifications. Before the RNAse treatment and denaturation step, slides were fixed in 4% paraformaldehyde buffered in 1 × PBS (10 × PBS: 1.3 M NaCl, 70 mM Na_2HPO_4, 30 mM NaH_2PO_4, pH 7.5) for 8 min and 10 min, respectively. The step of endogenous peroxidases inactivation was carried out by exposing the slides to 0.01 M HCl for 8 min. The hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 2 × SSC, 0.25% sodium dodecyl sulphate, 2.75 ± 1.00 ng/μl probe DNA. The mixture was denatured at 75°C for 5 min and subsequently placed on ice for 5 min. Sixty microliters of the mixture was added to the chromosome preparations, covered with a coverslip (22 × 32 mm), and denaturated for 5 min at 80°C. An 82% stringency washing was applied: slides were washed in 2 × SSC twice for 5 min at 37°C, in 25% (v/v) formamide in 0.4 × SSC twice for 10 min at 42°C, then in 2 × SSC for 3 min at 37°C. The tyramide detection solution was prepared by thoroughly mixing a 1:50 tyramide-Fluorescein stock solution in amplification diluent (Perkin Elmer, Inc., Waltham, Massachusetts, USA) with 10% (w/v) dextran sulfate.

FISH

FISH procedure with 5S rDNA and biotinylated $(AAC)_5$ as probes was carried out according to the protocol of Heslop-Harrison et al. [35] and Schmidt et al. [36] with slight modification of the slide preatreatment before adding the hybridization mix. Additional treatment with 4% buffered paraformaldehyde solution (BPS), pH 8.0, for 9 min before RNAse treatment was used and pepsin treatment was excluded.

Microscopy and image analysis

Slides were examined under a Zeiss Axio Imager microscope (Carl Zeiss MicroImaging, Jena, Germany). Selected images were captured using an Axio Cam MRm digital camera. Image processing and thresholding were performed using AxioVision ver.4.6 software (Carl Zeiss MicroImaging, Jena, Germany). Final image optimization was performed using Photoshop (Adobe Inc., San Jose, California, USA).

Results

Dynamics of chromosome spreading under steam

The experiment has been done using *Allium cepa*, a species with large chromosomes, and *Humulus japonicus*, a species with small chromosomes. Morphological changes of metaphase chromosomes during slide preparation under 50% relative humidity (RH) and room temperature (25°C) were observed. Phase-contrast microscopy was used to visualize chromosome structure with high resolution and to check the amount of remaining cytoplasm. We analyzed the dynamics of chromosome spreading in both the standard procedure and the proposed steam application procedure ("SteamDrop").

Using the standard protocol, a drop of cell suspension in ethanol:acetic acid fixative (3:1) was placed on the slide. During fixative evaporation (15–25 seconds) the meniscus formation (Figure 1A-B), cytoplasm hydrolysis (Figure 1C-D) and a slight cell swelling (Figure 1D-F) occurred. In most metaphases the chromosomes remained close together.

Using the "SteamDrop" method a drop of cell suspension in 96% ethanol was placed on the slide. When the surface became granule-like, a drop of fixative (3:1 ethanol:acetic acid) was added. During the next 25–35 seconds, the fixative evaporated and the granule-like surface again appeared (Figure 2A-B, 2A'-C'). The moment of granule-like surface appearance after fixative addition was the crucial stage for steam application and obtaining good chromosome spreading. The stage under influence of steam was the shortest stage, lasting only few (3–5) seconds. During this stage, cell swelling, fast chromosome spreading and hydrolysis of the cytoplasm occurred (Figure 2C–H, 2D'-H').

Slide drying conditions

The next step after steam exposure was slide drying. To check the influence of the slide drying condition on the chromosome spreading, we compared drying (1) at natural condition (50% RH, 22–25°C) and (2) with additional air flow using a fan. In the first experiment the drying process was slow (20–30 sec), resulting in a high number of 'overspread' metaphase plates with chromosome loss. Under air flow conditions, the slides dried faster, causing reduced overspreading and as consequence the preservation of complete chromosome sets in metaphases. Also microscopic observations of chromosome behavior during the drying process without intensified air-flow showed that chromosomes and whole cells floated away with fixative currents. The phenomenon became weaker when air-flow

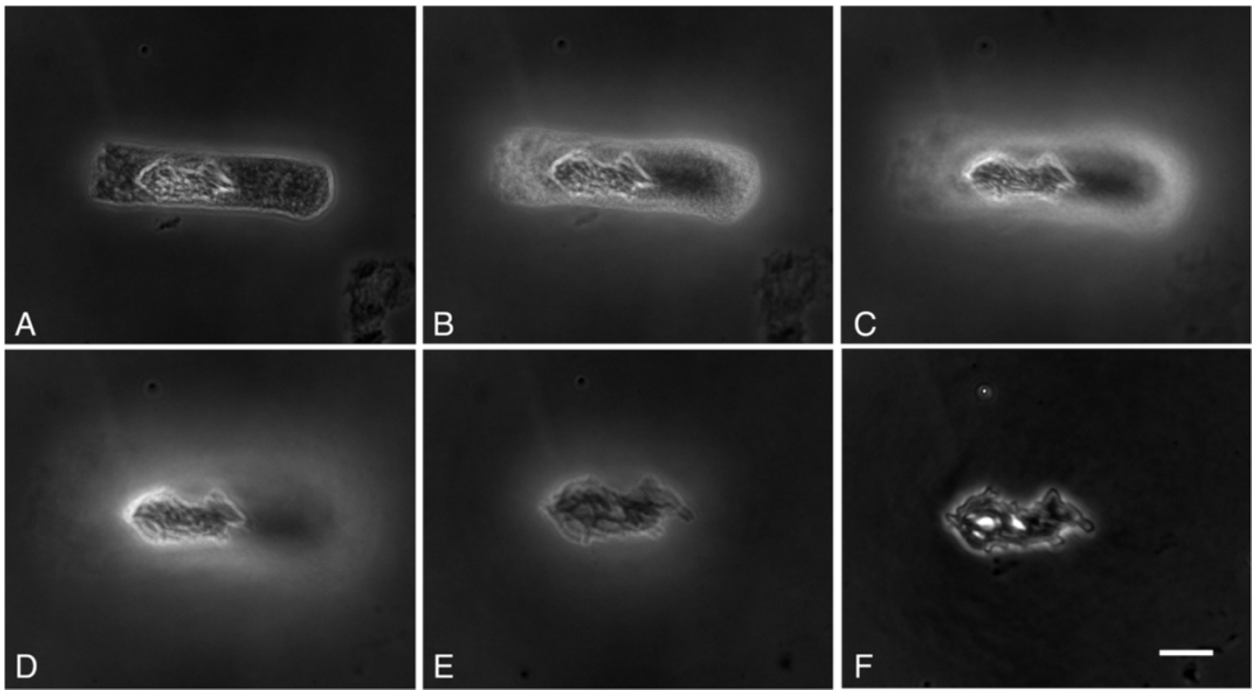

Figure 1 Dynamics of morphological changes of *A. cepa* metaphase chromosomes in the standard protocol. The meniscus formation **(A-B)** and a slight cell swelling **(C-F)** during fixative evaporation from the slide surface. Bar = 10 μm.

drying hastened the evaporation of fixative from the slide. Thus, cells kept their positions on the slide.

Effect of relative humidity on chromosome spreading and chromosome length

To estimate the effect of the relative humidity on chromosome spreading, different RH (25–30%, 50–55% and 65–70%) at room temperature (22–25°C) were applied. *Allium cepa* was used for this experiment. Adjustment of RH in an isolated room-box was done manually. The RH was measured by hygrometer Testo 625 (Testo AG, Lenzkirch, Germany).

Microscopic observation showed that under moderate RH (50–55%), the chromosome spreading was going on during the whole period of steam exposure, resulting in up to 60% of well spread chromosomes without cytoplasm. Under high RH (65–70%) in most cases the chromosomes were clumped, did not spread well after steam exposure and cytoplasm hydrolysis was often incomplete. Preparation under low RH (25–30%) resulted in early cytoplasm hydrolysis that prevented chromosome spreading.

The total chromosome length has been measured on slides prepared under moderate or high RH using a single cell suspension sample. The chromosomes prepared under high humidity were significantly smaller (163 ± 14 μm) than chromosomes prepared under moderate humidity (232 ± 1.0 μm, $P \leq 0.0001$). Under high RH the total chromosome length had a wide range, while under moderate RH it was almost uniform. The chromosomes prepared under high RH appeared as bright light structures of high optical density under a phase contrast microscope, while the chromosomes prepared under moderate RH were less condensed and appeared as grey structures (Figure 3).

Chromosome spreading using "SteamDrop"

In species with small chromosomes, most of the metaphase cells (85–97%) prepared with the "SteamDrop" method showed good chromosome spreading (<2 overlapped chromosomes, Figure 4 *Cannabis sativa*, *Humulus japonicus*, *Brassica oleracea*, *Rosa wichurana*). In species with large chromosomes only 15–20% of metaphases appeared well spread. Therefore, for these species a second drop of fixative with higher acetic acid concentration (e.g. 1:1 or glacial acetic acid) after the first drop of 5:1 or 3:1 ethanol:acetic acid were used (Figure 4 *Allium cepa*, *Allium fistulosum*, Triticale, *Triticum aestivum*). The second drop, containing a higher portion of acetic acid, completed the cytoplasm digestion and provided additional chromosome spreading before chromosome immobilization on the slide surface. Steam was applied after each drop of fixative. Microscopic analysis of chromosome spreading revealed that the use of two drops of fixative extended the time of chromosome spreading. Using a second fixative drop in onion increased the number of well spread metaphases two-fold as compared to a single drop treatment. It should be mentioned that the application of nitrous oxide gas as

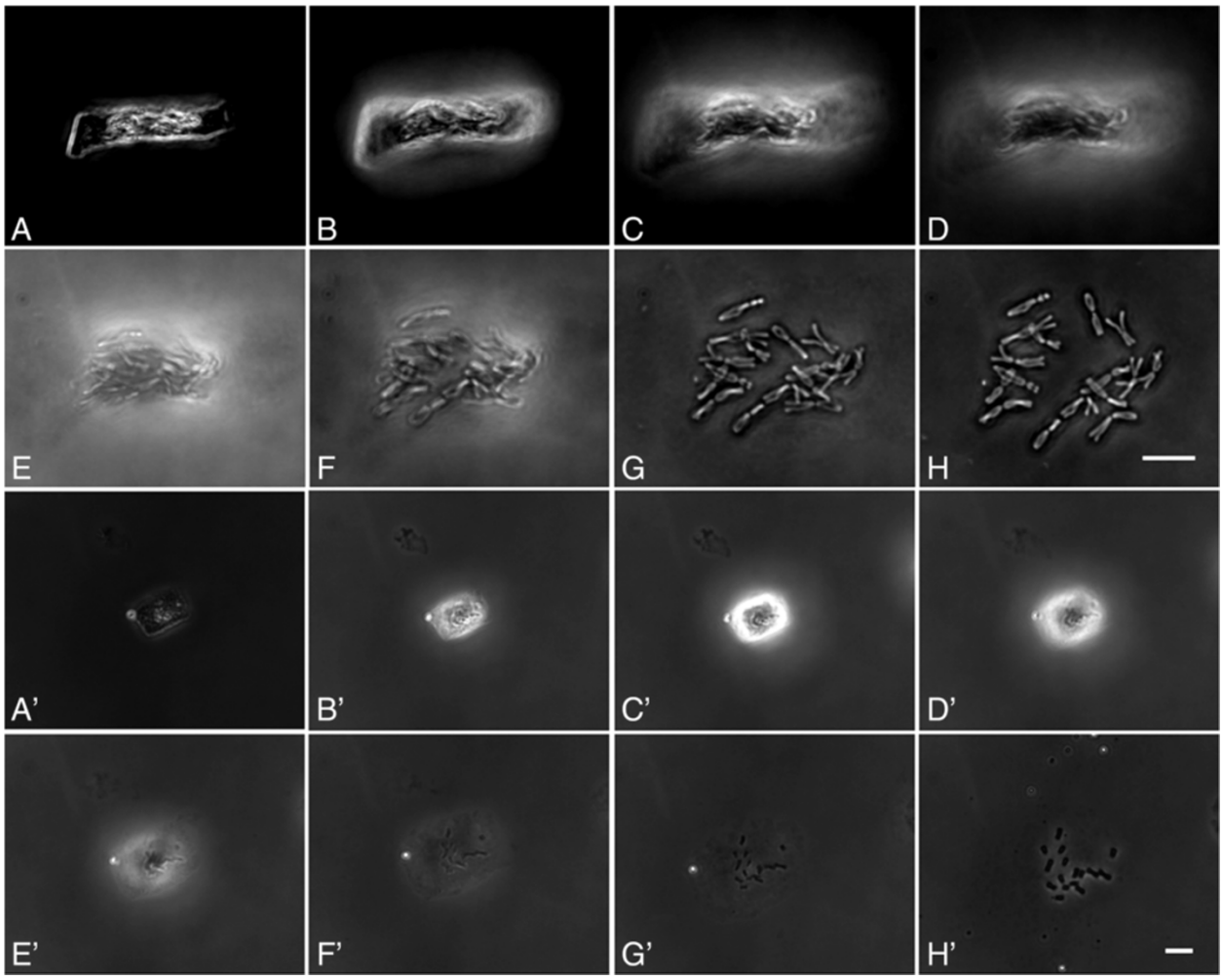

Figure 2 Dynamics of *Allium cepa* (A–H) and *Humulus japonicus* (A′-H′) chromosome spreading understeam. Meniscus formation **(A-B, A′- C′)**, cell swelling **(C-D, D′-E′)** and chromosome spreading **(E–H, F′-H′)**. **C–H** and **D′-H′** - steam application to the slides. Arrows indicate stretching of an *Humulus japonicus* chromosome. Bar = 10 μm.

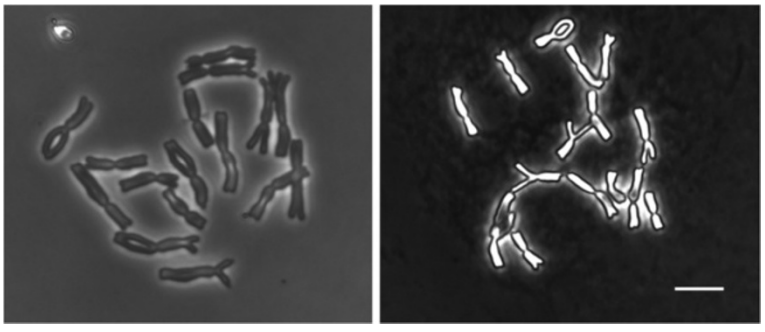

Figure 3 Metaphase chromosomes of *Allium cepa* prepared under moderate (50%) RH (left) and high (70%) RH (right). Bar = 10 μm.

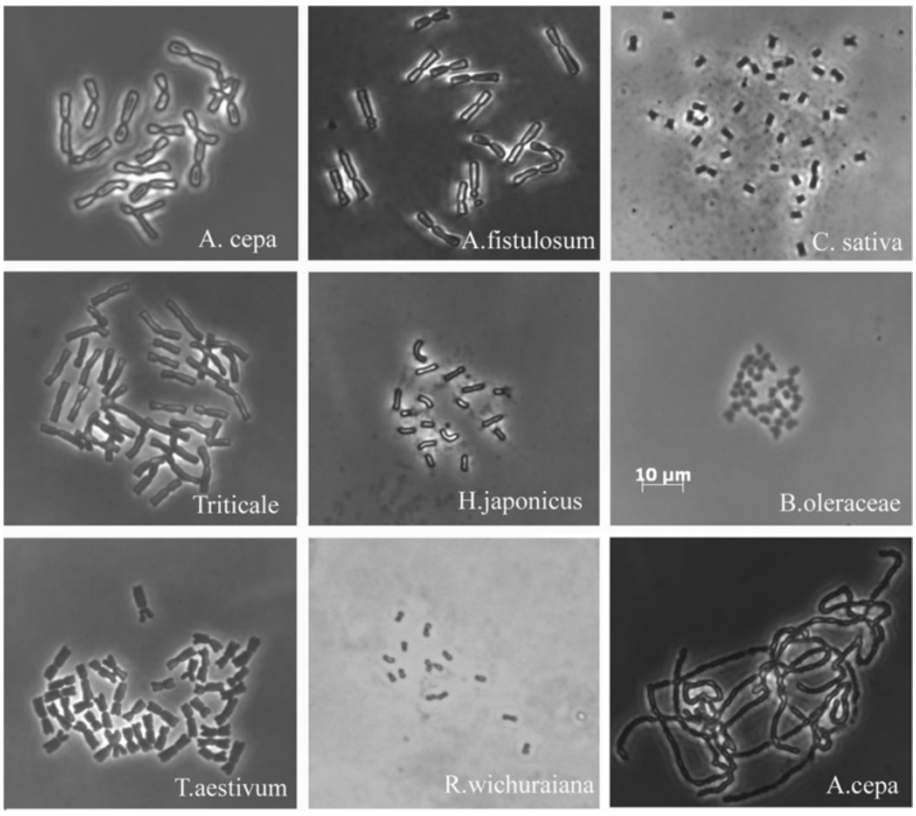

Figure 4 Mitotic metaphase plates of species with large (*Allium cepa, Allium fistulosum,* Triticale*, Triticum aestivum*) and small (*Cannabis sativa, Humulus japonicus, Brassica oleracea, Rosa wichurana*) chromosomes. The last figure is pachytene chromosomes of *A. cepa*. All photos were made using the same magnification. Bar = 10 μm.

metaphase arresting agent in combination with the second fixative drop increased the number of well spread metaphases up to 60% in *Allium* species. Application of a second drop in species with small chromosomes (*Cannabis sativa*, *Humulus japonicus*, *Humulus lupulus*, *Linum. usitatissimum*, *Populus nigra*, *Brassica oleracea*, *Ricinus communis*) resulted in a higher percentage of overspread metaphases and chromosome loss as compared to the single drop use. However, this was not true for *Rosa wichurana*, *Spathiphyllum wallisii*, *Syngonium auritum*, *Zantedeschia elliotiana*. These species have also small chromosomes but a rigid cell wall and the application of the second drop resulted in well spread metaphases with completely digested cytoplasm.

The application of the second drop was also successful for meiotic chromosome preparation using a higher concentration of enzymes (1.5% cellulase, 1.5% pectolyase, 1.5% cytohelicase) for 3 h and a higher concentration of acetic acid in fixative (1:1) for first drop and 100% acetic acid for second drop (Figure 4. *Allium cepa*).

Cell suspension storage

Our experiments showed that cell suspension of PMCs and root meristems in 96% ethanol can be used for chromosome preparation even after 6 months of storage in freezer (–20°C). The storage did not influence the chromosome preparation quality. Whereas, commonly used storage of anthers for several months in ethanol: acetic acid fixative or in 70% ethanol resulted in poor chromosome spreading of PMCs impaired by a high amount of cytoplasm. The "SteamDrop" method makes it possible to prepare mitotic and meiotic chromosomes independent of the season.

"SteamDrop" chromosome preparations are excellent for FISH

Chromosomes prepared by the "SteamDrop" method were evaluated for their applicability in FISH experiments. It was found that chromosome preparations are highly sensitive to denaturation in the hybridization mixture. Immediately after denaturation, DAPI stained chromosomes sometimes showed chromatin protrusion that often hampers signal detection and karyotyping. Application of an additional treatment with 4% buffered paraformaldehyde solution (BPS), pH 8, for 9 minutes before RNAse treatment helped to overcome this problem. Furthermore, chromosome preparation according to the "SteamDrop" protocol does not require pepsin pretreatment. The

"SteamDrop" preparations were largely free of cytoplasm and yielded a high signal to noise ratio. In our experience, pepsin pretreatment did not increase signal to noise ratio, but might damage chromatin structure.

FISH experiments on mitotic chromosomes with a 5S rDNA probe in *Allium fistulosum* (Figure 5B) or HJSR *Kpn*I probe in *Humulus japonicus* (Figure 5A) or the $(AAC)_5$ oligonucleotide probe in *Triticum aestivum* (Figure 5D) were analyzed. The chromosomal positions of the corresponding FISH signals coincided with results described earlier [37,32].

FISH on the pachytene chromosome of *Allium cepa* with the 5S rDNA revealed two hybridization sites (Figure 5C), in accordance with previously obtained data on mitotic chromosome of *Allium cepa* [37].

Detection of genes on plant chromosomes strongly depends on the quality of chromosome preparations. The applicability of the "SteamDrop" chromosome preparations for gene localization was evaluated in Tyramide-FISH experiments. Two genes of *Allium cepa*, the LFS (lachrymatory factor synthase) and the bulb alliinase, which are involved in the same biochemical pathway, were used for visualization on mitotic metaphase chromosomes. The LFS gene (550 bp) was detected in a proximal position on chromosome 5 (Figure 5E); the bulb alliinase gene fragment (1.1 Kb) was found in a distal position on chromosome 4 (Figure 5F), as reported previously [38,39].

Discussion

Steam stimulates chromosome spreading

Steam application at the moment of meniscus formation causes effective chromosome spreading. Steam hastens cell wall hydrolysis by heating the slide surface. This stimulates ethanol evaporation and increases the acetic acid concentration for cellulose hydrolysis [40]. Moreover, steam delivers water to the slide surface [5], providing rapid cell rehydration. Claussen et al. [6] showed the essential role of water in mammalian cell swelling. Kato et al. [20] also emphasized the importance of a high humidity treatment in order to spread plant chromosomes and thus proposed the use of a humidity chamber during slide preparation. We propose to use steam for efficient chromosome spreading. We suppose that during steam application three coinciding processes occur: (1) cytoplasm rehydration and swelling; (2) rapid cell wall hydrolysis, which aids cell burst due to cytoplasm swelling; (3) chromosome movement. It is very

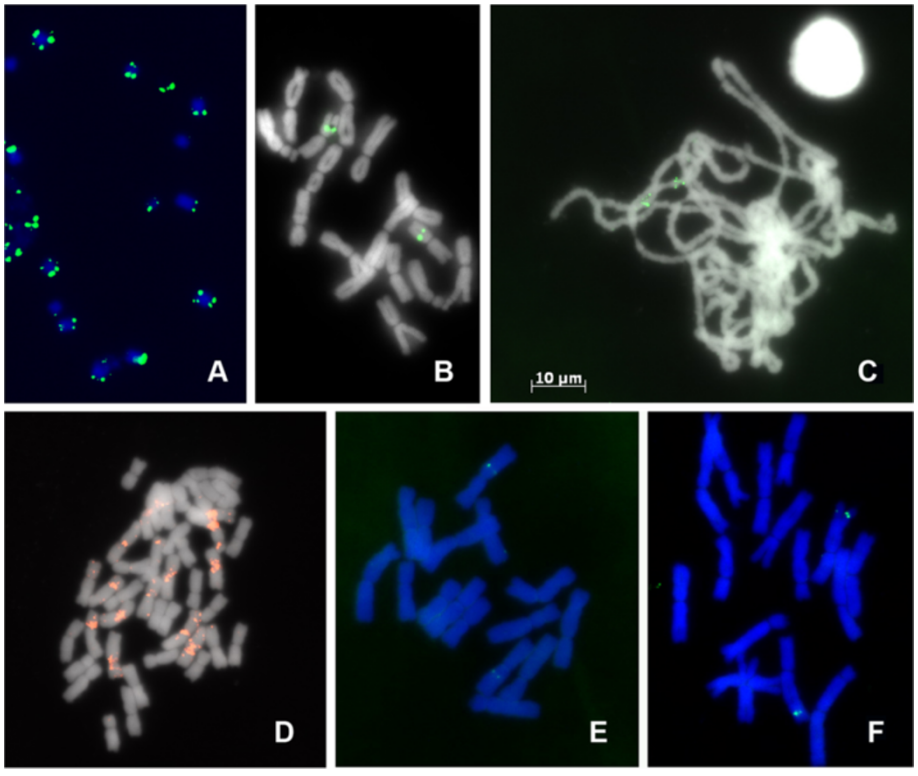

Figure 5 The "SteamDrop" chromosome preparations used in different cytogenetic techniques. (A) FISH - *Humulus japonicus* probing with the HJSR *Kpn*I repeat; **(B)** FISH - *Allium fistulosum* probing with 5S rDNA (pSCT7); **(C)** FISH - *Allium cepa* pachytene chromosomes probing with 5S rDNA (pSCT7); **(D)** FISH - *Triticum aestivum* probing with the $(AAC)_5$ oligonucleotide; **(E)** Tyramide-FISH - *A. cepa* probing with the LFS gene clone (550 bp); **(F)** Tyramide-FISH - *A. cepa* probing with bulb alliinase gene fragment (1.1 Kb).

important that the steam is applied at the moment a granule-like surface appears, when meniscus formation is occurring. Meniscus pressure and steam cause rapid plant cell swelling and, as a consequence, efficient chromosome spreading.

Effect of relative humidity

Mammalian chromosome spreading depends on relative humidity of the environment [3-7]. We found that RH influences on plant chromosome preparation as well. The optimal RH for plant chromosome preparation using our "SteamDrop" method was 50–55%, similar to that described for mammalian chromosome preparation [3]. We suppose that the impact of RH on chromosome spreading is mainly determined by slide drying time and water-induced cytoplasm swelling. Under low RH, quick ethanol evaporation with increasing acetic acid concentration occurs (fixative is not an azeotropic mixture, [6]) resulting in prompt cytoplasm digesting before steam application. Cytoplasm swelling does not take place and chromosomes remain close together. Under high RH, ethanol evaporates slowly while the process of fixative rehydration goes fast. This results in a low concentration of acetic acid. Therefore, undigested thick cytoplasm hampers steam-induced chromosome spreading. Under mid-level RH, the processes of the fixative rehydration and the ethanol evaporation are balanced. At the moment of steam application, cytoplasm density is sufficient for steam-induced chromosome spreading.

"SteamDrop" may cause chromosome stretching

Plant chromosome stretching was observed under steam action. It was found that the degree of chromosome stretching depends on RH of ambient condition. Thus chromosomes prepared under moderate humidity were 1.42 times longer that those prepared under high RH. Claussen et al. [6] showed chromosome preparation-induced changes in the lengths of human lymphocyte chromosomes. Authors proved that chromosomes have their own potential to swell and they suggested that some modifications of chromosome proteins promote chromosome stretching. Moreover, it was shown by real-time scan force microscopy that DNA molecules may decondense and lengthen under specific micro-environmental condition [41]. Thus, chromosome stretching could be caused by DNA relaxation itself or rehydration of chromatin proteins or combination of both processes. Claussen et al. [6] proposed the concept of chromosomal region–specific protein swelling. They showed that "G-banded chromosomal regions" are involved in chromosome stretching. Unstretched human chromosomes do not show any visible GTG-banding patterns [4,6]. Reproducible G-banding patterns on plant chromosomes are difficult to obtain [42]. Hliscs et al. [4] supposed that the absence of plant chromosome stretching is what causes a lack of G-bands. Probably, plant chromosome stretching produced by the "SteamDrop" protocol will help to obtain reproducible G-banding patterns on plant chromosomes. Further experiments will be done to check this assumption.

Conclusion

The results reported here demonstrate the effectiveness of "SteamDrop" method for high quality chromosome preparation of plant species with small and large chromosomes. The applicability of the chromosome preparation for FISH and Tyramide-FISH experiments was shown. The advantages and distinctions of our "SteamDrop" method from previously developed methods are (1) the steam application caused efficient chromosome spreading; (2) the minimization of washing steps reduce chromosome damage and cell loss; (3) instead of commonly used dropping onto the slide of cell suspension in ethanol-acetic acid fixative we propose to use cell suspension in 96% ethanol that allowed to regulate the chromosome spreading and amount of cytoplasm around chromosomes by adding ethanol-acetic acid fixative in proper ratio; (4) moreover, long-term storage of cell suspension in 96% ethanol does not impair the quality of chromosome preparation; (5) several slides can be prepared from a single root; (6) a simple protocol: the preparation of cell suspension excluding of metaphase arresting and enzyme treatment steps takes only 2–3 minutes; the chromosome slide preparation – 1 minute.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IK designed the study, performed the research and wrote the manuscript. KVL, AS and MD performed the research. LK designed and coordinated the study and wrote article. All authors read and approved the final manuscript.

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Erratum: “An easy “SteamDrop” method for high quality plant chromosome preparation” [Molecular cytogenetics, 7(1), 1-10]

1. Page 42. The sentence “In the first experiment the drying process was slow (20–30 sec), resulting in a high number of ‘overspread’metaphase plates with chromosome loss.” should be replaced by the following sentence: “In the first experiment the drying process was slow (20–30 sec), resulting in 30 – 40 % metaphase plates were ‘overspread’ (1 or more chromosomes lost).”

2. Fig. 4. The scale bar is the same for all pictures.

CHAPTER 2

EVALUATION OF DIRECT AND INDIRECT DETECTION SYSTEMS FOR EFFICIENT PHYSICAL MAPPING OF GENES

The Tyramide-FISH technique has previously been applied on model species for cytogenetic studies (onion, maize, wheat, oat) having large chromosomes but has never been used for species with small chromosomes to perform physical mapping of the genes. In our preliminary experiments, the effectiveness of the Tyramide-FISH physical mapping on *R. wichurana* chromosomes using the described detection system (direct detection) was very low. Therefore, the optimization of an alternative indirect detection system was required and was the goal for this chapter.

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Anchoring Linkage Groups of the Rosa Genetic Map to Physical Chromosomes with Tyramide-FISH and EST-SNP Markers

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Abstract

In order to anchor *Rosa* linkage groups to physical chromosomes, a combination of the Tyramide-FISH technology and the modern molecular marker system based on High Resolution Melting (HRM) is an efficient approach. Although, Tyramide-FISH is a very promising technique for the visualization of short DNA probes, it is very challenging for plant species with small chromosomes such as *Rosa*. In this study, we successfully applied the Tyramide-FISH technique for *Rosa* and compared different detection systems. An indirect detection system exploiting biotinylated tyramides was shown to be the most suitable technique for reliable signal detection. Three gene fragments with a size of 1100 pb–1700 bp (*Phenylalanine Ammonia Lyase*, *Pyrroline-5-Carboxylate Synthase* and *Orcinol O-Methyl Transferase*) have been physically mapped on chromosomes 7, 4 and 1, respectively, of *Rosa wichurana*. The signal frequency was between 25% and 40%. HRM markers of these 3 gene fragments were used to include the gene fragments on the existing genetic linkage map of *Rosa wichurana*. As a result, three linkage groups could be anchored to their physical chromosomes. The information was used to check for synteny between the *Rosa* chromosomes and *Fragaria*.

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Introduction

Genome structure and function may be studied when comparing the genetic positions of genes with their physical locations on chromosomes. In former times, to assign linkage groups to physical chromosomes it was needed to create monosomic addition lines, nullisomic lines, chromosome substitution lines or translocation lines [1–3]. This is a very time consuming task. Nowadays, a more efficient approach exists by direct visualization of genetically mapped markers on chromosomes using fluorescent in situ hybridization (FISH) to locate large genomic clones (BAC, YAC, cosmids etc.) containing the markers. However, FISH with large genomic DNA fragments often results in many non-specific hybridization due to the presence of huge amounts of repetitive DNA in plant genomes [4,5]. To overcome this problem, FISH using direct labeled individual genes can be applied [6–8]. This approach however still is very challenging for most ornamental species and in particular for woody species, such as *Rosa*.

The genus *Rosa*, a member of the Rosaceae, consists of approximately 200 species and 20000 cultivars, most of complex hybrid origin. The genus has a wide phenotypic variability and a high level of genetic heterozygosity [9]. Despite the crop's long domestication history, intensive breeding and economic importance, relatively little is known about the genetics and cytogenetics of roses [10,11]. Nevertheless, several characteristics of rose make it a worthy candidate for a model system for genomic research in woody species [11].

Performing cytogenetic analyses for roses is difficult because of their genome size (the diploid genome size is 0.83 to 1.30 pg/2C; [12]) and very small chromosomes. The mitotic index is generally low in shoot and root tips, root development is weak and roots are thin in mature individuals for several *Rosa* species [13]. The basic chromosome number of roses is 7 [14,15] and ploidy levels range from diploid ($2n = 2x = 14$) to octoploid ($2n = 8x = 56$) [16]. A number of basic cytogenetic studies, including chromosome counts and karyotyping, have been done on roses [14–31]. A karyotype with indication of 45 S and 5 S rDNA sites was constructed for some wild species [24–27]. Repetitive sequences, such as 45 S and 5 S rDNA, are rather easy to map, compared to low-copy genes. Reports of physical mapping of low copy genes are found in several genera, such as tomato [32], rice [33], barley [34], wheat [35], sugar beet [36], *Sorghum* [37], maize [7], *Populus trichocarpa* [38] and safflower [39], among others. However, physical mapping of low-copy genes remains a problem in lots of other species and genera and also in *Rosa*. Moreover, in most reports showing conventional FISH results, the target DNA sequences were over 10 kb. Since EST-markers are good candidates to anchor linkage groups to physical chromosomes, lowering the

probe-size detection limit should be obtained. Significant improvements in detection limits have been reported, such as the use of a cooled-charge-coupled device (CCD) camera and primed *in situ* DNA labeling (reviewed by Figueroa and Bass [40]). An alternative FISH method used to detect very small probes is tyramide signal amplification (TSA)-FISH, or Tyramide-FISH, a multi-step procedure involving (1) *in situ* hybridization with a labeled probe, (2) signal amplification by streptavidin-horseradish peroxidase (SA-HRP) and tyramides and (3) detection and imaging of the amplified signal [41]. This method was originally introduced by Bobrow et al. [42] for microplate immunoassays. Raap et al. [43] introduced the use of fluorescent tyramide conjugates as substrates for Horse Radish Peroxidase (HRP) into FISH technology. With Tyramide-FISH, the detection sensitivity can be increased up to 100 times compared to the conventional FISH procedures [44]. Tyramide-FISH has been successfully used in human genetics for single-copy gene detection [41,45–52]. In plants, however, Tyramide-FISH has only been used in a few studies [53–56].

Molecular markers have been developed in roses to enhance breeding efficiency through the identification and characterization of genes controlling important traits [9,57,58]. Major efforts for the construction of genetic linkage maps in the *Rosa* genus have been concentrated at the diploid level [57,59–63,]. Four mapping populations allowed the construction of an integrated consensus map consisting of about 600 markers distributed across 7 linkage groups, with an overall length of 530 cM [58]. Recently, interest in mapping at the tetraploid level has been renewed [64,65]. Some major rose traits have been located on the rose genetic maps, such as flower color and double corolla [59] and resistance to powdery mildew [61,62,63]. To date, no genome sequence is available for the *Rosa* genus that allows validation of the positions of markers located to linkage maps. But *Rosa* is well-supported by the closest sister taxon, which contains the genus *Fragaria*, and also shows sequence homology with *Malus* and *Prunus* [65–68]. Developing markers in EST fragments of genes can be based on this sequence homology with other Rosaceae. Although SSRs are widespread in the plant genome, the number of ESTs containing an SSR motif can be quite limited [69]. EST-SNPs have more potential as a functional marker. Due to the conserved nature of the coding sequence, these markers are also appropriate for the comparison of genetic maps between species [70,71]. High Resolution Melting (HRM) analysis is the method of choice for EST-SNP genotyping, because SNP sequence information is not a prerequisite [72]. HRM was originally introduced as a method for mutation scanning in human genetics [73] and has the ability to simultaneously detect and genotype DNA polymorphisms [74]. The use of HRM for EST-SNP marker development and consecutive mapping in plants has already been reported in several crops such as barley [72], alfalfa [75] and apple [76] but not yet in rose.

The combination of the opportunities of Tyramide-FISH and the HRM molecular marker system may result in an effective integration of physical and genetic maps. The present study had two main aims: 1) to optimize the Tyramide-FISH technology for roses in order to cytogenetically map single-copy genes and 2) to connect their physical position with their genetic position on the linkage groups of *Rosa wichurana* (Moghaddam et al. 2012) using HRM technology.

Materials and Methods

Plant Material

The plant material used in this study was *Rosa wichurana, Rosa* 'Yesterday' and 90 F1 hybrids of *Rosa* 'Yesterday' x *Rosa wichurana*. Both parent plants and the hybrid progeny are diploid (2n = 2x = 14). The plants were own-rooted and grown in the field. For chromosome slide preparations, cuttings of *Rosa wichurana* were made. Rooted cuttings were transferred to terracotta stone pots and grown in the greenhouse without artificial light or temperature regulation. The conditions inside the greenhouse were thus dependent on the moderate climatic conditions typical for the East Flanders region of Belgium.

Chromosome preparation

Somatic metaphase chromosome spreads were prepared from shoot meristems collected and pretreated according to [13]. Briefly, young shoot meristems (2–3 mm) from which upper green leaves were removed, were collected in ice-cold 1 mM 8-hydroxyquinoline and 0.1% colchicine solution and incubated for 3.5 hours at room temperature in the dark. Afterwards, meristems were fixated in 3:1 ethanol:glacial acetic acid for 45–60 minutes and stored in 70% ethanol at −20°C. Chromosome slide preparation was carried out according to the spreading protocol of Pijnacker and Ferwerda [77] or to the "SteamDrop" method of Kirov et al. [78].

Primer and probe design

DNA of *Rosa wichurana*, *Rosa* 'Yesterday' and their hybrids was extracted from young leaves using the Qiagen DNeasy Plant Mini Kit (Chatsworth, CA). The genes *PAL*, *P5CS* and *OOMT* were isolated according to Razavi et al. [79] starting from ESTs available in the Genome Database of Rosaceae [80]. These genes are known to be involved in abiotic stress response (*Phenylalanine Ammonia Lyase* (*PAL*) and *Pyrroline-5-Carboxylate Synthase* (*P5CS*), [81,82]) and rose scent production (*Orcinol O-Methyl Transferase* (*OOMT*), [83]), which are important traits for roses.

To have good probes to use in Tyramide-FISH, we designed primers in order to obtain PCR fragments of about 1500 bp (see Table 1). Plasmid DNA of the cloned gene fragments was labeled using the Biotin Nick Translation Mix (Roche) according to the manufacturer's instructions. As a control, the pTA71 plasmid (containing a 9 kb fragment of 45 S rDNA, [84]) was labeled with biotin.

To generate EST-SNPs for HRM, we searched for SNPs between *Rosa wichurana* and *Rosa* 'Yesterday' in the sequences of the cloned genes *PAL*, *P5CS* and *OOMT*. Primers flanking a single SNP were developed for amplification of the EST-SNPs (Table 2). Primers were tested on the parents and 5 siblings of the mapping population *Rosa* 'Yesterday' x *Rosa wichurana*. Good primers were then applied to the entire mapping population.

Tyramide-FISH optimization

Probe hybridization was performed according to Khrustaleva and Kik [53] with minor modifications. Slides were fixed in 4% buffered paraformaldehyde in 1xPBS (10xPBS: 1.3 M NaCl, 70 mM Na2HPO4, 30 mM NaH2PO4, pH 7.5) for 8 min before the RNAse treatment and 10 min before denaturation. Inactivation of endogenous peroxidases was done by incubating the slides in 0.01 M HCl for 8 min. Pepsin treatment was performed during 30 sec at room temperature. The hybridization mixture contained 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 2xSSC, 0.25% sodium dodecyl sulphate and 2.00 ng/µl probe DNA. The hybridization mix was denatured at 80°C for 5 min, subsequently placed on ice for 5 min, and added to the chromosome slides. Slides were then denatured for 5 min at 80°C and hybridization was carried out at 37°C overnight. A 82% stringency washing was attained by washing the slides twice in 2xSSC for 5 min at 37°C, twice in 25% (v/v) formamide in

Table 1. Overview of the primers used to isolate the genes PAL (Phenylalanine Ammonia Lyase), OOMT (Orcinol O-Methyl Transferase) and P5CS (Pyrroline-5-Carboxylate Synthase).

Gene	Primers (5′-3′)	Tm (°C)	Source sequence	Amplicon (bp)
PAL	ACCACTGGKTTTGGTGCWAC CCYTTGAASCCATAATCCAA	*59.9*	*Prunus persica*	1700
OOMT	TGCACTACCAATCCATCCAA TGCCAAGTAACATTTGGCTTT	*59.9*	*Rosa chinensis 'Old Blush'*	1100
P5CS	GCTGGCATCCCTGTTGTTAT CTTCGGATCGCTAATGAAGC	59.9	*Prunus persica*	1700

The length of the obtained amplicons is indicated as well as the Tm and source sequence.
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0.4xSSC for 10 min at 42°C, and finally in 2xSSC for 3 min at 37°C.

For probe detection, three tyramide amplification systems were used: direct detection (modified from Schriml et al. [47] and Khrustaleva and Kik [53]), indirect detection (modified from Schriml et al. [47] and Perez et al. [32]) and indirect detection with two rounds of amplification. The incubation time with the tyramide solution vary from 5 to 10 min. In the direct detection system, tyramide-FITC (Tyr-FITC) or tyramide-Cy3 (Tyr-Cy3) was used in dilutions 1:50. In the indirect detection system, biotinylated tyramides (Tyr-Bio, PerkinElmer, Belgium) were used in the dilutions 1:25 and 1:50 and the antibodies (Strepatavidin-Cy3, or Streptavidin-Cy3) were 1:100 and 1:300 diluted. The concentration of Tyr-Bio and Streptavidin-HRP (SA-HRP) antibodies used in the first round of the indirect detection with two rounds of amplification system were the same as in the indirect detection system. In the second round of amplification SA-HRP was diluted 1:300 or 1:200 and Tyr-Cy3 was used in dilutions 1:100, 1:300, 1:500 or 1:1000.

Images were taken using a fluorescence microscope Zeiss AxioImager M2 (400x and 1000x magnification) equipped with an AxioCam MRm camera and using Zen software (Zeiss, Zaventem, Belgium). Calculation of chromosome size, centromere index and signal positions was performed using the freeware computer application Micromeasure software, version 3.3 [85].

Karyotype Analysis

A karyotype was constructed after measurement of five well-spread metaphases using Micromeasure version 3.3 (http://rydberg.biology.colostate.edu/Micromeasure) [85]. Measurements were performed on DAPI stained images and chromosomes were characterized on the basis of chromosome length and centromeric index [86]. Chromosomes were then arranged in order of decreasing length. The condensation index [(genome size 1C (Mbp)/mean length of total chromosome complement (μm)] was also calculated. The FISH signal position (RD) was calculated according to the formula: RD = distance from signal to centromere ×100%/length of the chromosome arm.

Genotyping and linkage mapping of EST-SNP markers

HRM was performed as described in [87] but using only the 0.8× LightCycler 480 High Resolution Melting Master Mix (Roche). LightCycler 480 Gene Scanning software was used for genotyping. Three EST-SNPs for the candidate genes *PAL*, *OOMT* and *P5CS* were amplified in the mapping population. A scoring matrix was calculated in Microsoft Excel. Segregation patterns of the new marker sets based on the HRM profiles for the offspring plants of the mapping population were added to the already existing mapping data described in Moghaddam et al. [63]. Estimation of the linkage groups and regression mapping was performed as described in De Keyser et al. [88] using JoinMap 4.0 [89]. Calculation settings for the mapping were: using linkages with a recombination frequency smaller than 0.49 and LOD higher than 1; goodness-of-fit jump threshold for removal of loci 5 and performing a ripple after adding 1 locus. Markers with severe segregation distortion (Chi-square test significance higher than 0.005) and markers creating "tension" in the maps (according to the Nearest Neighbours Fit) were removed from the final maps.

Determination of the position of OOMT, PAL and P5CS genes on *Fragaria vesca* pseudo-chromosomes

Positions of the *PAL* and *P5CS* genes on the pseudochromosomes of *Fragaria vesca* (FraVesHawaii_1.0) were determined in the gene database at NCBI. Localization of the *OOMT* gene was identified by an alignment of a *Rosa chinensis OOMT1* partial gene sequence (AJ786302) with each of the *F. vesca* pseudochromosome (CM001053.1-CM001059.1) using the BLASTN tool [90]. The E-value threshold was fixed at e-15. To identify the closest strawberry orthologous to the *Rosa wichurana* genes used in our Tyramide-FISH experiments, a BLASTN search against distinct copies of the strawberry genes was performed. As a query, the parts of the *Rosa wichurana* sequences of the *OOMT*, *PAL* and *P5CS* genes corresponding to the gene fragments used in the Tyramide-FISH were used.

Results

Tyramide-FISH optimization

Using the direct detection system to detect the single-copy gene *PAL*, many nonspecific signals were observed, although for the control probe pTa71, 45 S rDNA sites could be detected (Fig. 1D). Therefore, the indirect detection and indirect detection with two rounds of amplification systems were optimized for single-copy gene detection. In the indirect detection system, *PAL* (1700 bp) could be observed when using a 1:25 dilution rate for Tyr-Bio, 8–10 minutes tyramide incubation time and a 1:100 dilution rate for SA-Cy3. These conditions gave the best signal-to-noise ratio as determined by visual inspection. In the indirect detection with two rounds of amplification system, signals for *PAL* became visible under the following conditions: a first round using SA-HRP (1:100), Tyr-Bio (1:25), 5 min tyramide incubation time and a second round using SA-HRP (1:300), Tyr-Cy3 (1:500), 6 min tyramide incubation time. Changing the concentration of SA-HRP (1:200, 1:300) and Tyr-Cy3 (1:100, 1:300, 1:500 or 1:1000) in the second round of amplification in the indirect detection with two rounds of amplification system, resulted in slight differences in the signal-to-noise ratio. The optimized indirect detection and indirect detection with two rounds of amplification systems both allowed visualization of the *PAL* signals in 30–40% of the observed metaphases. Because indirect detection is more time consuming than indirect detection, we used indirect detection for the subsequent physical mapping of the genes.

Table 2. Overview of HRM primers for PAL (Phenylalanine Ammonia Lyase), OOMT (Orcinol O-Methyl Transferase) and P5CS (Pyrroline-5-Carboxylate Synthase).

Gene	Primers (5′-3′)	Amplicon (bp)	N° of introns	N° of SNP's
PAL	TTGGAGGTTCAAGGAATTTACC CCAAGAAGCGAAAAAGCTCA	227	1	/[z]
OOMT	GTTTGAGGCAGTTCCTCCTG GGTCTTGGTCCAGATCGAGT	223	1	1
P5CS	GTGCTTGCAAACATGGAAGA TGGTGCTCTAGTTGGCAAAA	204	1	1

Amplicon length, amount of introns present in the amplicon and the number of SNPs in the amplicon are indicated.
[z]no sequence information is available for *Rosa wichurana*.
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Physical mapping of genes using ID

To be able to link the Tyramide-FISH signals to a certain chromosome and to identify the NOR-bearing chromosome, the karyotype of *Rosa wichurana* was constructed for the first time (Table 3; Fig. 2). The karyotype contains 7 pairs of chromosomes with the chromosome formula 5M+1SM+1ST. The length of the chromosomes ranges between 2.2 µm and 3.7 µm (Table 3). The smallest chromosome bears a NOR-satellite, as confirmed by Tyramide-FISH with 45 S rDNA (Fig. 1 D). Chromosomes 1 and 7 can be easily distinguished based on their size and centromeric indexes. In addition, it is also possible to discern the only submetacentric chromosome 4. The condensation index of *Rosa wichurana* is 28.1±2 Mbp μm^{-1}, based on the genome size of *Rosa wichurana* (1C = 562 Mbp; [91]) and the mean total length of the metaphase chromosomes (1n = 20±1 µm).

The three genes used in this study were mapped on different chromosomes (Fig. 1; Fig. 2). Signals from hybridization of the *PAL* gene were visualized in the distal part of the long arm of the smallest *Rosa wichurana* chromosome 7 (Fig. 1C, Fig. 2) (RD = 77.0±2.1%). The signals were detected in 25–30% of the analyzed metaphases. The *OOMT* gene was visualized in the proximal position of the long arm of chromosome 1 (Fig. 1A, Fig. 2) (RD = 22.6±3.2%). The signals were observed in 30–35% of the analyzed metaphase cells. Tyramide-FISH for the *P5CS* gene resulted in signals on chromosome 4 in 30–40% of the analyzed chromosome spreads. The signals were localized in the distal position (RD = 72.7±3.8%) on the long arm of this chromosome (Fig. 1B, Fig. 2).

Positioning of EST-SNP on the genetic linkage map

HRM profiles of *OOMT* and *PAL* yielded different melting curves between the parents; melting curves of the offspring were identical to either one of both parental curves. Both markers were scored as <lmxll> according to JoinMap 4.0 [89]. The segregation for *PAL* was slightly distorted (p = 0.005); 64% of the offspring plants were scored as <lm>. For *OOMT*, no segregation distortion was detected. The HRM profiles of *P5CS* also differed between the parents and segregated as 4 profiles in the offspring plants (2 of them were identical to the parental profiles; Fig. 3). Hence, this marker was scored co-dominantly as <efxeg> according to JoinMap 4.0 [89] in a ratio of 23:19:19:29 for ee:ef:eg:fg, respectively. No segregation distortion was present for *P5CS*. Segregation pattern-derived EST-SNP markers for *PAL*, *OOMT* and *P5CS* were integrated in the existing genetic linkage maps of Moghaddam et al. [63] (Fig. 4). *P5CS* was inserted into consensus linkage group RwLG-B1; *OOMT* in group RwLG-B2 and *PAL* into group RwLG-B3 (Fig. 4). The OOMT gene was previously mapped on linkage group 2 [58,105] that correspond to our RwLG-B2. Two morphological traits, "flower size" (*Rosa* 'Yesterday' has double flowers, *Rosa wichurana* has simple flowers) and "flower color" (*Rosa* 'Yesterday' has pink flowers, *Rosa wichurana* has white flowers), were recorded as qualitative traits in the mapping population [63]. "Flower size" and "flower color" are very old and well-known loci in rose linkage maps. The traits were scored in the mapping population *Rosa* 'Yesterday' x *Rosa wichurana* during 3 years in a qualitative manner [63]. A close linkage between *PAL* and "Flower size" (3 cM) was observed. *OOMT* and "Flower color" are on the same linkage group but with a larger linkage distance (36 cM). Genetic mapping and Tyramide-FISH results are in concordance as the three genes were mapped on three different chromosomes and linkage groups. The position of the *PAL* and *P5CS* genes near the end of the linkage groups correspond with their positions on the chromosomes, which is also relative to the telomeric ends (Fig. 4). The relative position of *OOMT* is central on RwLG-B1 and has a proximal position on chromosome 1 (Fig. 4).

Anchoring of linkage groups to *Rosa wichurana* chromosomes and *Fragaria vesca* pseudochromosomes

Searching for orthologous genes for *OOMT*, *P5CS* and *PAL* genes in strawberry genome revealed that they are represented in 3, 4 and 2 genes paralogous, respectively (Table 4). Sequence alignment showed that sequence diversity between the paralogous ranges from 66% (for *OOMT*) to 91% (for *P5CS*) (Table 4). Two paralogous *OOMT* genes are located on strawberry pseudochromosome 6 (FvChr6) and one on FvChr3. Two paralogous *P5CS* genes are located close to each other on FvChr7 and two on FvChr6. Paralogous for the *PAL* genes were found on FvChr6 and FvChr7. BLASTN comparison between the sequences of OOMT, P5CS and *PAL* from *Rosa wichurana* and all found paralogous in strawberry, revealed that three strawberry paralogues (highlighted in Table 4) show a high similarity and/or sequence coverage to the rose genes. These paralogues are used for making a comparison between the physical locations of *OOMT*, *P5CS* and *PAL* genes on the strawberry pseudochromosomes and the *Rosa wichurana* chromosomes (Fig. 4). *OOMT* is located in the centre of FvChr6 (Fig 4) and, as revealed in our Tyramide-FISH, in the centromeric region on chromosome 1 of *Rosa wichurana* (RwChr1; Fig 4). *PAL* is located distally on FvChr6 (Fig 4) and distally on chromosome 7 of *Rosa wichurana* (RwChr7; Fig 4)). *P5CS* is located distally on pseudochromosome FvChr7 (Fig 4) and on the distal part of *Rosa wichurana* chromosome 4 (RwChr4; Fig 4).

Discussion

Short DNA fragments could be visualized on physical chromosomes using Tyramide-FISH

To the best of our knowledge this study reports the first successful use of Tyramide-FISH in a plant genus with small chromosomes. Previously, Tyramide-FISH has been applied to

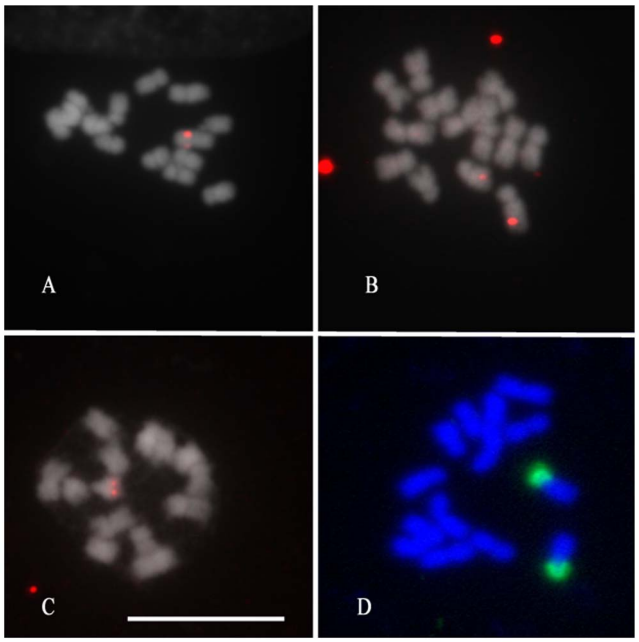

Fig. 1. Tyramide-FISH with indirect detection (A, B and C) and direct detection (D) systems on metaphase chromosomes of *Rosa wichurana*. Chromosomes were hybridized with *OOMT* (A), *P5CS* (B), *PAL* (C) and pTA71 plasmid (D). (Bar - 10 μm).
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visualize short DNA fragments for large chromosomes of several monocots including onion [53,92], barley [54], wheat [55] and oat [56]. Despite the difficulty of using rose as a cytogenetic object, we successfully visualized short DNA fragments (1.1–1.7 Kb) of genes using Tyramide-FISH. Although rose chromosomes are very small, the degree of chromosome condensation is rather low (28.1 ± 2 Mbp μm^{-1}). This value is comparable with tomato (40.6 Mbp μm^{-1}, [93]) and humans (26.6 Mbp μm^{-1}, [94]), but is more than seven times lower than in onion (249.6 Mbp μm^{-1}, [95]). The nature of chromosome structure and chromatin compaction influences the accessibility of target DNA. Low chromatin compaction may positively influence the Tyramide-FISH sensitivity by improving the probe penetration into the chromosomes. On the other hand, less compact chromatin theoretically can have a negative impact on Tyramide-FISH because it contains smaller amounts of proteins (e.g., histones) and electron rich amino acids (e.g., tyrosine, tryptophan) around the site of hybridization. Tyramides, used for signal amplification, are phenolic compounds that react and bind with these electron rich moieties in the presence of HRP and hydrogen peroxide. Therefore, a smaller amount of electron rich amino acids can hamper a successful tyramide-conjugate coupling reaction after oxidation by HRP [42].

We found that the commonly-used direct detection system with fluorescent labeled tyramides (Tyr-FITC, Tyr-Cy3) was not suitable for rose chromosomes. In that system, many nonspecific signals hampered the identification of signals from the *PAL* gene. Optimization using the indirect detection and indirect detection with two rounds of amplification overcame this problem. The indirect detection system has previously been applied to detect the *Rad51* gene on wheat chromosomes [55] and several EST clones on human chromosomes [47]. In the study of Schriml et al. [47], the indirect detection system using avidin-FITC provided the best results, i.e., clear, distinct signals on one or both of the homologues; whereas both the Tyr-Cy3 and Tyr-FITC (direct detection) resulted in high background [47]. The frequency of signal detections was about 30–40% in our study. This is comparable with previous studies. In the study of Perez et al. [55], the Tyramide-FISH procedure using Tyr-Bio was able to detect target DNA sequences as small as 2 kb with a frequency of 37.5%. These frequencies are high enough to unequivocally locate small sequences (<2 kb) using a few metaphase cells and shows the effectiveness of our Tyramide-FISH detection system. In most cases, we observed the Tyramide-FISH signals only on one homologous. The same results were obtained on wheat [55] and *Allium* (Kirov et al. unpublished data) where short DNA probes were used. Since chromatin structure significantly influences FISH results, the unequal distribution of the signals among the homologous and the low frequency of the signals may be the results of variation in chromatin accessibility and/or chromatin disorder between chromosomes and metaphase plates, caused by chromosome preparation procedure.

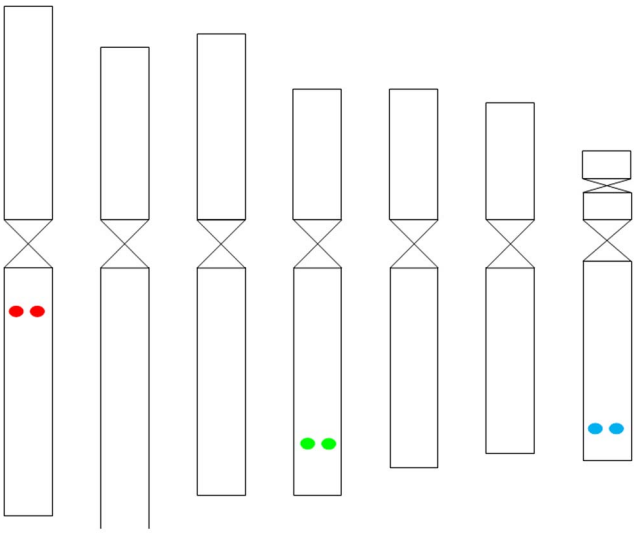

Fig. 2. Ideogram of *Rosa wichurana* chromosomes with an indication of the physical position of the candidate genes for *OOMT* (red), *PAL* (blue) and *P5CS* (green).
doi:10.1371/journal.pone.0095793.g002

The HRM technology for EST-SNP marker generation has several advantages

We successfully visualized the position of the *OOMT*, *P5CS* and *PAL* genes on the *Rosa wichurana* chromosomes 1, 4 and 7, respectively. Using EST-SNP markers for these genes, we could anchor three linkage groups of *Rosa wichurana* to their physical chromosomes for the first time. EST-SNP markers made it possible to connect the physical position of the *OOMT*, *P5CS* and *PAL* genes with their position on the genetic map. The HRM technology allowed detecting SNPs in a fast and efficient way. Unlike other technologies for gene mapping, HRM can be applied immediately after PCR without further handling [73]. During a single two-hour assay we amplified all 3 genes in a single-step procedure on a 384-well plate. This dramatically increases the genotyping throughput in a mapping population. Curve shapes cannot always be assigned to specific alleles [96], but this was not the case here. EST-SNP markers are situated in functional genes, therefore these markers are a valuable tool for the integration of the physical and genetic position of genes.

Table 3. Size and centromere index of the *Rosa wichurana* chromosomes.

Chromosome number	Chromosome Length (μm)	Relative Length (%)	Centromere Index (%)
1	3.70±0.30	17.80±0.20	46.00±1.20
2	3.20 0.60	17.00±0.20	40.30±1.30
3	3.00±0.50	15.20±0.20	44.30±1.00
4	2.80±0.40	14.00±0.10	36.90±0.70
5	2.60±0.40	13.60±0.10	41.40±0.70
6	2.50±0.40	12.40±0.20	41.80±1.10
7	2.20±0.50	10.00±0.10	23.40±0.90

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Tyramide-FISH showed single loci for members of multigene families

Surprisingly, by using Tyramide-FISH we only observed single loci for each gene even though they were described as members of multigene families [97,98]. To estimate the copy number of genes in a plant genome, a collection of EST sequences can be used [99]. For roses, more than 20000 rose EST sequences were uploaded in NCBI [83,100–102] of which only 1936 EST sequences [101] belong to *Rosa wichurana*. This number of EST sequences is not enough for the estimation of the copy number of the three genes that we studied *in Rosa wichurana* even not if EST sequences from another *Rosa* species would be used in our analysis. Variations in EST sequences can be explained by the copy numbers of a gene but also by allelic variations. Some *Rosa* species may have up to 16 allelic variants (for ploidy level 2n = 8x) per gene. Therefore, for a correct estimation of the copy number of the genes in *Rosa wichurana* using a database of EST sequences, it should contain more sequences (e.g. 120892 ESTs were used for tomato [99]) of cDNA clones isolated from different tissues. Moreover, an EST library represents only expressed genes and does not include pseudogenes that can be visualized by Tyramide-FISH.

To clarify our result we performed BLASTN searches of all *PAL*, *OOMT* and *P5CS* genes known in *Fragaria*, the closest relative of *Rosa* [65,103]. It has a completely sequenced genome [104]. We found 2, 4 and 3 hits for the *PAL*, *P5CS* and *OOMT* genes, respectively, distributed along 3 *Fragaria* pseudochromosomes 3, 6 and 7. However, the similarity between the *Fragaria* orthologous genes (66–76%) for *OOMT* and *PAL* genes is low. The 4 *Fragaria* orthologous genes for *P5CS* genes showed a higher level of intragenic similarity, but a pairwise alignment with the rose gene fragment for *P5CS* used in our Tyramide-FISH indicated only one strawberry orthologous gene with a high similarity (82%) and query coverage (99%). Therefore, if the rose genome contains a similar copy number of *PAL*, *OOMT* and *P5CS* and with similar intragenic differences as in the *Fragaria vesca* genome, with the hybridization and washing stringency we used in our study, we can specifically detect the particular orthologues PAL, OOMT and P5CS genes with high homology to the probe DNA sequence. Thus, for each orthologue we can get a clear locus on the chromosomes, which is a very important feature for anchoring linkage groups to physical chromosomes.

Comparative analysis of physical gene positions between *Rosa wichurana* and *Fragaria vesca*

A comparison of the physical position of the three genes between the *Rosa wichurana* chromosomes and the *Fragaria vesca* pseudochromosomes revealed that FwChr6 contains both orthologous *PAL* and *OOMT* genes, although they are located on different chromosomes of *Rosa wichurana*. Previously, Gar et al. [65] genetically mapped a set of orthologous EST markers on *Rosa* and compared this with their position on the *Fragaria vesca* chromosomes. They showed 10 rearrangements including 4 translocations and 6 inversions changing the gene order between *Rosa* and *Fragaria vesca* chromosomes. One of these rearrangements involved FwChr6, which was shared by markers from 2 *Rosa* linkage groups. Our results are thus in accordance with Gar et al. [65]. Physical mapping on the rose chromosomes of additional genes present on FwChr6 will shed light on the nature and the scale of this rearrangement.

In conclusion, our results demonstrate that Tyramide-FISH is a useful tool for physical mapping of short DNA fragments of genes on *Rosa* chromosomes. We could physically map 3 genes on the

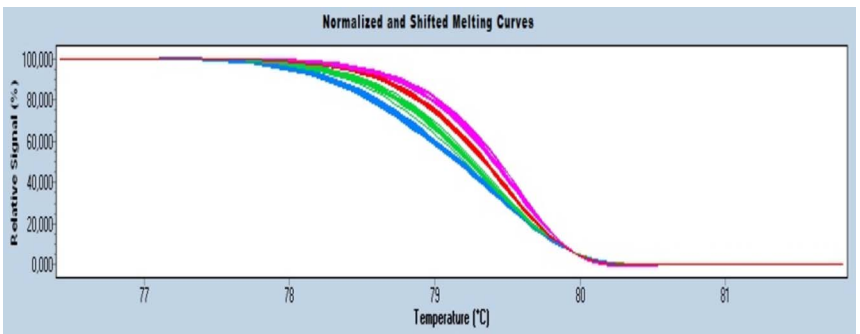

Fig. 3. HRM melting profiles for *P5CS*. The melting curve for *Rosa wichurana* is part of the green cluster; *Rosa* 'Yesterday' is part of the red cluster. Both clusters also contain curves of the siblings. Blue and pink clusters contain only the melting curves of siblings.
doi:10.1371/journal.pone.0095793.g003

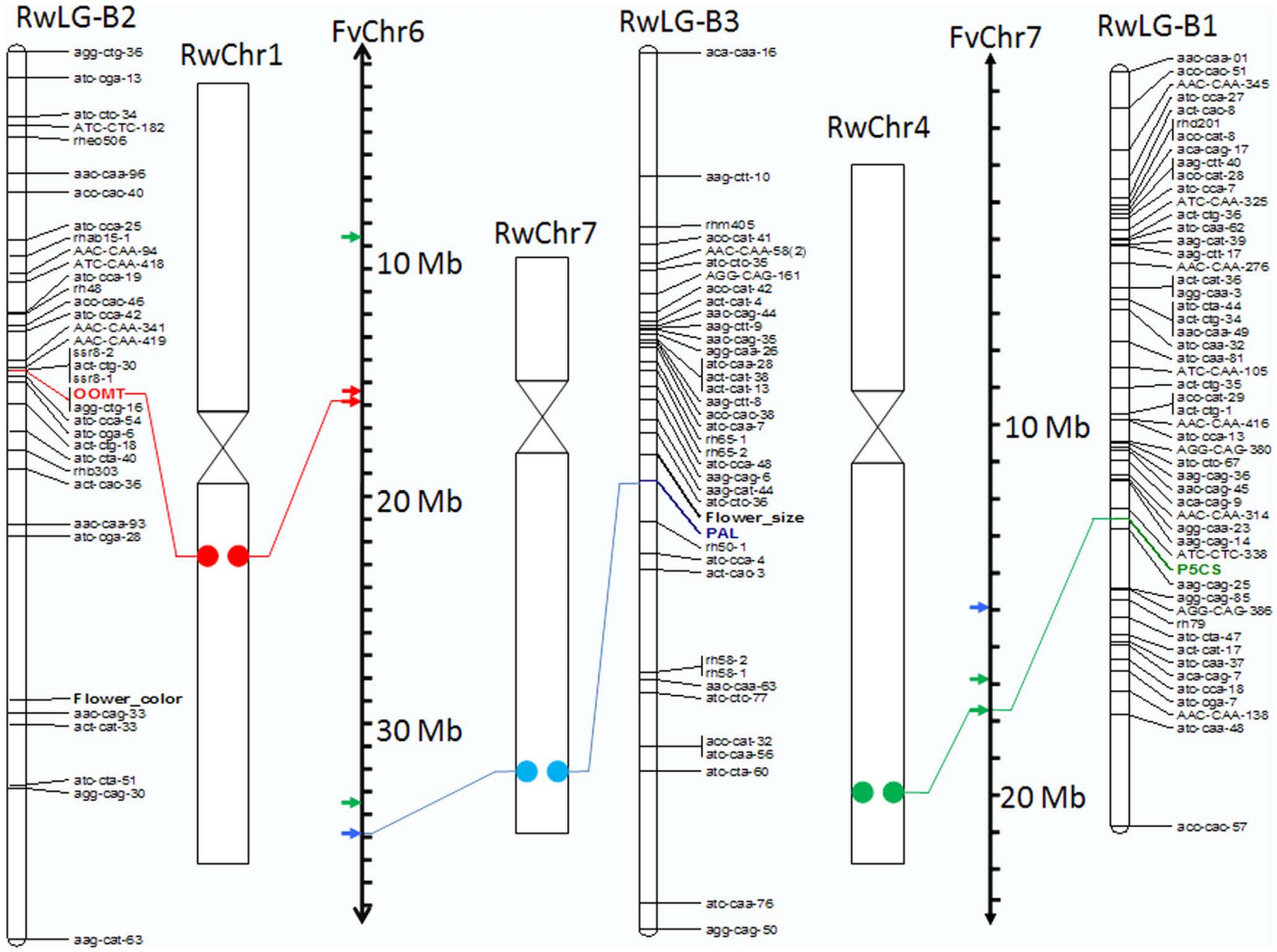

Fig. 4. Integration of the gene position on the genetic map (RwLG) (partially) obtained by regression mapping in Joinmap 4.0 showing the consensus linkage groups with indication of the map position of *P5CS* (green), *OOMT* (red), *PAL* (blue) and the physical chromosomes of *Rosa wichurana* (RwChr) and the pseudochromosomes of *Fragaria vesca* (FvChr). Framework of the genetic linkage map follows Moghaddam et al. (2012).
doi:10.1371/journal.pone.0095793.g004

chromosomes of *Rosa wichurana*. Using the opportunities of the Tyramide-FISH and the HRM technology, 3 linkage groups could be anchored to 3 physical chromosomes of *Rosa wichurana*. An integration of a cytogenetic and genetic map of rose is an indispensable tool for assistance in map based cloning. Moreover, the information obtained from the physical mapping of individual rose genes can be applied for contig and pseudochromosome

Table 4. Divergence among members of *PAL*, *P5CS*, *OOMT* orthologous genes of *Fragaria* and their similarity to *Rosa wichurana* gene fragments used in this study.

Gene	Number of orthologous genes found in *Fragaria*	*Fragaria* orthologous gene localizations	Similarity between *Fragaria* orthologous genes	% similarity to *Rosa wichurana* gene fragments (E-value; % coverage)
PAL	2	FvChr7:15014006–15017322 FvChr6:34874086–34877587	76%	75% (3e-35; 20%) 83% (0.0; 65%)
P5CS	4	FvChr7: 17624431–17630820 16924786–16929803 FvChr6: 8598452–8605103 33424492–33427031	78%–91%	82% (0.0;99%) 88% (2e-52;37%) 79% (2e-15; 21%) Not significant[z]
OOMT	3	FvChr3: 7085125–7086298 FvChr6: 15275992–15277245 15267146–15267850	66–67%	70% (8e-37; 87%) 91% (0.0; 88%) 44% (8e-47; 62%)

Genes that were selected for the comparative analysis are highlighted.
[z]: Not significant: according to BLAST search.
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anchoring to physical chromosomes which will assist future genome sequencing in *Rosa*.

Author Contributions

Conceived and designed the experiments: IK KVL EDK JDR LK. Performed the experiments: IK KVL EDK JDR. Analyzed the data: IK KVL JDR NVR LK. Contributed reagents/materials/analysis tools: IK KVL EDK JDR NVR LK. Wrote the paper: IK KVL EDK JDR LK.

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Erratum: "Anchoring linkage groups of the Rosa genetic map to physical chromosomes with Tyramide-FISH and EST-SNP markers" [*PloS one*, *9*(4), e95793].

1. Page 54. The sentence "In most cases, we observed the Tyramide-FISH signals only on one homologous." should be replaced by the following sentence: "In most cases (70-80% of the cells), we observed the Tyramide-FISH signals only on one homologous."

CHAPTER 3

DEVELOPMENT OF CYTOGENETIC MARKERS FOR ROSA AND ALLIUM CHROMOSOME IDENTIFICATION

For efficient physical mapping, the chromosomes need to be unambiguously distinguishable to be able to assign FISH signals to certain chromosomes. In this chapter, we aimed to identify DNA sequences for *R. wichurana* and *A. fistulosum* which can be used as cytogenetic markers. For *R. wichurana* the conservative repetitive DNA sequences such as 45 rDNA, 5S rDNA and an *Arabidopsis*-type telomeric repeat were used (Paper 3). To develop cytogenetic markers for *A. fistulosum* next generation sequencing data and a bioinformatic search for tandem repeats were applied (Paper 4).

PAPER 3:

ILYA V. KIROV, KATRIJN VAN LAERE, NADINE VAN ROY AND LUDMILA I. KHRUSTALEVA (2016) TOWARDS A FISH-BASED KARYOTYPE OF *ROSA* L. *COMPARATIVE CYTOGENETICS*, *10*, 543.

PAPER 4:

ILYA V. KIROV, ANNA V. KISELEVA, KATRIJN VAN LAERE, NADINE VAN ROY, LUDMILA I. KHRUSTALEVA (2016) TANDEM REPEATS OF ALLIUM FISTULOSUM ASSOCIATED WITH MAJOR CHROMOSOMAL LANDMARKS (ACCEPTED IN MOLECULAR GENETICS AND GENOMICS)

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Towards a FISH-based karyotype of *Rosa* L. (Rosaceae)

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Abstract

The genus *Rosa* Linnaeus, 1753 has important economic value in ornamental sector and many breeding activities are going on supported by molecular studies. However, the cytogenetic studies of rose species are scarce and mainly focused on chromosome counting and chromosome morphology-based karyotyping. Due to the small size of the chromosomes and a high frequency of polyploidy in the genus, karyotyping is very challenging for rose species and requires FISH-based cytogenetic markers to be applied. Therefore, in this work the aim is to establish a FISH-based karyotype for *Rosa wichurana* (Crépin, 1888), a rose species with several benefits for advanced molecular cytogenetic studies of genus *Rosa* (Kirov et al. 2015a). It is shown that FISH signals from 5S, 45S and an *Arabidopsis*-type telomeric repeat are distributed on five (1, 2, 4, 5 and 7) of seven chromosome pairs. In addition, it is demonstrated that the interstitial telomeric repeat sequences (ITR) are located in the centromeric regions of four chromosome pairs. Using low hybridization stringency for ITR visualization, we showed that the number of ITR signals increases four times (1–4 signals). This study is the first to propose a FISH-based *R. wichurana* karyotype for the reliable identification of chromosomes. The possible origin of *R. wichurana* ITR loci is discussed.

Keywords

Cytogenetic markers, fluorescence in situ hybridization, interstitial telomeric repeat (ITR), 5S rDNA, 45S rDNA, *Rosa wichurana*

Introduction

Rosa Linnaeus, 1753 is an economically important ornamental genus belonging to the Rosaceae. Of the approximately 200 described *Rosa* species (Wissemann and Ritz 2005), only 8 to 15 species contributed to the original germplasm of the modern rose cultivars. *Rosa* is one of the most widely cultivated ornamental plants worldwide, but few basic molecular cytogenetic studies in *Rosa* have been performed, including chromosome counts and karyotyping (Wylie 1954, Price et al. 1981, Liu and Li 1985, Subramanian 1987, Ma et al. 1997, Fernandez-Romero et al. 2001, Akasaka et al. 2002, 2003, Jian et al. 2013a, 2013b). Performing molecular cytogenetics in roses is a big challenge due to their very small genome size (the diploid genome size is 0.83 to 1.30 pg/2C, Roberts et al. 2009), small chromosomes (Kirov et al. 2014a), low mitotic index in roots and shoots, and weak root development (Ma et al. 1996). Moreover, most wild roses are polyploids (Vamosi and Dickinson 2006), ranging from diploid (2n = 2x = 14) to decaploid (2n = 10x = 70) (Roberts et al. 2009, Jian et al. 2010).

Rosa wichurana (Crépin, 1888) is a valuable model species for molecular cytogenetic studies in *Rosa* genus (Kirov et al. 2015b). It is a diploid species (2n = 2x = 14) with suitable apical and root meristems that can be used for chromosome preparations. *Rosa wichurana* is involved in the origin of modern rose cultivars and is one of the parental species used for the construction of several rose genetic maps (Crespel et al. 2002, Dugo et al. 2005, Shupert et al. 2007, Spiller et al. 2011, Moghaddam et al. 2012). To increase the efficiency of FISH experiments, we recently developed the "SteamDrop" protocol for the preparation of high quality chromosome slides (Kirov et al. 2014b). Using this "SteamDrop" protocol and Tyramide-FISH it was possible to physically map several single-copy genes on the mitotic and meiotic chromosomes of *R. wichurana* (Kirov et al. 2014a, Kirov et al. 2015a) and to anchor three linkage groups of the genetic map (Moghaddam et al. 2012) to three *R. wichurana* chromosomes.

Because the chromosomes are difficult to distinguish, further progress in cytogenetic mapping depends on the development of cytogenetic markers useful for chromosome identification. The conservative tandemly organized repetitive sequences 5S and 45S rRNA genes are valuable sources of cytogenetic markers, and have been used for chromosome identification in many plant species including *Rosa* species (Ma et al. 1997, Fernandez-Romero et al. 2001, Akasaka et al. 2002, 2003, Lim et al. 2005, Jian et al. 2012, Kirov et al. 2014a). Other conservative repeats, such as the *Arabidopsis*-type telomeric repeat (Fuchs et al. 1995, He et al. 2013) might be used for chromosome identification. Typically, telomeric repeats (TRs) occupy the end (telomere) of the chromosomes (Fuchs et al. 1995). However, the location of TRs on plant chromosomes is not restricted to the telomere ends and telomere-like sequences have been found in centromeric, subtelomeric and interstitial regions in several genera (Fuchs et al. 1995, Uchida et al. 2002, Tek and Jiang 2004, Mlinarec et al. 2009, Mandakova et al. 2010, Gong et al. 2012, He et al. 2013, Sousa et al. 2014). The unique position of these interstitial telomeric repeats (ITRs) on some chromosomes and their high copy number make them valuable cytogenetic markers. The position of ITR on chromosomes can also reflect ancient chromosomal rearrangement as telomeric sequences and

their remnants are involved in chromosomal rearrangements via illegitimate recombination between centromeric/telomeric repeats (Murat et al. 2010) and can be associated with fragile sites of chromosomes (Grabowska-Joachimiak et al. 2015). In addition, the chromosomal location of ITR can be used to detect descending dysploidy (Sousa and Renner 2015).

Development of an effective cytogenetic marker system is an important step in answering many biological questions (Jiang and Gill 2006). FISH-based markers have shown their effectiveness and ease-to-use. The modern methods of probe labeling and the application of directly labeled oligonucleotides make FISH-based chromosome identification a robust and fast procedure (Kato et al. 2004, Fu et al. 2015, Tang et al. 2014, Cuadrado et al. 2009). Up-to-date FISH based karyotyping was established for many plant species including wheat, maize, rice, soybean, common bean and others (Cheng et al. 2001, Kato et al. 2004, Findley et al. 2010, Iwata-Otsubo et al. 2015). Cytogenetic markers are widely used to trace individual chromosomes in hybrids accelerating transferring of desirable traits from wild relatives (Szinay et al. 2010). FISH-based karyotyping is used to shed light on speciation and allopolyploid formation (Badaeva et al. 2016). And a relatively new application came with the development of a FISH-based chromosome sorting procedure, allowing individual chromosome identification, sorting and further sequencing (Giorgi et al. 2013). These and other applications clearly demonstrate the importance of having a system of cytogenetic markers enabling chromosome identification.

This study aims to explore the opportunities of ITRs, 5S and 45S rDNA as cytogenetic markers allowing to distinguish individual chromosomes of *Rosa*. FISH with 5S rDNA, 45S rDNA and the *Arabidopsis*-type telomeric repeat was performed. These FISH results were combined with chromosome morphology measurements (Kirov et al. 2014a), in order to identify all seven mitotic chromosomes of *R. wichurana*. In addition, we also attempted to identify pachytene bivalents by FISH using the 45S rDNA and *Arabidopsis*-type telomeric repeat probes.

Materials and methods

Plant material

Rosa wichurana plants were grown in the field. For chromosome slide preparations, cuttings were made. Rooted cuttings were transferred to terracotta stone pots and grown in the greenhouse (moderate climatic conditions, East Flanders, Belgium). To prepare mitotic chromosome slides, young meristems were harvested. For meiotic (pachytene) chromosome slides, flowers buds with a hypanthium size of 3 mm were harvested.

Probe labeling

Plasmids containing 5S rRNA genes of rye (pSCT7, Lawrence and Appels 1986) and 45S rRNA genes of wheat (pTA71, Gerlach and Bedbrook 1979) were labeled by Digoxigenin-

and Biotin- Nick Translation Mix (Roche, Germany), respectively, according to the manufacturer's protocol. The *Arabidopsis*-type telomere repeat $(CCCTAAA)_3$, labeled by TAMRA at the 5' end (Syntol, Russia) was used.

Chromosome preparation and fluorescence *in situ* hybridisation

Pachytene and mitotic chromosomes were prepared according to the "SteamDrop" protocol (Kirov et al. 2014b).

For FISH we used the protocol described in Heslop-Harrison et al. (1991) with some modifications. Briefly, slides were incubated overnight at 37°C. Chromosomes were pretreated with 4% paraformaldehyde in 2xSSC (pH 8.3–8.5) for 6 min and dehydrated in ethanol (70%, 90% and 100%). Hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 2xSSC, 0.25% sodium dodecyl sulphate, 2.00 ±1.00 ng/μl probe DNA. The mixture was denatured at 75°C for 5 min, placed on ice for 5 min and 60 μl was applied on each slide. Slides were denaturated at 75°C for 5 min and incubated in a humid chamber for 15–16 hours at 37°C (the common hybridization condition) or at 23–25°C (the low stringency hybridization condition). For stringency washing 0.1xSSC solution was used at 48°C (2 times 7 minutes). Biotin and digoxigenin labeled probes were detected by Streptavidin-Cy3 (Sigma-Aldrich, USA), diluted 1:200 in TNB buffer, and anti-digoxigenin-FITC (Roche, Germany), diluted 1:200 in TNB buffer, respectively.

For sequential FISH experiments, the slides were washed in the series of ethanol (70%, 90% and 100%) after the first round of FISH and then the above-mentioned FISH procedure was applied.

Microscopy and image analysis

Images were acquired using a Zeiss AxioImager M2 fluorescence microscope (400× and 1000× magnification) equipped with an AxioCam MRm camera and Zen software (Zeiss, Belgium). Final image adjustments were performed using Photoshop (Adobe Inc., USA). Measurements of chromosome lengths and karyotyping was done in MicroMeasure version 3.2 (Reeves and Tear 2000) for at least 10 well-spread metaphases.

Results

FISH using Arabidopsis-type telomere repeat, 5S rDNA and 45S rDNA allows unambiguous identification of 3 *Rosa wichurana* mitotic chromosomes

FISH using the common hybridisation temperature of 37°C with 45S rDNA revealed a signal on chromosome 7, while the *Arabidopsis* type telomere-based probe hybridized on chromosome 5 (Fig. 1A).

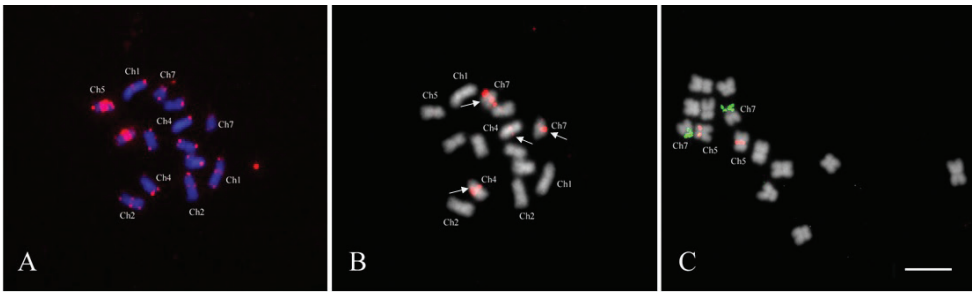

Figure 1. FISH on the chromosomes of *R. wichurana*. **A** FISH with *Arabidopsis*-type telomere probe (red) and 45S (green) under hybridization at 37°C **B** FISH with *Arabidopsis*-type telomere probe under the low hybridization stringency condition (23-25°C). Arrows indicate the major ITRs on chromosome 5 and arrowheads show the ITRs which are visible under the low hybridization stringency condition **C** The same metaphase as in 1B rehybridized with 5S rDNA under the common hybridization stringency (37°C). Arrows indicate the 5S rDNA signals. Sacale bar: 5 μm.

To further evaluate the value of the telomeric repeat (TR) as a cytogenetic marker, FISH was carried out at room temperature (the low hybridization temperature). We observed the *Arabidopsis*-type TR signals on all chromosome ends (Fig. 1B). Besides the telomeric signals, a bright fluorescent signal in the centromeric region on chromosome 5 and weak signals in the centromeric region on three other chromosomes 1, 2 and 7 were observed. Remarkably, the weak centromeric signals on chromosomes 1, 2 and 7 were not observed when performing a hybridization at 37°C (Fig. 1A). No ITRs were present on chromosomes 3, 4 and 6. FISH with 5S rDNA using the common hybridization temperature of 37°C showed fluorescent signals on the long arm of chromosomes 4 and 7 (Fig. 1C) but the signal frequency across the metaphases was low (20–40%).

Sequential FISH at the low hybridization temperature with the *Arabidopsis*-type telomere-based probe and 5S rDNA showed co-localization of these signals on chromosome 7. We also performed double-color FISH with the *Arabidopsis*-type telomere repeat-based probe and the 45S rDNA probe under the low temperature of hybridization (Fig. 2) which confirmed the identification of four (1, 2, 5 and 7) out of seven chromosomes.

A summary of the karyotypic features and distribution of FISH probes is given in Fig. 3. Taken together, three chromosomes (4, 5 and 7) of *R. wichurana* could be unambiguoulsy identified by 5S rDNA, 45S rDNA and the *Arabidopsis*-type TR using common FISH hybridisation conditions (Fig. 3).

All the other chromosomes can only be distinguished at this time based on their morphological parameters. Differentiation between chromosome 1 and 2 is possible by their centromeric indices which are 46.00 ±1.2% and 40.30 ±1.3%, respectively (Kirov et al. 2014a) and by the presence of an ITR when using FISH at low temperature hybridization conditions. Chromosomes 3 and 6 have centromeric indices on the level of 44.3 ±1.0% and 41.8 ±1.1%, respectively (Kirov et al. 2014a). However, these chromosomes still remain very difficult to distinguish from each other.

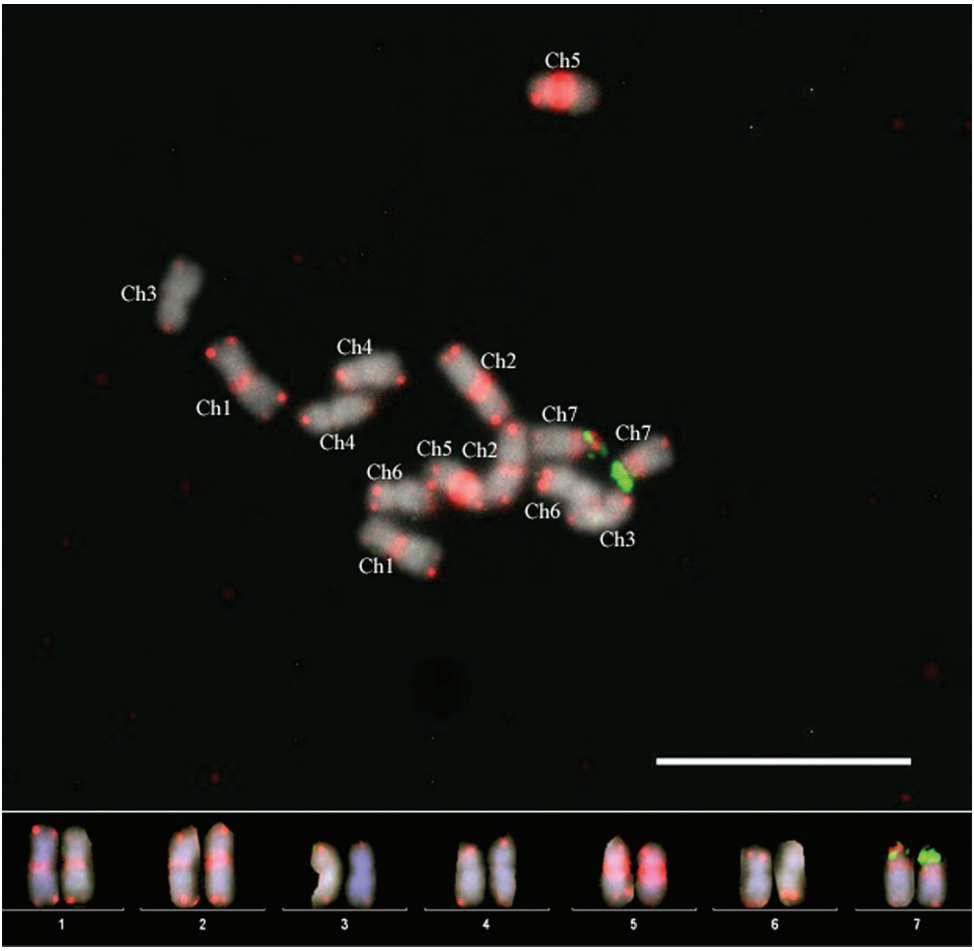

Figure 2. Double-color FISH under the low hybridization conditions using the *Arabidopsis*-type telomere repeat-based (red) and 45S rDNA (green) probes to *R. wichurana* mitotic chromosomes. Scales bar: 10 μm.

ITRs are located on the centromere of chromosome 5

FISH experiments with 5S rDNA, 45S rDNA, and the *Arabidopsis*-type TR on rose pachytene chromosomes provide a much higher resolution of the mapped sequences. 5S rDNA-FISH on pachytene chromosomes did not reveal any reliable signals, while FISH with the 45S rDNA probe resulted in a clear signal at the subtelomeric region of the NOR-bearing chromosome (Fig. 4). FISH with the *Arabidopsis*-type TR probe resulted in signals on all ends of pachytene chromosomes and one bright signal on the centromeric region of chromosome 5 (Fig. 4). Since centromeres of rose pachytene bivalents are clearly visible after DAPI staining as being the weakest part of the chromosomes (Kirov et al. 2015a), comparison between the DAPI stained chromosomes (Fig. 4B') and the ITR signal positions (Fig. 4A') revealed that the ITRs are located exactly on the centromere of chromosome 5.

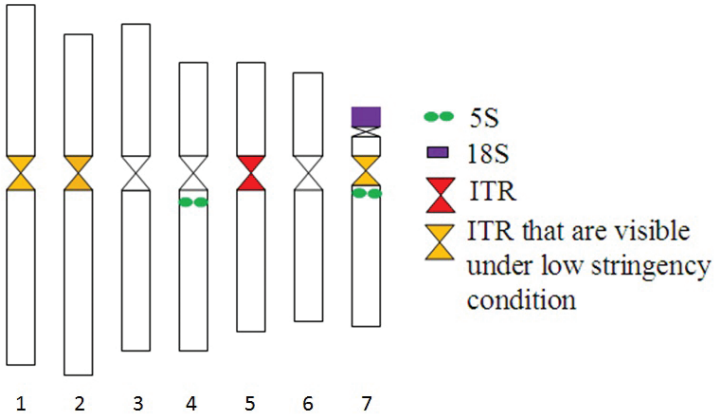

Figure 3. Distribution of the repetitive sequences on the mitotic *R. wichurana* chromosomes. [1] – ITR1: signals that are visible under hybridization at 37°C as well as at low temperature (23–25°C). [2] – ITR2: signals that are visible only under hybridization at low temperature (23–25°C).

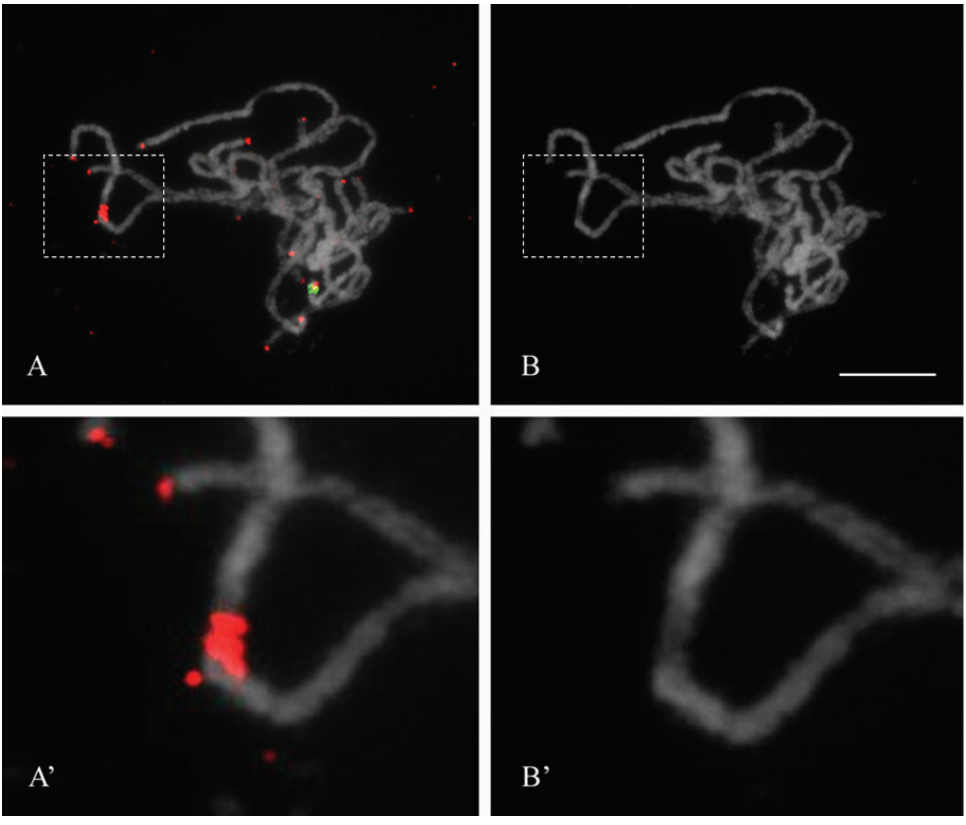

Figure 4. High resolution physical mapping of ITR on *R. wichurana* pachytene chromosomes. FISH with the *Arabidopsis*-type telomere repeat probe (red) and 45S (green). Merged (**A**) and the DAPI gray scale (**B**) pictures are shown. FISH was performed under the low hybridization stringency condition. Dotted lines show the regions that were digitally enlarged (A' and B'). Scales bar: 5 μm.

Discussion

Rosa mitotic and meiotic chromosomes are difficult to distinguish by common karyotype analysis (Kirov et al. 2014, Kirov et al. 2015a). The development of cytogenetic markers is necessary for individual chromosome identification and further cytogenetic studies in *Rosa*. In our study, we positively evaluated the use of the conservative tandem repeats, *Arabidopsis*-type telomere, 45S and 5S probes, as FISH-based cytogenetic chromosome markers for *R. wichurana*. However, the 5S rDNA probe cannot be considered as a good cytogenetic marker for *R. wichurana* chromosomes due to the low reliability of the FISH-signals. Application of FISH with the 5S rDNA probe to chromosome slides prepared by an alternative method (spread protocol of Pijnacker and Ferwerda (1984)) and using FAM labeled 5S oligos or a *R. wichurana* 5S clone as probes, did not improve FISH results (data not shown). Thus the reason for weak 5S rDNA FISH signals on *R. wichurana* chromosomes remains unclear. FISH with the *Arabidopsis*-type TR under low hybridization conditions (hybridization at 23-25°C instead of 37°C) provided us an additional tool for identification of *Rosa* chromosomes.

In this study, FISH with the 45S rDNA and the *Arabidopsis*-type telomere probe, reliably identified 2 (chromosome 5 and 7) of the 7 pachytene bivalents of *R. wichurana*. These markers will accelerate the ongoing physical mapping of pachytene chromosomes of *R. wichurana* as their identification by morphological parameters or specific heterochromatin patterns is impossible (Kirov et al. 2015a).

ITRs can be used to trace ancient chromosomes rearrangements such as chromosome fusions, Robertsonian translocations and duplications resulting in dysploidy (Mandakova et al. 2010, Sousa et al. 2014). However, *Rosa* species have a basic chromosome number n = 7, suggesting that no descending dysploidy, which usually results in basic chromosome number changes, has occurred. Therefore, it seems unlikely that the observed ITRs are the indications of such chromosome fusions or translocations. ITRs might also be the traces of intrachromosomal rearragements implicating telomeres (e.g., inversions and duplications) (Murat et al. 2010). In our study, the *Arabidopsis* telomere-like motif was found in centromeric repeats of *Rosa wichurana*, as is also observed in several other genera (Tek and Jiang 2004, He et al. 2013, Emadzade et al. 2014). The FISH signal from ITRs on chromosome 5 is significantly stronger than those observed in the telomeres of *R. wichurana* chromosomes. Thus, we hypothesize that the occurrence of ITRs in the centromeric regions of *R. wichurana* chromosomes is the result of insertion of *Arabidopsis* telomere-like sequence into centromeric sequence followed by massive amplification of centromeric tandem repeat(s) containing an *Arabidopsis* telomere-like motif. To check this hypothesis identification of centromeric repeats of *R. wichurana* should be done (Tek and Jiang 2004). The events leading to insertion of ITR sequences into centromere are unknown.

Interestingly, FISH under the low hybridization temperature – and thus low stringency – revealed more chromosomes possessing the telomeric repeat compared to FISH performed under the common hybridization temperature. This result suggest that these chromosomes (1, 2 and 7) may contain truncated or diverged telomere motifs. As a consequence for our experiments, the telomeric probe may be much more informative as cytogenetic marker when hybridized at a lower temperature than at 37°C (Fuchs et al.

1995, Tek and Jiang 2004, Sousa et al. 2014, Sousa and Renner 2015). However, the application of ITR markers under the low-hybridization stringency and simultaneous mapping of other probes (e.g. genes) can be challenging as non-specific hybridization signals may occur due to low stringency. In this case sequential FISH can be applied.

High-resolution FISH on pachytene chromosomes with the telomere probe resulted in a signal in the centromere of chromosome 5, indicating that the telomere-like motifs may be the components of the *R. wichurana* functional centromere as it has been shown for potato (Tek and Jiang 2004).

This is the first report describing valuable cytogenetic markers for four mitotic chromosomes and two pachytene bivalents of *R. wichurana*. Moreover, by combining our FISH results with the chromosome morphology measurements (Kirov et al. 2014a), all 7 mitotic chromosomes of *R. wichurana* could be identified. Because *R. wichurana* has many advantages as a model species for cytogenetic studies of the *Rosa* genus, the development of a complete set of cytogenetic markers should facilitate the physical mapping of its genome. Designing new DNA probes based on NGS data covering all chromosomes of *R. wichurana* is a scope for our future research. These markers will be indispensable for high-resolution physical mapping experiments (Kirov et al. 2015a) that are currently ongoing for this species.

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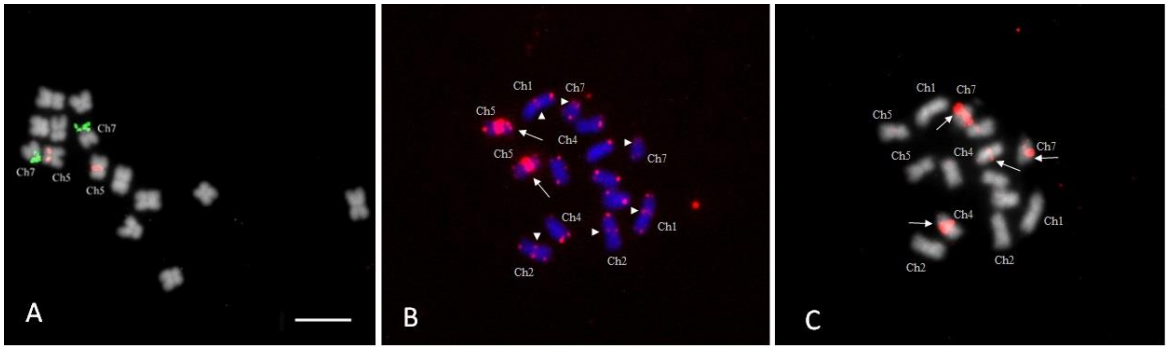
Erratum: Towards a FISH-based karyotype of Rosa L.(Rosaceae). *Comparative Cytogenetics*, *10*, 543. **[**Kirov, I. V., Van Laere, K., Van Roy, N., & Khrustaleva, L. I. (2016)]

1. Order of the picture on Figure 1 is wrong therefore this Figure should be replaced by following one:



Tandem repeats of *Allium fistulosum* associated with major chromosomal landmarks

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Abstract

Tandem repeats are often associated with important chromosomal landmarks such as centromeres, telomeres, subtelomeric and other heterochromatic regions and can be good candidates for molecular cytogenetic markers. Tandem repeats present in many plant species, demonstrate dramatic differences in unit length, proportion in the genome and chromosomal organization. Members of genus Allium with their large genomes represent a challenging task for current genetics. Using the next generation sequencing data, molecular and cytogenetic methods, we discovered two tandemly organized repeats in the *Allium fistulosum* genome (2n=2C=16), HAT58 and CAT36. Together these repeats comprise 0.25% of the bunching onion genome with 160,000 copies/1C of HAT58 and 93,000 copies/1C of CAT36. Fluorescent *in situ* hybridization (FISH) and C-banding showed that HAT58 and CAT36 associated with the interstitial and pericentromeric heterochromatin of the *A. fistulosum* chromosomes 5, 6, 7 and 8.

FISH with HAT58 and CAT36 performed on *A. cepa* (2n=2C=16) and *A. wakegi* (2n=2C=16), a natural allodiploid hybrid between *A. fistulosum* and *A. cepa*, revealed that these repeats are species specific and produced specific hybridization patterns only on *A. fistulosum* chromosomes. Thus, the markers can be used in interspecific breeding programs for monitoring of alien genetic material. We applied non-denaturation-FISH that allowed detection of the repeat bearing chromosomes within three hours. A polymorphism of the HAT58 chromosome location was observed. This finding suggests that the rapid evolution of the HAT58 repeat is still ongoing.

Keywords

Allium fistulosum, *Allium wakegi*, FISH, heterochromatin, pachytene, pericentromeric region, satellite DNA

Abbreviations

FISH	Fluorescence *in situ* hybridization
ND-FISH	Non-denaturing Fluorescence *in situ* hybridization
TRs	Tandem repeats
TAMRA	6-carboxytetramethylrhodamine
DAPI	4,6-Diamidino-2-phenylindole
PMCs	Pollen mother cells

Introduction

Tandem repeats (TRs) have been widely discovered in plant genomes (Schmidt and Heslop-Harrison1998; Hemleben et al.2007; Mehrotra and Goyal 2014). A huge variation in unit length, genome

proportion, chromosomal organization and epigenetic modifications of TRs has also been shown (Fesenko et al. 2002; Sharma and Raina 2005; Mehrotra and Goyal 2014; Zhang et al. 2014), suggesting a rapid evolution mode in the plant genome. Usually, TRs are associated with important chromosomal landmarks such as centromeres, telomeres, subtelomeric and other heterochromatic regions and have been widely studied during the last few decades (Henikoff et al. 2001; Jiang et al. 2003; Koo et al. 2011). TRs are a valuable source of cytogenetic markers for distinguishing individual chromosomes (Albert et al. 2010). FISH with tandem repeats have been successfully used for chromosome identification and for studying plant chromosome evolution in wheat (Komuro et al.2013), maize (Albert et al. 2010), radish (He et al. 2015), bulb onion (Do et al. 2001) etc. Although the function of many TR families is not fully understood, it was demonstrated that some TRs play an important role in the function of centromeres (Jiang et al. 2003), regulation of gene expression (Verstrepen et al. 2005) and maintenance of telomeric ends (Blackburn 2001). It is also well known that many TRs form heterochromatin (McClintock 1951; Ananiev et al. 1998; Wallrath 1998; Alkhimova et al. 2004), and it was proposed that TRs are involved in heterochromatin formation via an RNA-mediated pathway (Reinhart and Bartel 2002, Cohen and Jia 2014; Holoch and Moazed 2015). Since heterochromatin is involved in regulation of gene expression (Grewal and Moazed 2003), 3D genome organization (Fransz et al. 2002; Wang et al. 2014), sister chromatid cohesions (Oliveira et al. 2014), and speciation (Ferree and Barbash 2009), a possible role of TRs in these processes might be hypothesized. Next generation sequencing, together with use of a bioinformatic tool, such as RepeatExplorer (Novak et al. 2010, 2013), enables to identify TRs in species in which genomes have been sequenced with low coverage.

The genus *Allium* consists of many economically important plant species, including bulb onion (*Allium cepa*, 2n=2x=16), Japanese onion (*A. fistulosum*, 2n=2x=16), leek (*A. porrum*, 2n=4x=32), and garlic (*A. sativum*, 2n=2x=16). *Allium* species have huge genomes and large mitotic chromosomes, making them good cytological plant models. *Alliums* have a basic chromosome number x=7, 8 (observed in most species) or 9 (Ricroch et al. 2005). Genome sizes can display 4.5-fold differences among *Allium* species from7 pg/1C in *A. altyncolicum* (Ricroch et al. 2005) to 31.49 pg/1C in *A. ursinum* (Ohri et al. 1998). Multiple genome duplication events and amplification of repetitive DNA have a major influence on the *Allium* genome sizes (King et al. 1998). Several repetitive DNA families were discovered in *Allium* species including retrotransposons (Pearce et al. 1996, Vitte et al. 2013; Kim et al. 2014; Kiseleva et al. 2014), tandem repeats (Barnes et al. 1985; Irifune et al. 1995; Seo et al. 1999; Fesenko et al. 2002; Fajkus et al. 2015) and non-tandem repeats (Shibata and Hizume, 2002; Nagaki et al. 2012).

A. fistulosum (bunching onion) has a genome of 11.5 Gb/C (Ricroch et al. 2005). A significant portion of the genome consists of repetitive elements. Our knowledge about qualitative and quantitative content of the *A. fistulosum* repeatome (ensemble of all repetitive DNA families of a genome) is limited. It was shown that 4.5% of its genome is occupied by a 380-bp tandem repeat located in the major heterochromatic blocks of all subtelomeric regions (Irifune et al. 1995). Several centromeric repeat sequences (Afi) were identified for *A. fistulosum* by the chromatin immunoprecipitation (ChIP) method using antibodies against CENH3 histone (Nagaki et al. 2012). However, the sequences did not show any characteristics similar to TRs or centromeric retrotransposons, which are typical elements of plant centromeres.

The aim of this study was to analyze repeatome content of *A. fistulosum* genome and to identify novel TRs in *A. fistulosum* genome that can be used as a cytogenetic marker. Using next generation sequencing data, we were able to determine two novel TRs: HAT58 and CAT36, which together occupy 0.25% of the *A. fistulosum* genome. FISH analysis showed that CAT36 is located in the pericentromeric regions of chromosomes 5 and 6 of *A. fistulosum*. HAT58 occupied intercalary heterochromatin of chromosome 6, 7 and 8 associated with C-banding patterns. HAT58 and CAT36 are species specific tandem repeats that were shown by FISH on chromosomes of *A. wakegi* (2n=2C=16), a natural allodiploid hybrid between *A. fistulosum* and *A. cepa.*

Material and methods

Plant material, chromosome preparation and DNA isolation

Seeds of *A. fistulosum* L. 'Russkiy Zimniy'(2n=2x=16) and *A. cepa* L. 'Haltsedon' (2n=2x=16) were purchased from Gavrish seed company (Moscow, Russian Federation). Bulbs of *A.* x *wakegi*, a natural hybrid between *A*. *cepa* and *A*. *fistulosum* (2n=2x=16), were kindly provided by prof. M. Shigyo (Yamaguchi University, Japan). Genomic DNA was isolated from 5-day-old seedlings and young leaves of *A. cepa* and *A. fistulosum* according to the protocol of Rogers and Bendich (1985). Mitotic and pachytene chromosomes were prepared according to the SteamDrop protocol (Kirov et al. 2014).

Tandem repeat identification and repeatome characterization

RepeatExplorer (Novak et al. 2013) was used to explore tandem repeats based on the Illumina reads of *A. fistulosum* from NCBI (SRX268217). Totally 5,101,906 Illumina reads (80bp) of *A. fistulosum* were used for RepeatExplorer analysis. RepeatExplorer performs all-to-all similarity comparison of the NGS reads and represents the results as graph-based clusters of similar reads. The shape of the cluster is used for target isolation of different repeat families. Contigs are assembled from reads belonging to the clusters. A minimum overlapping length of 55 bp and 40 bp was used for clustering and assembly. All contigs from clusters with a globula-like structure (corresponding to tandemly organized repeats) were compared with known sequences from NCBI by BLASTN. For repeatome characterization, the html output file after RepeatExplorer clustering, only the clusters with a genome portion value of more than 0.005%, were used. This file was analyzed by a home-made script written in python (v3.4) programming language. The script allowed us to generate a summary report containing information about a type of repeat based on RepeatExplorer annotation and the genome portion occupied by the repeat.

PCR amplification, cloning and sequencing of CAT36 and HAT58

Tandem repeat units of HAT58 and CAT36 were identified by Tandem Repeat Finder Software (Benson, 1999). Amplification of the determined tandem repeats (named as HAT58 and CAT36) and of Afi11, a previously identified centromeric repeat of *A. fistulosum* (Nagaki et al. 2012), were performed using specific primers (Table 1) designed by Primer 3.0 plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). The PCR conditions were 94°C – 1 min, 35 cycles : 94°C – 1 min; 58°C – 1 min; 72°C – 1 min; final elongation: 72°C – 3 min.

The sequences of the repeats were verified by cloning of the repeat monomer obtained by PCR product into pGEM-T Easy vector (Promega, Madison, WI, USA) in E. COLI strain DH10B according to the manufacturers' instruction. The individual clones were sequenced using ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instruction. The sequences of the oligomeric units were submitted to NCBI GenBank, accession number for CAT36 is KX137122.1 and for HAT58 is KX137121.1.

Table 1 Primers used for the repeat amplification and the expected length of the PCR product.

Tandem repeats	Primers, 5'-3'	Accession number in NCBI	Size of repeat unit	Expected length of PCR product (bp)
HAT58	F:AAAAACATCTTCCAACAGCATAAA R:TGCATGAAAAGACAGCGTTT	KX137121	65	50
CAT36	F:TCCCACCTAAATTACGGACA R:AAATAGCGGCTTCTGCACTA	KX137122	197	154
Afi11	F: AAAGGTTCATGCCTGCTTTC R: TTTTACGGCATGCGATACCT	AB735740	158	139

Probe labelling

Probes for HAT58, CAT36 and Afi11 were obtained by PCR labeling using Biotin-16-dUTP (Roche, Mannheim, Germany) or Digoxigenin--dUTP (Roche, Mannheim, Germany). pSCT7, containing 5S rRNA genes of rye (Lawrence and Appels 1986), was labeled with Biotin -Nick Translation Mix according to the manufacturers' protocol (Roche, Mannheim, Germany).

Genomic DNA of *A. fistulosum* was labeled with Biotin-Nick Translation Mix according to the manufacturers' protocol (Roche, Mannheim, Germany).

HAT58 (5'-TGCATGAAAAGACAGCGTTTAGAGTTTTTATGC-3') oligonucleotide was designed and labeled at the 5'-end using TAMRA (6-Carboxytetramethylrhodamine) manufactured by Syntol company (Moscow, Russian Federation).

FISH, GISH and Non-denaturing (ND)-FISH

The chromosome slides were dried overnight at 37°C and pretreated with 4% buffered paraformaldehyde in 2xSSC (pH 8.0 – 8.5), followed by dehydration in 70%, 90% and 100% ethanol. The hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 2xSSC, 0.25% sodium dodecyl sulphate, 2.75±1.00 ng/µl probe DNA (in GISH experiment: 1.25 ng/µl probe - labeled genomic DNA of *A. fistulosum* and 37.5 ng/µl block – non-labeled genomic DNA of *A. cepa*). The mixture was denatured at 75°C for 5 min and subsequently placed on ice for 5 min. 60 µl of the mixture was added to the chromosome preparations, covered with a coverslip (22x32 mm), and denatured for 5 min at 80°C. Posthybridization washing included: 2xSSC twice for 5 min at 37°C, 0.1xSSC twice for 10 min at 48°C; then washing in 2xSSC for 3 min at 37°C. The Biotin-labeled probe was detected with streptavidin-Cy3 and signal amplification using anti-streptavidin-biotin, and subsequent incubation with streptavidin-Cy3 (Sigma-Aldrich, St. Louis, MO, USA). The Digoxigenin-labeled DNA was detected with anti-digoxigenin-fluorescein raised in sheep (Roche, Mannheim, Germany) and amplified with fluorescein anti sheep raised in rabbit (Vector Laboratories, USA). Chromosomes were counterstained in 5 µg/ml DAPI in Vectashield anti-fade (Vector Laboratories,USA).

A non-denaturing FISH (ND-FISH) procedure was carried out according to Cuadrado and Jouve (2010). ND-FISH allows for detection of tandem repeats without denaturation of the chromosome DNA, and shortens the FISH procedure dramatically.

C-banding/DAPI

The C-banding procedure was based on protocols described earlier (Barros and Guerra 2010, Grabowska-Joachimiak et al. 2011). The chromosome slides were incubated for 2 min in 0.2 M HCl in a water bath at 60°C, rinsed under tap water and in distilled water, then incubated in a saturated solution of $Ba(OH)_2$ at 50° C for 1 min, then rinsed under tap water until completely clear. The next step was incubation in 2xSSC buffer for 2 h at 60 ° C, then rinsing under tap water and in distilled water, and storage of air-dried slides for at least 1 day at RT and overnight at 37° C before staining with 4',6-diamidino-2- phenylindole (DAPI) in Vectashield-mounting medium (Vector Laboratories, http://www.vectorlabs.com).

Fluorescent microscopy and image analysis

Slides were checked using a Zeiss Axio Imager microscope (Carl Zeiss MicroImaging, Jena, Germany). 5-10 metaphases per slide were used for the analysis. Images were captured using an Axio Cam MRm digital camera. Image processing was performed using AxioVision version 4.6 software (Carl Zeiss MicroImaging, Jena, Germany). Final image adjustments were made using Photoshop (Adobe Inc., San Jose, CA, USA). The captured images of the chromosomes and position of FISH signals and C-bands were measured using the program MicroMeasure version 3.2 (Reeves and Tear 2000).

Results

A. fistulosum repeatome characterization and identification of tandem repeats

To identify clusters corresponding to tandem repeats, we carried out a search for a globula-like (CL2, CL5, CL6, CL7 and CL58) or ring-like (CL36) structure, both of which are typical for tandemly-organized repetitive sequences (Novák et al. 2010, Macas et al. 2011; Renny-Byfield et al. 2012). Contigs from CL2, CL5, CL6 and CL7 showed similarity to the known subtelomeric repeat of *A. fistulosum* (Irifune et al. 1995). These four clusters together comprise 7.12% of the genome. The other two clusters, CL58 (Fig. 1A) and CL36 (Fig.1B), comprise respectively 0.09% and 0.16% of the total reads used. Analysis in TandemRepeat finder (Benson, 1999) showed that the length of the monomers for CL36 and CL58 were 197 bp and 65 bp, respectively. After FISH analysis of the repeat chromosomal location we named these repeats as HAT58 (Heterochromatin-associated ***A****llium* tandem repeat from CL58) and CAT36 (periCentromeric ***A****llium* tandem repeat from CL36). The PCR products of CAT36 and HAT58 were cloned and sequenced: accession numbers at the GenBank for CAT36 is KX137122.1 and for HAT58 is KX137121.1. The DNA sequence of HAT58 did not show similarity to annotated genomic sequences in NCBI databases. In contrast, 6 genomic sequences (in GSS database of NCBI) of *A. cepa* were found by BLASTN to be similar to the CAT36 sequence.

PCR with HAT58 primers (Table 1) and genomic DNA of *A. fistulosum* and *A. wakegi*, a natural diploid hybrid between *A. fistulosum* and *A. cepa* (Hizume, 1994), resulted in ladder-like PCR products but no

PCR products were found for *A. cepa* (Fig. 1). PCR with CAT36 primer pairs resulted in ladder-like PCR products for *A. fistulosum*, *A. wakegi* and *A. cepa* (Fig. 1). Thus, both the BLASTN search and PCR results confirmed the tandem organization of these repeats in *A. fistulosum* and *A. cepa* genomes.

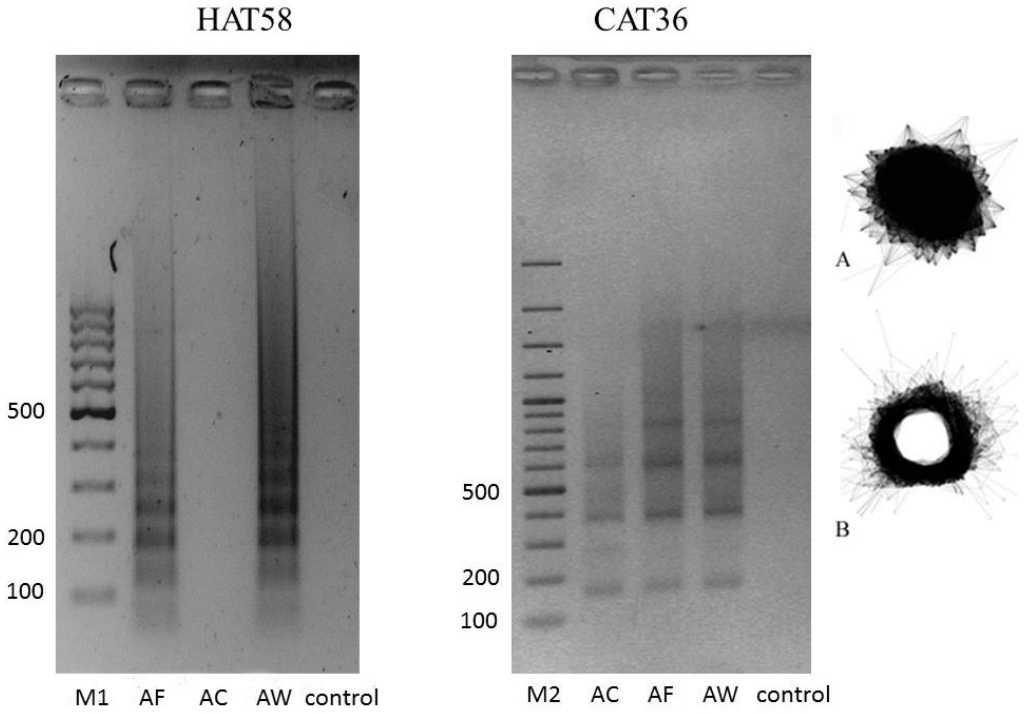

Fig. 1 Results of PCR amplification with primers for HAT58 and CAT36 repeats. AF – *A. fistulosum*, AC - *A. cepa*. AW –*A. wakegi,* control – negative control with water instead of DNA, M1 – 100bp ladder, M2 100bp Plus ladder **A**. The shape of the HAT58 (CL58) cluster; **B.** The shape of the CAT36 (CL36) cluster.

Chromosome localization of HAT58 and CAT36

FISH with HAT58 on *A. fistulosum* mitotic chromosomes showed fluorescent signals on three pairs of homologs which were identified by karyotype analysis: NOR bearing chromosome 6 (relative length,RL – 11.9±0.3; centromeric index, CI - 18.2±2.6), the smallest metacentric chromosome 7 (RL- 10.3±0.5; CI- 47.1±0.7) and the smallest submetacentric chromosome 8 (RL- 10.3±0.3; CI – 38.2±3.0 (de Vries and Jongerius 1988). On NOR-bearing chromosome 6, the FISH signal was located in the pericentromeric region of the long arm (Fig. 2A-C). On chromosome 8, FISH signals were revealed on the short arm (2-3 loci) and one locus in a proximal position on the long arm of chromosome 8 (Fig. 2A-C). The locus of HAT58, located on chromosome 7, demonstrated polymorphisms between plants, and three types of

FISH patterns on chromosome 7 were identified (Fig. 3A-C): 1) absence of the signals on both homologs (Fig.2A); 2) presence of the signal on one homolog (Fig. 3B); 3) presence of the signals on both homologs (Fig. 2C). Chromosome 7 is a metacentric chromosome and possesses 5S rRNA genes on the short arm (Hizume 1994). To determine whether 5S rRNA genes and HAT58 are located on the same chromosome arm, we employed two-color FISH with HAT58 (digoxigenin labeled) and 5S rRNA genes (biotin labeled), which revealed that the HAT58 locus and 5S rRNA genes are located on different arms of chromosome 7 (Fig. 2D). Therefore, we could conclude that HAT58 is located on the long arm of chromosome 7.

FISH with CAT36 probes on *A. fistulosum* revealed signals in the pericentromeric regions of chromosome 5 (RL- 11±1.5; CI - 47.9±1.9) and chromosome 6 (Fig. 4E). Because CAT36 and HAT58 repeats are both located in the pericentromeric region of chromosome 6, we checked whether they are co-localized or not. Two-color FISH with HAT58 and CAT36 on metaphase (Fig. 2E) and prophase (Fig. 2F) chromosomes of *A. fistulosum* demonstrated that CAT36 is located closer to the centromere than HAT58.

FISH analysis of HAT58 and CAT36 locations on *A. cepa* was performed in order to compare their location in two closely related species. According to recent phylogenetic and biogeographic analysis *A. cepa* and *A. fistulosum* are classified in the same section Cepa, subgenus Cepa (Friesen et al. 2006, Li et al.2010). The PCR products obtained with the CAT36 and HAT58 primers (Table 1) and genomic DNA of *A. cepa* and *A. fistulosum*, respectively, were used as a probe in the FISH experiment on *A. cepa* chromosomes. No FISH signals were observed with HAT58, while CAT36 produced only weak dispersed signals on the *A. cepa* chromosomes (Fig. 3D). Thus, FISH analysis of two closely related species showed that these repeats are species specific and produced specific hybridization patterns only on *A. fistulosum* chromosomes. An analysis of the chromosomal location of these polymorphic sites was performed in *A.* x *wakegi* (2n=2x=16), a natural allodiploid hybrid between *A. cepa* and *A. fistulosum* that possesses eight chromosomes of *A. cepa* and eight chromosomes of *A. fistulosum* (Tashiro 1980, 1984, Hizume 1994, Shibata and Hizume 2002). Simultaneous GISH and FISH showed hybridization patterns of HAT58 and CAT36 only on chromosomes belonging to *A. fistulosum* and no signals were detected on chromosomes belonging to *A. cepa.* The chromosomal locations of both tandem repeats were the same as in *A. fistulosum*: HAT58 – on the long arm of chromosome 6, on the long arm of chromosome 7, on the short

arm and the long arm of chromosome 8 (Fig. 2H); CAT36 - in the pericentromeric region of chromosomes 5 and 6 (Fig. 2I).

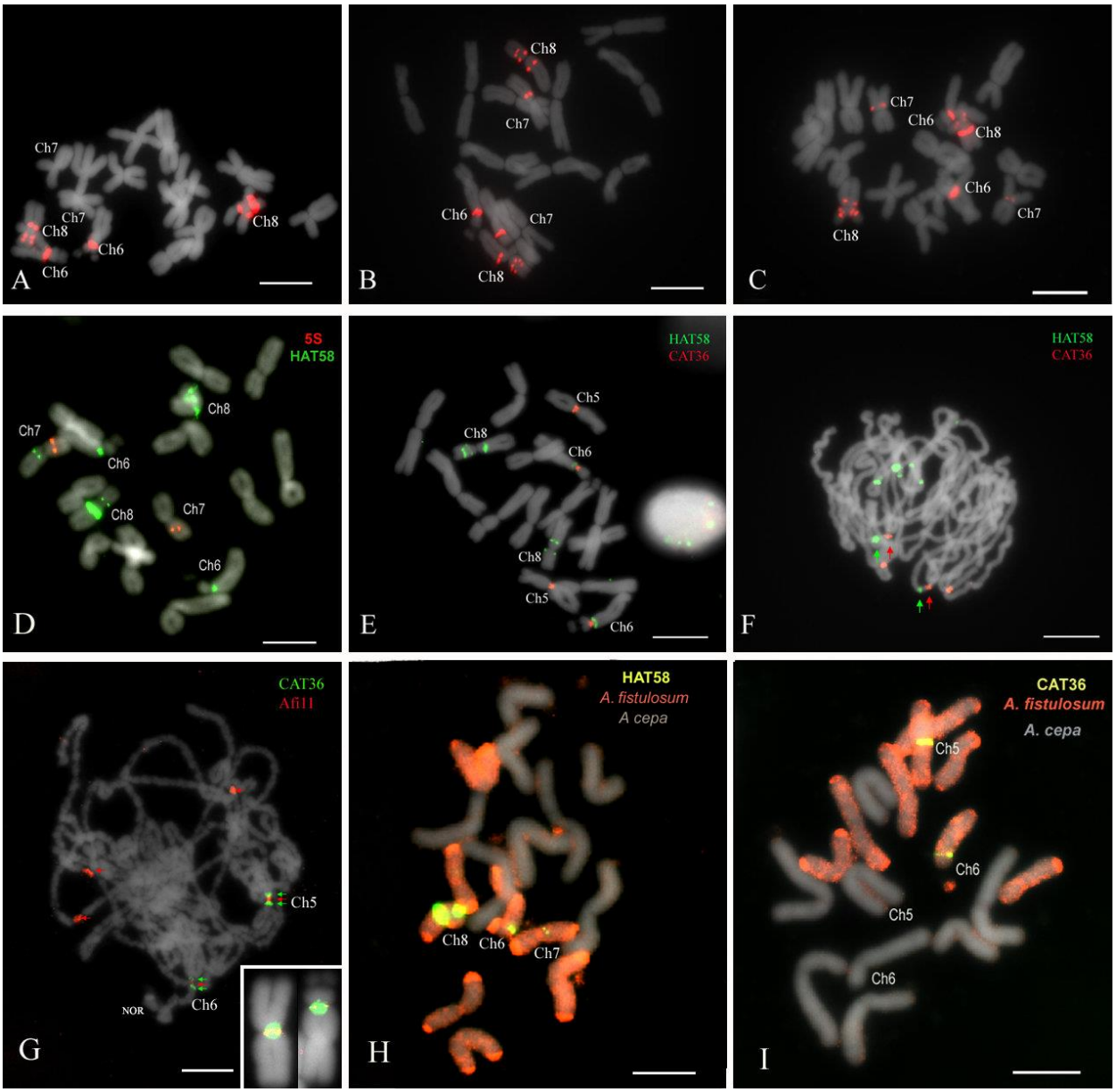

Fig. 2 A-C FISH with HAT58 tandem repeat on *A. fistulosum* chromosomes. Metaphase with no signals on chromosome 7 (**A**), with a signal on one homolog (**B**) and signals on both homologous chromosomes 7 (**C**). **D** Two-color FISH with HAT58 and 5S rRNA genes on *A. fistulosum* metaphase chromosomes; **E-F** Two-color FISH with HAT58 and CAT36 on *A. fistulosum* metaphase chromosomes (**E**) and *A. fistulosum* prophase chromosomes (**F**). Red and green arrows indicate CAT36 and HAT58 signals on chromosome 6, respectively. **G.** FISH with CAT36 and Afi11 repeats on pachytene chromosomes of *A. fistulosum*. Insert

shows co-localization of CAT36 and Afi11 on the *A. fistulosum* mitotic metaphase chromosomes 5 (left) and 6 (right). H. Simultaneous GISH and FISH with HAT58 on mitotic chromosomes of *A.* x *wakegi*. I. Simultaneous GISH and FISH with CAT36 on mitotic chromosomes of *A.* x *wakegi*. Bar - 10µm

Two-color FISH with HAT58 and CAT36 probes on *A. fistulosum* meiotic chromosomes at diakinesis allowed reliable detection of bivalents formed by homologous chromosomes 5, 6, 7 and 8 (Fig. 3A).

In an attempt to simplify the identification of individual chromosomes of *A. fistulosum*, ND-FISH with TAMRA-labeled oligonucleotides designed on HAT58 was performed. ND-FISH allows detection of tandem repeats without denaturation of the chromosome DNA and makes the FISH procedure much more rapid (Cuadrado and Jouve 2010). ND-FISH with HAT58 showed pronounced chromosome-specific patterns (Fig. 3B) that fully corresponded to FISH results (Fig 2A-C).

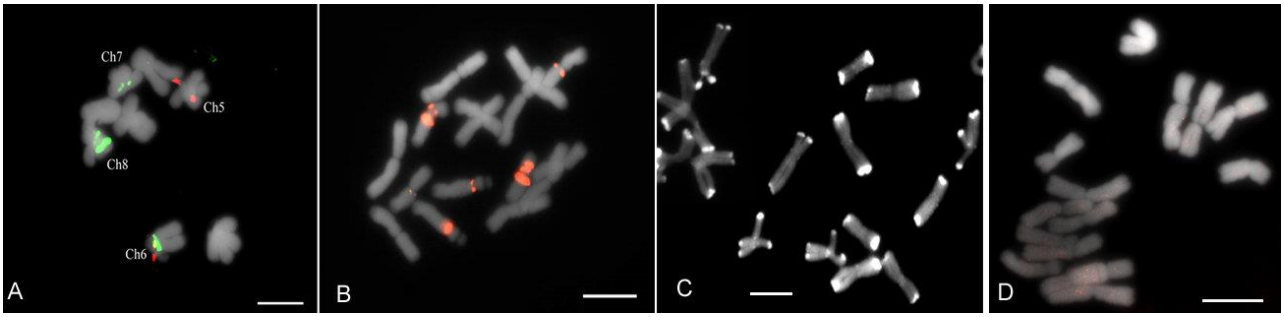

Fig. 3 A 8 cross bivalents of *A. fistulosum* at diakinesis after FISH with HAT58 (green) and CAT36 (red). B ND-FISH with TAMRA-labeled oligonucleotide probe designed for HAT58 on *A. fistulosum* mitotic metaphase chromosomes; C C-banding/DAPI on *A. fistulosum* mitotic metaphase chromosomes; D FISH with HAT58 (red) on *A. cepa* mitotic metaphase chromosomes. Bar – 10µm

HAT58 is associated with C-banding/DAPI patterns

In Allium the C-banding treatments usually reveal pronounced telomeric bands, different intensity centromeric bands, and rare and less often clearly seen intercalary bands. Analysis of five cultivars of *A. fistulosum* revealed the presence of intercalary C-bands on chromosome 6, 7 and 8 (Inada and Endo

1994). In order to clarify the association of these C-bands with HAT58 hybridization sites, C-banding/DAPI was applied (Fig.3C). We found the correspondence of HAT58 location with C-bands location on the arms of chromosome 6, 7 and 8 (Fig. 4). We measured the distance from telomeric end to the HAT58 hybridization sites and C-bands, and calculated the relative position of these signals on the chromosome arm. Statistical analysis confirmed the co-localization of the HAT58 fluorescent signals and the corresponding C-bands (Table 2).

Table 2. The relative position of HAT58 FISH-signals and C-bands on the mitotic metaphase chromosomes of *A. fistulosum*

Chromosome/arm	FISH	C-banding	t-test**	
	Medium ± SD	Medium ±SD	t-stat	t-critical
Chr6 long arm	0.9*± 0.03	0.9 ± 0.01	0.97	2.78
Chr7 long arm	0.8 ± 0.01	0,8 ± 0.04	-2.47	2.78
Chr8 short arm	0.8 ± 0.03	0.8 ± 0.07	1.21	2.14
short arm	0.7 ± 0.07	0.7 ± 0.10	-0.66	2.20
long arm	0.4 ± 0.06	0.4 ± 0.05	1.91	2.36

*- The relative position of FISH signals/ C-bands on chromosomes was calculated as the ratio of the distance between the site of hybridization/C-band and the telomeric end to the length of the chromosome arm.

**- Statistical analysis was performed using t-Test: Two-Sample Assuming Unequal Variances

Previously, it was reported that C-banding/DAPI patterns are similar to the Giemsa-stained C-bands, and it was proven that fluorescent bands observed with DAPI after C-banding are not necessarily AT-rich regions but also heterochromatin regions with another composition of nucleotides (Barros and Guerra 2010). Our result indicates, at least, that HAT58 is a constituent portion of the intercalary heterochromatin of chromosome 6, 7 and 8 of *A. fistulosum.*

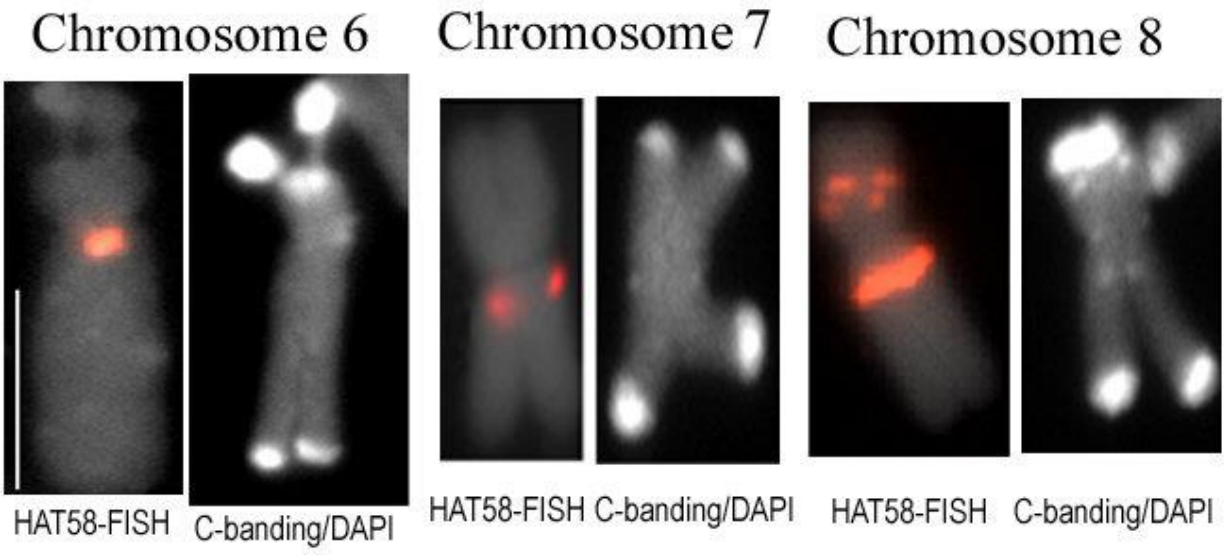

Fig. 4 Extracted chromosomes 6, 7 and 8 from mitotic metaphases of *A. fistulosum* after FISH probing with HAT58 tandem repeats and C-banding/DAPI. Bar -5µm

The functional centromeric repeat loci (Afi) of chromosomes 5 and 6 are flanked by CAT36

Recently, Nagaki et al (2012) discovered a functional centromeric sequence of *A. fistulosum* (Afi) and the authors proved with FISH that Afi is present in the centromeric region of all chromosomes. We found that CAT36 was located in the pericentromeric region of chromosomes 5 and 6 of *A. fistulosum.* To order the locations of Afi and CAT36, two-color FISH was performed on mitotic metaphase chromosomes of *A. fistulosum.* Primers were designed using Afi sequence (Nagaki et al 2012; Table 2) and Afi11 PCR product, obtained with the genomic DNA of *A. fistulosum*, was used as a probe in the FISH experiment. Two-color FISH with Afi11 and CAT36 revealed that signals from Afi11 (red) and CAT36 (green) overlap on chromosomes 5 and 6 (Fig. 2G insert). The level of metaphase chromosome condensation in Allium is on average 10 times higher than that in human and 5 times that in tomato (Khrustaleva and Kik 2001). To increase the resolution limit of FISH, high resolution FISH on an early pachytene chromosome of *A. fistulosum* was performed. The high resolution FISH showed that CAT36 is located in the pericentromeric part of the chromosomes flanking the Afi11 clusters (Fig. 2G).

The location of identified (HAT58 and CAT36) and previously known (45S rDNA, 5S rDNA; Hizume 1994) TRs, as well as the location of a 380 bp subtelomeric repeat (Irifune et al. 1995) and the centromeric repeat (Nagaki et al. 2012) on *A. fistulosum* chromosomes, are summarized in Fig. 5.

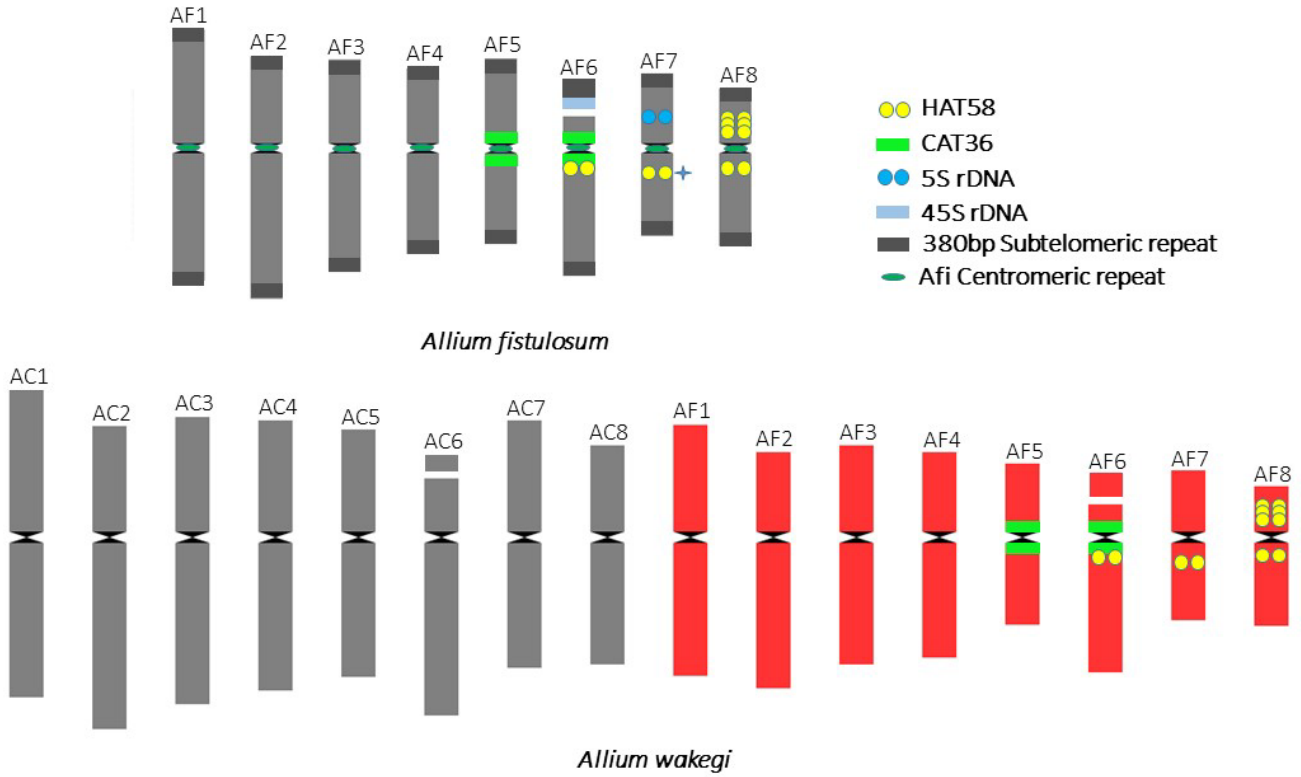

Fig. 5 Idiograms of *A. fistulosum* chromosomes (top) and *A. wakegi* chromosomes (bottom) with marked localization of HAT58 and CAT36. For *A. fistulosum,* localization of 45S and 5S rDNA genes (Hizume 1994), Afi centromeric repeat (Nagaki et al. 2012) and a 380bp subtelomeric tandem repeat (Irifune et al. 1995) are shown. Polymorphic site of HAT58 on chromosome 7 is marked by a cross.

Discussion

We used the combination of sequencing, bioinformatic tools and molecular cytogenetic methods for rapid development of cytogenetic markers for identification of individual chromosomes in *A. fistulosum.* We isolated two tandem repeats, HAT58 and CAT36, in the *A. fistulosum* genome that: 1) allowed the identification of half of the chromosome complement, specifically chromosomes 5, 6, 7 and 8; 2) are specific for *A. fistulosum* relative to *A. cepa* and showed species specific hybridization patterns in *A.*

wakegi, a natural allodiploid hybrid between *A. fistulosum* and *A. cepa*; 3) are associated with heterochromatin and/or pericentromeric regions; 4) CAT36 flanks the functional centromeric sequence Afi11 (Nagaki et al. 2012) on chromosome 5 and 6; and 5) can be easily detected by FISH and ND-FISH. We revealed polymorphisms for chromosome locations of HAT58 in *A. fistulosum*. This finding suggests that the HAT58 repeat is still undergoing rapid evolution.

Tandem repeats are an important source of cytogenetic markers

Tandem repearts (TRs) have been used in numerous studies for developing FISH markers (Do et al. 2001, Albert et al.2010, Komuro et al.2013, He et al. 2015). The *A. fistulosum* chromosomes are large and can be distinguished by their length and centromere position with exception of chromosomes 3 and 4. However, morphological characteristics cannot be used for identification of aberrant chromosomes or recombinant chromosomes in interspecific hybrids because the length of chromosome arms could be changed. Species-specific FISH markers are useful for determining the location of alien genetic material in the recipient genome because they reveal both genetic origin and chromosomal location simultaneously. HAT58 and CAT36 reveal species specific patterns of hybridization that was demonstrated using FISH for two closely related species *A. cepa* and *A. fistulosum*. It is significant that until now only two FISH markers, 45S rDNA and 5S rDNA have been used for identification of individual chromosomes in *A. fistulosum*. However these rDNA markers exist in all plants and may not be useful for monitoring of alien introgressions in interspecific hybrids. Allium species of subgenus Cepa share a very similar tandem repeat in subtelomeric region of all 8 chromosomes (Pich et al. 1996). These authors conclude that this tandem repeat existed in progenitor forms and remained unusually well conserved during speciation. In contrast, HAT58 and CAT36 are species specific cytogenetic markers. HAT58 is present only in the *A. fistulosum* genome (Fig.1) and exists as one polymorphic site on chromosome 7: plants with three type location patterns of this repeat were observed (Fig.2A-C). Tandem repeats undergo rapid evolution and burst-like evolutionary modes (Garrido-Ramos et al. 2015). Therefore TRs can quickly spread to new genomic regions resulting in polymorphic sites (Emadzade et al. 2014). In this respect, FISH visualization of HAT58 and CAT36 on chromosomes of *A. wakegi*, a natural allodiploid hybrid between *A. fistulosum* and *A. cepa*, was of interest. The parental origin of *A. wakegi* was proven by genomic study, karyotype analysis (Tashiro 1980, 1984) and GISH probing with genomic DNA of *A. cepa* and *A. fistulosum* (Hizume 1994, Shibata and Hizume 2002). Simultaneous GISH and FISH in *A.*

wakegi showed that a chromosomal location of these TRs has been preserved in the same position as in the parental species. A FISH study on *A. wakegi* probing with 5S rDNA, which differ in number of loci between the parental species, did not show differences for the chromosomal locations of FISH patterns (Hizume 1994). In contrary, extensive chromosomal reorganization was documented for homoploid hybrids of the desert sunflowers *Helianthus anomalus, Helianthus deserticola* and *Heliantus pradoxus* (Lai et al.2005). These hybrids were maintained through sexual reproduction and their speciation occurred through rapid karyotypic evolution (the recombinational speciation model) and spatial separation from their parental species. *A. wakegi* is sterile and propagated vegetatively. It has been grown in western Japan, China, and countries of southeastern Asia since ancient times (Inden & Asahira, 1990). *A. wakegi* originated by interspecific hybridization of two closely related species *A. cepa* and *A. fistulosum,* presumably thousands years ago, and survived due to the adaptive advantage over parental species and sterility of allodiploid form. Hybrid sterility caused the reproductive isolation of *A. wakegi* from its parental species. The *A. cepa* genome has 27% more DNA than *A. fistulosum* genome (Jones and Rees, 1968) and the *A. cepa* chromosomes are on average 12% longer than *A. fistulosum* chromosomes (Albini and Jones 1990). The chiasma distribution is different between these two species: in *A. cepa* chiasmata occur mainly in interstitial and distal chromosome regions and in *A. fistulosum* they are localized adjacent to centromeres (Albini and Jones 1990). These cytogenetic differences between parental species contributed to hybrid sterility and prevented subgenome homogenization via recombination. A question arises: would we expect any homogenization of chromosomes in an asexually propagated plant that has not undergone meiosis and generations of reproduction via seed ? However, *A. wakegi* may flower in some rare cases and allowed Iwasa (1960) to study meiosis in PMCs. This author reported bivalent, univalent and multivalent formations and young pollen-grains degradation. Synthetic hybrid between *A. cepa* and *A. fistulosum* are also sterile. Although, recombination among chromosomes of *A. cepa* and *A. fistulosum* was demonstrated in a GISH study of the second generation bridge cross [*A. cepa* x (*A. fistulosum* x *A. roylei*)] (Khrustaleva and Kik 2000). Therefore, it remains to be determined using more cytogenetic markers whether these two genomes in an allodiploid hybrid undergo homogenization or they cohabit a single nucleus without any change.

In attempt to simplify the procedure of signal detection we applied ND-FISH with TAMRA-labeled oligonucleotides designed on HAT58. Previously, it was shown that tandem repeats can be detected by FISH without chromosome denaturation (Cuadrado and Jouve 2010). When using ND-FISH, two hours of hybridization are sufficient for probe penetration and hybridization with chromosomal DNA.

Moreover, the time for detection can be further reduced by application of probes directly labeled with fluorochromes. Here we demonstrated that ND-FISH, with a fluorochrome labeled oligonucleotide probe, allows the detection of the repeat bearing chromosomes within three hours.

Rapid evolution of the identified tandem repeats

In our study, we used Illumina reads (80bp) of *A. fistulosum* covering an equivalent of 3.6% of the genome (1C=11.5 Gb, Ricroch et al. 2005) to characterize the repeatome of bunching onion tandem repeats. The results of the annotation of 482 clusters, representing 42.03% of the genome, showed that a huge part of the *A. fistulosum* genome (26.93%) is shaped by unknown repeats which are not similar to any known repeats present in the databases. Based on the cluster shapes corresponding to these repeats, it can be suggested that most of them are dispersed repeats. Previously, many dispersed repeats were discovered in the *A. cepa* genome (Shibata and Hizume, 2002). Based on the globula-like shape of clusters CL58 and CL36, we discovered two tandem repeats, named HAT58 and CAT36. The tandem nature of the repeats was confirmed by PCR and FISH analyses. HAT58 and CAT36 together comprise 0.25% of the *A. fistulosum* genome. The calculated copy number of HAT58 and CAT36 in the genome of bunching onion is about 160,000 copies/1C and 93,000 copies/1C, respectively. FISH mapping of the HAT58 and CAT36 repeats to *A. cepa* revealed no signal and weak dispersed FISH signals, respectively. The result suggests that the *A. cepa* genome does not contain HAT58 and may contain a much lower proportion of CAT36 repeats that are dispersed along the entire chromosome in contrast to the localized position in pericentromeric region of the *A. fistulosum* chromosomes 5 and 6. These results are in accordance with the rapid evolution mode of TRs previously observed in *Beta* (Schmidt and Heslop-Harrison 1993), *Nicotiana* (Renny-Byfield et al. 2012), *Solanum* (Zhang et al. 2014) and *Zea* (Albert et al. 2010). Unequal crossing over, illegitimate recombination, conversion-like events, replication slippage and extrachromosomal circular DNA are thought to be responsible for the fast satellite DNA turnover in the genome (Charlesworth et al. 1994; Cohen et al. 2008). Earlier on, we demonstrated that the centromeric regions of *A. fistulosum* contained Ty3/gypsy retrotransposons (Kiseleva et al. 2014), which belong to a number of lineages of the chromovirus family of Ty3/gypsy LTR (long terminal repeat) retrotransposones (Neumann et al. 2011). It can be hypothesized that the insertion of CAT36 into the pericentromere-specific retrotransposon sequence, and its subsequent amplification in the genome of *A. fistulosum*, may cause the accumulation of CAT36 in the

pericentromeric region of *A. fistulosum* chromosomes. Based on our results, two hypotheses could be put forward: (1) HAT58 is younger than CAT36 because HAT58 is a species-specific tandem repeat, and therefore was formed after the divergence of *A. cepa* and *A. fistulosum* from a common ancestor, whereas CAT36 was present in the ancestor genome; (2) HAT58 was present in the ancestor genome and was subsequently eliminated from the *A. cepa* genome. The observed polymorphism of HAT58 suggests that the rapid evolution of this repeat is still ongoing, which may support the first hypothesis.

The TRs markers presented in our study are species specific and associated with constitutive heterochromatin. Further analysis of the tandem repeats will clarify their role in speciation, heterochromatin formation and function of a high packaged Allium chromosome. Knowledge of the chromosome organization of TRs may help to fill in sequence gaps that can arise during plant genome assembly, as TR arrays are difficult to sequence and assemble (Treangen and Salzberg 2011). FISH markers can accelerate the ongoing genome sequencing project of *A. fistulosum.* These chromosomal markers can be used as a reference resource in onion breeding as well as in chromosomal evolution studies in general.

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CHAPTER 4

PHYSICAL MAPPING OF TARGET GENES ON CHROMOSOMES OF ALLIUM AND ROSA SPECIES

Our developed and optimized protocols can be used to physically map genes on *R. wichurana* and *Allium*. Since *R. wichurana* mitotic chromosomes are small and provide limited resolution for physical mapping, we performed physical Tyramide-FISH on pachytene chromosomes, allowing to increase the resolution up to 20 times (Paper 5). We also describe the results of physical mapping of EST clones and two important genes, alliinase and LFS, on *Allium* chromosomes (Paper 6).

PAPER 5: KIROV, I. V., VAN LAERE, K., & KHRUSTALEVA, L. I. (2015). HIGH RESOLUTION PHYSICAL MAPPING OF SINGLE GENE FRAGMENTS ON PACHYTENE CHROMOSOME 4 AND 7 OF ROSA. BMC GENETICS, 16(1), 1 - 10.

PAPER 6: KHRUSTALEVA, L., KIROV, I., ROMANOV, D., BUDYLIN, M., LAPITSKAYA, I., KISELEVA, A., & KARLOV, G. (2012). THE CHROMOSOME ORGANIZATION OF GENES AND SOME TYPES OF EXTRAGENIC DNA IN ALLIUM. *ACTA HORT*, *969*, 43-51.

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RESEARCH ARTICLE Open Access

High resolution physical mapping of single gene fragments on pachytene chromosome 4 and 7 of *Rosa*

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Abstract

Background: Rosaceae is a family containing many economically important fruit and ornamental species. Although fluorescence in situ hybridization (FISH)-based physical mapping of plant genomes is a valuable tool for map-based cloning, comparative genomics and evolutionary studies, no studies using high resolution physical mapping have been performed in this family. Previously we proved that physical mapping of single-copy genes as small as 1.1 kb is possible on mitotic metaphase chromosomes of *Rosa wichurana* using Tyramide-FISH. In this study we aimed to further improve the physical map of *Rosa wichurana* by applying high resolution FISH to pachytene chromosomes.

Results: Using high resolution Tyramide-FISH and multicolor Tyramide-FISH, 7 genes (1.7–3 kb) were successfully mapped on pachytene chromosomes 4 and 7 of *Rosa wichurana*. Additionally, by using multicolor Tyramide-FISH three closely located genes were simultaneously visualized on chromosome 7. A detailed map of heterochromatine/euchromatine patterns of chromosome 4 and 7 was developed with indication of the physical position of these 7 genes. Comparison of the gene order between *Rosa wichurana* and *Fragaria vesca* revealed a poor collinearity for chromosome 7, but a perfect collinearity for chromosome 4.

Conclusions: High resolution physical mapping of short probes on pachytene chromosomes of *Rosa wichurana* was successfully performed for the first time. Application of Tyramide-FISH on pachytene chromosomes allowed the mapping resolution to be increased up to 20 times compared to mitotic metaphase chromosomes. High resolution Tyramide-FISH and multicolor Tyramide-FISH might become useful tools for further physical mapping of single-copy genes and for the integration of physical and genetic maps of *Rosa wichurana* and other members of the Rosaceae.

Keywords: Fluorescence In Situ Hybridization, Pachytene, Tyramide-FISH, *Rosa*, Physical map

Background

Rosa is a genus of the Rosaceae family consisting of approximately 90 genera and approximately 3000 species. Many of these are economically important such as *Malus*, *Prunus*, *Pyrus*, *Fragaria*, *Rubus*, *Sorbus*, *Cotoneaster* and *Crataegus* [1–5]. Approximately 150 species and more than 20.000 cultivars of *Rosa* are described [6]. Most species have a complex origin [7]. Interestingly, only 7 to 15 species have contributed to the original germplasm of the modern rose cultivars [8]. *Rosa* species have small genomes and a high level of heterozygosity. Basic chromosome number is $x = 7$ [1], but ploidy levels range from diploid ($2n = 2x = 14$) to decaploid ($2n = 8x = 56$). Genomes of *Pyrus* [9], *Prunus* [10], *Fragaria* [4] and *Malus* [11] recently have been sequenced, providing valuable information for comparative genomics, gene cloning, marker development, QTL mapping and marker-assisted selection. Comparative analysis of these sequenced genomes has shed more light on the mode of evolution of some Rosaceae genera and species. In contrast, the organization of the *Rosa* genome has only been poorly investigated and the knowledge about the macro-synteny and collinearity of the Rose genome with other sequenced genomes Rosaceae family is poor.

Genetic maps have been widely used for comparative genomic and genome organization studies [12, 13]. The distance between markers in genetic maps expressed in

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recombination frequencies, or centimorgans (cM) is known to be unequally distributed along the chromosomes [14–17]. Gene order in genome regions with extremely low recombination frequency (e.g. centromeres, heterochromatin) cannot be revealed because of the low resolution of genetic mapping in these regions [18]. In addition, lack of collinearity between parental genomes used for development of the mapping population can cause inaccuracy in genetic maps [19]. In contrast to genetic maps, physical maps show real positions of DNA sequences on the chromosomes. Physical mapping using fluorescence in situ hybridization (FISH) does not depend on recombination frequency, therefore it can be used for gene mapping in "cold spot recombination" regions [18]. But FISH mapping has a lower efficiency than genetic mapping. The integration of physical and genetic maps provides a unique tool combining advantages of both types of maps. FISH-based physical maps have been developed and successfully integrated with genetic maps for many plant species (see review [20]). Direct visualization of DNA sequences on chromosomes by FISH is also a valuable for genome sequencing. FISH mapping improves the quality of genome assembly as demonstrated in tomato [21], cucumber [22] and Amborella [23].

Until now most of the cytogenetic studies in *Rosa* have been dedicated to karyotyping, chromosome number evaluation and rRNA (5S and 45S) gene mapping [24–28]. Further progress in FISH using individual genes or DNA clones (e.g. ESTs, BACs) is required for efficient cytogenetic map construction. Physical mapping of individual genes as small as 1.1 kb has proven to be possible on mitotic metaphase chromosomes of *Rosa wichurana* using Tyramide-FISH [29]. However, the resolution of Tyramide-FISH on the small mitotic *Rosa* chromosomes is very low which significantly hampers the construction of a physical map and the determination of the order of DNA sequences. The use of pachytene chromosomes would be an improvement for physical mapping [30]. Pachytene chromosomes are 7–40 times longer than mitotic metaphase chromosomes and therefore provide a higher resolution [21, 30]. Moreover, heterochromatic and euchromatic regions are distinguishable at the pachytene stage [31, 32]. Pachytene bivalents consist of 8 DNA strands instead of 4 in mitotic chromosomes, which also increases the sensitivity of FISH. Also important is that meiotic cells (pollen mother cells, or PMC) synchronously divide providing many cells in the same stage. High resolution FISH mapping on pachytene chromosomes has been used successfully in tomato [14, 21, 33–35] and *Arabidopsis* [31, 36–39]. However, for many plant species, pachytene preparation is still very challenging [30, 32, 40, 41].

Rosa wichurana is a diploid species (2n = 2x = 14) that was involved in the origin of modern rose cultivars. This species is a valuable source for resistance to powdery mildew [42, 43] and has been used for construction of several genetic maps [42–46]. Moreover, *R.wichurana* is attractive object for molecular cytogenetic studies because it has intensively growing apical meristems and simple corolla, providing many anthers to be used for pollen mother cells (PMC) isolation and pachytene chromosome preparation. Therefore *Rosa wichurana* is a good model for the *Rosa* genus,

In this paper, we improved the SteamDrop protocol [47] for preparation of high quality pachytene chromosomes of *Rosa wichurana*. We performed physical mapping of 7 genes on pachytene chromosomes 4 and 7 of *Rosa wichurana* using a high resolution Tyramide-FISH and multicolour Tyramide-FISH. For the first time, multicolor Tyramide-FISH was applied to plant chromosomes. The protocol for multicolor-FISH allowed simultaneous visualization of the physical positions of three genes closely located on chromosome 7 of *Rosa wichurana*.

Methods

An orthology-based approach for probe design resulted in FISH probes with a length between 1.7 kb and 3 kb

To isolate gene sequences on specific chromosomes of *R. wichurana* we used orthologous sequences of *Fragaria vesca* as a reference. The *Rosa wichurana* chromosomes 4 and 7 correspond to *Fragaria vesca* pseudochromosomes 7 (FvChr7) and 6 (FvChr6), respectively [29, 48]. Several genes from *Fragaria vesca* pseudochromosomes 6 and 7 (FvChr 6 and FvChr 7) were randomly selected using the NCBI Map Viewer tool. These candidate genes were then used for BLASTN analysis against the genome of *F. vesca* (FraVesHawaii_1.0) in order to estimate their copy number in the *Fragaria* genome and to select only the single-copy genes. Seven genes [MLO-like proteins (MLO2 and MLO3), ATPase (AAA-2), Ubiquitin protein ligase (RIN-2), monodehydroascorbate reductase (MDAR), Villin-2-like, mannosylglycoprotein endo-beta-mannosidase (MGM)] which showed significant similarity only to the original sequences of *Fragaria vesca* were chosen for further analysis. The selected genes were used for BLASTN against nucleotide and EST databases of *Rosa* at NCBI. Full mRNA sequences of *Rosa* (*Rosa multiflora*) MLO genes (JX847132.1, JX847133) were used for primer design to isolate MLO-like genes from *R.wichurana*. Pairwise alignment of rose MLO mRNA sequences with orthologous *Fragaria* full length MLO sequences was performed to prevent primer annealing at the intron/exon border.

The other 5 *Fragaria* full-length gene sequences were used for BLASTN against transcriptome reads of *R.* x hybrid (NCBI accession number: SRX097578) or *R. chinensis* transcriptome reads or/and clusters

(https://iant.toulouse.inra.fr/). In this way, rose reads with significant similarity to different parts of the *Fragaria* genes were found and used for primer design.

Amplification and labeling of gene fragments

Primers were designed using CLCbio Genomics Workbench version 7.0 to amplify gene fragments with a length more than 2 kb based on known *Fragaria* gene sequences and their location on the pseudochromosomes (Table 1). PCR products were generated using designed primers and genomic DNA of *R.wichurana*. PCR products were obtained with all 7 primer combinations. PCR with primers for *villin* resulted in a short PCR product of 900 bp, which is too short to be used in Tyramide-FISH experiments on *Rosa*. The other PCR products which ranged between 1.7 kb and 3 kb were cloned (Table 1) by pGEM-T easy (Promega, Madison, WI, USA) or by pPCR-TOPO kit (Invitrogen, Carlsbad, CA, USA). At least two clones were sequenced for each primer combination. The partial sequences of the clones used in this study are available as Additional file 1.

For physical mapping of PAL and P5CS genes, previously cloned gene fragments [29], were used.

Plasmid DNA was isolated by the PureLink Quick Plasmid Miniprep Kit (Invitrogen, CA, USA) and labeled by Biotin-Nick translation mix (Roche, Mannheim, Germany), Digoxigenin-Nick Translation mix (Roche) or by a "home-made" Biotin-Nick translation Mix using a "home-labeled" dUTP nucleotide. "Home labeled" nucleotides were prepared according to the previously described protocol [49] using aminoallyl-dUTP (Thermo Scientific) and Biotinamidohexanoic acid N-hydroxysuccinimide ester (Sigma-Aldish Co., LLC, France). Our "home-made" Nick translation mix (30 μl) consists of 500 ng DNA, 83 μM dATP, dGTP, dCTP, 10.6 μM dTTP, 69 μM biotin-dUTP, 300 mU rDNAse I (Ambion) and 5U DNA Polymerase I (Invitrogen) in NT buffer (50 mM Tris–HCl, pH 7.8, 5 mM $MgCl_2$) containing 10 mM beta-mercaptoethanol. The reaction

Table 1 Sequences of primers for gene fragment isolation and PCR results

Gene	Abbreviation	Primers, 5′-3′	Location on *Fragaria* pseudo-chromosomes	Expected length PCR product (bp)	Length obtained PCR products (bp)
MLO-like protein	MLO3	F: AAAACACCAACATGGGCAGT	FvChr 7: 15397809..15393519	1675	1700
		R: TTCCGAAAATCAAAGGTCGT			
MLO-like protein	MLO2	F: AGGATTTCAAGGTCGTGGTG	FvChr6: 34503533..34507423	1852	1800
		R: TGGTCGGCTAGCATTTTTCT			
ATPase	AAA-2	F: GTTCCCTTTGTCATTGCAG	FvChr7: 21485846.. 21481090	2718	3400
		R: ACGGCCTCTTCATCAATT			
Ubiquitin protein ligase	RIN-2	F: TCCTTCAGCTACACCATTGAC	FvChr7: 19866961..19871497	2228	2500
		R: AAATTGCGCGTTCCTACT			
Monodehydroascorbate reductase	MDAR	F: GAGGCGGTATGGTTAATTT	FvChr6: 12864594..12867898	2417	2800
		R: AAACTTGGGCTTTGGTGA			
Villin-2-like	Villin	F: CTCGCTTCTTCACAACATACT	FvChr6: 33309900..33321407	3851	900
		R:TTCACTGCCATTTTCATCCT			
Mannosylglycoprotein endo-beta-mannosidase	MGM	F: CGGCATGGAAAATGAGTCAA	FvChr6: 5180627..5186123	3017	3000
		R: GAACAAAGGGATCTGCCA			
Phenylalanine ammonia lyase[a]	PAL	F: ACCACTGGKTTTGGTGCWAC	FvChr6:34874086–34877587	-	1700
		R: CCYTTGAASCCATAATCCAA			
Pyrroline-5-Carboxylate Synthase[a]	P5CS	F: GCTGGCATCCCTGTTGTTAT	FvChr7: 17624431–17630820	-	1700
		R: CTTCGGATCGCTAATGAAGC			

[a]Data from [29]

was done at 15 °C for 2 h and stopped by heating at 80 °C for 5 min.

Clone pTa71 containing a 9-kb *Eco*RI fragment of the 45S rDNA from wheat [50], was labeled using the Digoxigenin-Nick Translation Mix (Roche).

Pachytene chromosome preparation

Rosa wichurana plants were grown in a glass greenhouse under natural conditions in the temperate climate of East Flanders, Belgium. Five to ten anthers from each flower bud were placed on the slide and a drop of 60 % acetic acid was added. Anthers were then disrupted by a needle, heated at 55 °C for 1 min and squashed with a coverslip. The meiotic stages were observed under phase contrast (Zeiss AxioImager M2, Zeiss Company, Germany). Flower buds with most of the anthers containing PMCs in pachytene stage were fixed in Carnoy's solution (3:1, ethanol:acetic acid) for at least 3 h and transferred into 70 % ethanol for storage at −20 °C.

Flower buds were washed in water for 30–40 min and transferred into 10 mM citric buffer (citric acid, sodium citrate dehydrate, pH 4.7–4.8) for 15–30 min. Anthers were then separated using a pincet and transferred into 50 μl enzyme mixture. One-third to one-fourth of all anthers from a single flower bud were used for the preparation of one tube of cell suspension. The enzyme mixture contained 0.6 % Pectolyase Y-23 (Kikkoman, Tokyo, Japan), 0.6 % Cellulase Onozuka R-10 (Yakult Co. Ltd., Tokyo, Japan) and 0.2 % Cytohelicase (Sigma-Aldish Co. LLC, France). A modified SteamDrop protocol [47] was used for cell suspension and chromosome preparation. Briefly, 10 μl of cell suspension were added on the slide; after 8–10 s, a drop of 22–28 μl of 1:1 ethanol:acetic acid was applied. When the slide surface became granule-like (50–80 s) steam (3–5 s) was applied to the slides and a second drop of 15–18 μl of 1:2 ethanol:acetic was added. When the granule-like surface appeared again on the slide, steam was applied and 300 μl of 45 % or 60 % acetic acid was added. Then slides were incubated on a heating plate (42 °C) for 15–30 s. A needle was used to spread the drop of acetic acid over the ring of cells on the slide. Acetic acid was removed by positioning the slide vertically on filter paper.

Tyramide-FISH

We used the Tyramide-FISH protocol with an indirect detection system previously optimized for *R. wichurana* mitotic chromosomes [29]. Prehybridization procedures for single color and two color Tyramide-FISH included overnight incubation at 37 °C, 4 % paraformaldehyde treatment (6–7 min) and dehydration in an ethanol series (70 %, 90 % and 100 %). Probe hybridization and detection for single color Tyramide-FISH were carried out as described [29]. For two color Tyramide-FISH probe hybridization was conducted according to Kirov et al. [29] with a hybridization mixture containing two labeled probes: digoxigenin labeled MLO2 gene and biotin labeled PAL gene. The two probes were detected sequentially. First, the PAL gene was detected by Streptavidin-HRP (1:100, PerkinElmer, Belgium) followed by application of tyramide-biotin (1:25 in plus amplification buffer (PerkinElmer)) application. Then HRP (Horse Radish Peroxidase) was inactivated using 3 % hydrogen peroxide (20 min). After this slides were simultaneously incubated with streptavidin-Cy3 (1:100, Sigma-Aldrich)) and anti-digoxigenin-HRP (1:100, Roche) diluted in blocking buffer (Roche) followed by tyramide-biotin amplification. Biotin from the second layer was detected by Streptavidin-FITC (Vector Laboratories, Burlingame, CA).

Zeiss AxioImager M2 (400× and 1000× magnification) equipped with an AxioCam MRm camera and Zen software (Zeiss, Zaventem, Belgium) were used to analyse the hybridisation signal and to capture all images. Relative distance to the signal was calculated by the following formula:

Distance from telomere of the long arm to the signal × 100 % / length of whole arm.

Images were analyzed using ImageJ software. Signal position was measured using the Micromeasure software [51], version 3.3 (http://sites.biology.colostate.edu/MicroMeasure/). Pachytene chromosomes with a similar length were chosen for calculation of centromere index and percentage of heterochromatin. At least 9 pachytene chromosomes were used to determine the location of the Tyramide-FISH signals (Table 2).

Results

High quality pachytene chromosome preparations suitable for Tyramide-FISH were obtained for Rosa wichurana

Flower buds with a hypanthium size of 3–5 mm had the most PMCs in pachytene stage although also PMC in metaphase I and tetrad stages could be observed in the same flower bud (Fig. 1).

DAPI staining of *R wichurana* pachytene chromosomes did not reveal strong heterochromatin blocks that can be used as cytogenetic markers. All centromeres are the weakest stained parts of the chromosomes flanked by pericentromeric heterochromatin. Only chromosomes 4 and 7 are distinguishable by their clear presence of heterochromatin patterns on the short arms (Fig. 2, Table 2). In addition chromosome 7 possesses NOR (nucleolar organizing regions) on the short arm.

To prepare pachytene chromosomes, the SteamDrop protocol was modified by adding a drop of 60 % acetic acid after the second steam application and before drying the cells. This modification improved the chromosome spreading and resulted in a clear 45S

Table 2 Characteristics of pachytene chromosome 4 and 7 of R. wichurana

Chr. Nr.	Chromosome length (μm)	Short arm (μm)	Long arm (μm)	Short arm heterochromatin (%)[a]	Long arm heterochromatin (%)[b]	Total heterochromatin (%)[c]	Centromere index	NOR	n[d]
4	45.6 ± 5.4	11.4 ± 1.4	34.0 ± 4.0	62.0 ± 6.0	21.0 ± 4.5	31.0 ± 4.0	25.0 ± 0.5		7
7	38.4 ± 4.2	4.0 ± 0.6	33.3 ± 3.5	88.0 ± 6.7	16.8 ± 4.4	24.3 ± 2.3	10.3 ± 0.8	+	9

[a]– calculated by the formula: length of heterochromatin of the short arm × 100%/total length of the short arm
[b]– calculated by the formula: length of heterochromatin of the long arm × 100%/total length of the long arm
[c]– calculated by the formula: (length of heterochromatin of the short arm + length of heterochromatin of the long arm) × 100 %/total length of the chromosome
[d]– Number of pachytenes used in the measurements of chromosome lengths and calculation of % heterochromatin

rDNA signal on chromosome 7 of *Rosa wichurana* (Fig. 3a). Moreover, application of Tyramide-FISH on pachytene chromosomes revealed differences in signal frequency between the slides prepared by 15, 30 and 60 s of acetic acid treatment on the heating plate (the final step in modified SteamDrop procedure). The maximum signal-to-noise ratio (visual observation) and signal frequency (60–70 %) were obtained on the slides prepared by 30 s of acetic acid treatment. Both 15 s and 60 s of acetic acid treatment resulted in low signal frequency (10–15 %). In addition the level of background was higher for 15 s and very low for 60 s.

Pachytene chromosomes of Rosa wichurana provide up to 20 times higher resolution compared to mitotic chromosomes

The total pachytene chromosome length varied between 235 μm and 411 μm, which is 10–20 times longer than the mitotic chromosomes (20 μm ± 1 μm; [29]). The chromatin compactization of pachytene chromosomes of *R. wichurana* is 2.4 - 1.4 Mbp μm^{-1}, calculated based on the genome size of 562 Mbp [52]. Taken into account the 0.2 μm - resolution limit for fluorescence microscopy, it can be concluded that the spatial resolution of FISH on *R. wichurana* pachytene chromosomes is between 300–500 kb.

High resolution physical mapping of gene fragments using Tyramide-FISH

Seven gene fragments resulted in clear Tyramide-FISH twin signals on one pachytene bivalent (Fig. 3, Table 3). Only Tyramide-FISH for the gene RIN-2 resulted in multiple signals distributed over all the chromosomes.

Three genes were mapped on the long arm of pachytene chromosome 4. The AAA-2 gene was mapped in the distal part (relative distance of 8.0 ± 1.0 %) of chromosome 4 (Fig. 3c). The MLO3 and P5CS genes were mapped on more proximal positions, with a relative distance of 44.0 ± 1.5 % and 30.0 ± 2.0 %, respectively (Fig. 3b).

Four genes (MLO2, MDAR, MGM and PAL) were mapped on chromosome 7. MGM was located in the distal part of the chromosome (relative distance of 18.0 ± 1.5 %) (Fig. 3d). PAL, MDAR (Fig. 3e) and MLO2 genes were physically mapped in the middle of the long arm of the the pachytene chromosome 7 with relative positions of 46.6 ± 1.0 %, 52.0 ± 1.5 % and 44.8 ± 0.5 %, respectively. Tyramide-FISH with pairs of the genes (MGM + MLO2, MLO2 + PAL and PAL + MDAR) confirmed the order and location of all genes on the same chromosome.

Tightly linked genes can be distinguished using high resolution multicolor Tyramide-FISH

Because PAL and MLO2 are very closely located on chromosome 7, Tyramide-FISH with PAL and MLO2 resulted in one large signal. Therefore, to determine the order of PAL and MLO2 genes on chromosome 7, multicolor Tyramide-FISH was applied (Fig. 3f). Two-color Tyramide-FISH revealed the order of MLO2, PAL and MGM genes on the same chromosome. In 58 % of the observed pachytene cells ($n = 17$) closely located red (PAL) and green (MLO2) signals were observed, while the red and green signals in the other 42 % of pachytene cells partially overlapped. These results suggest that the distance between MLO2 and PAL genes is on the border of the spatial resolution of Tyramide-FISH on pachytene

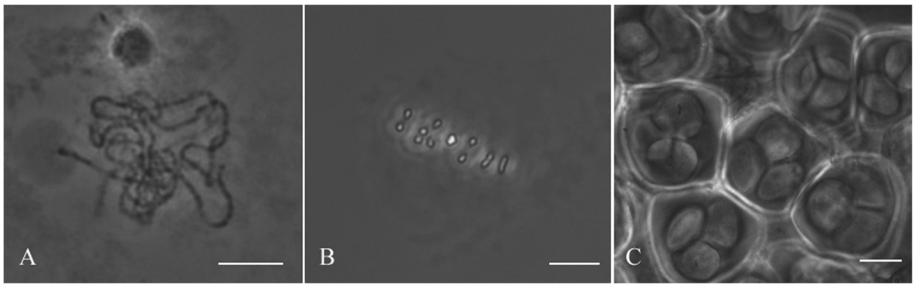

Fig. 1 Pachytene (**a**), Metaphase I (**b**) and Tetrad (**c**) stages found in a *R. wichurana* flower bud. Bars = 10 μm

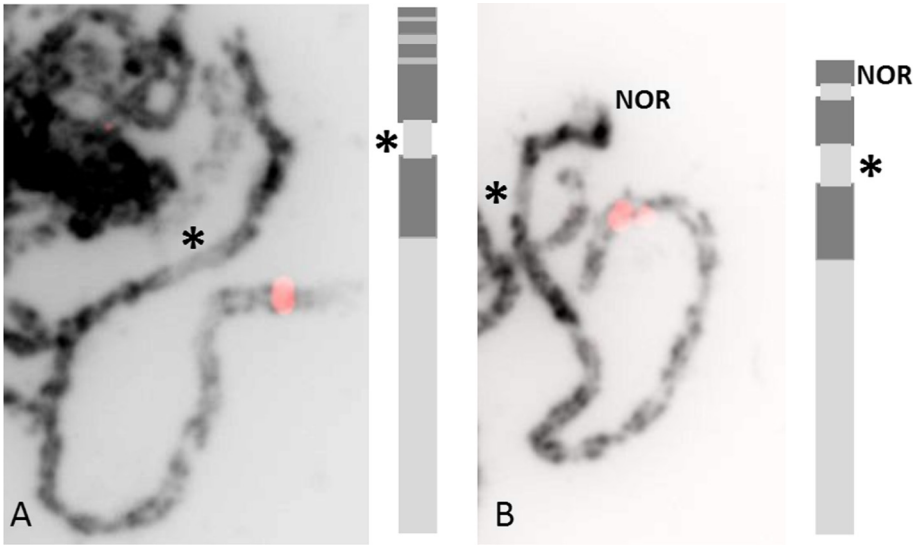

Fig. 2 Inverted DAPI pictures and ideogram of chromosome 4 (**a**) and 7 (**b**). Red signals showed the location of AAA-2 (**a**) and MGM (**b**) genes after Tyramide-FISH to verify the correct chromosome number. Stars indicate the centromere position on the chromosomes

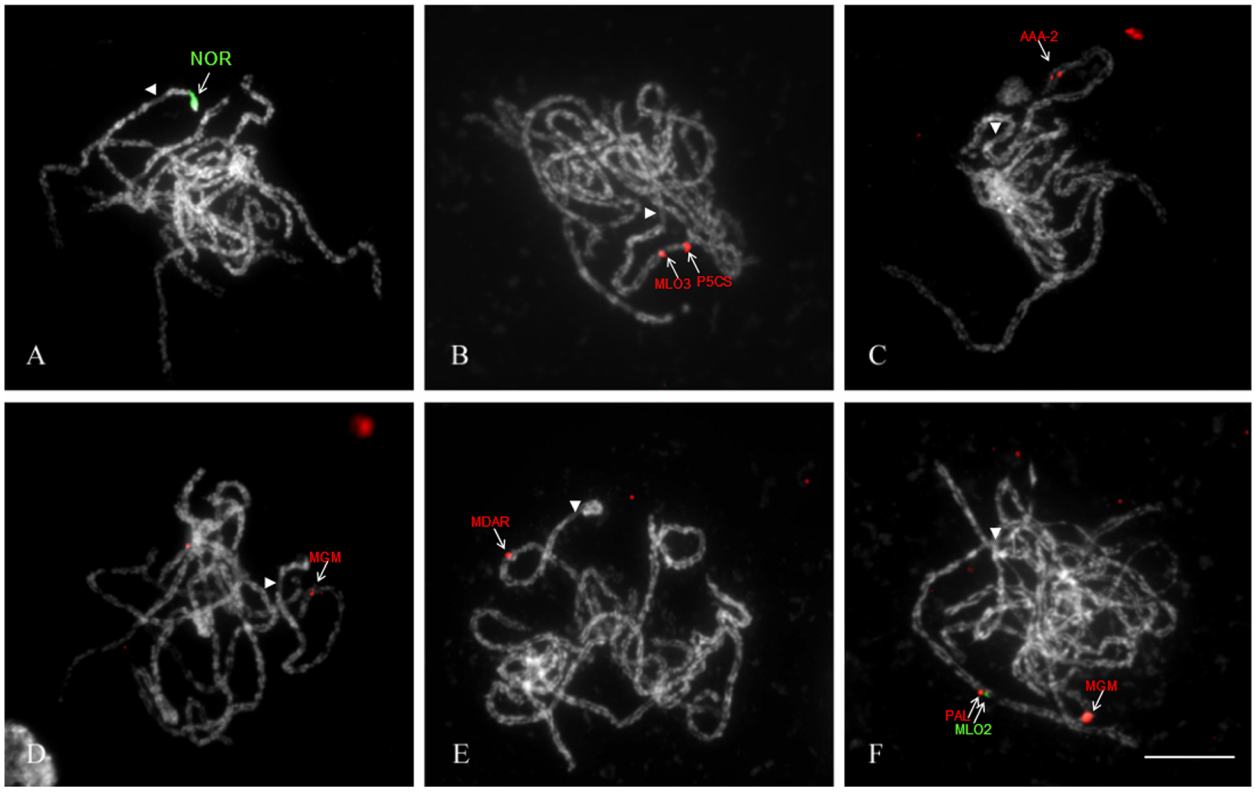

Fig. 3 *In situ* physical mapping of genes on pachytene chromosomes of *Rosa wichurana*. FISH with Dig-labeled 45S rDNA (pTA71 plasmid) (**a**); Tyramide-FISH with MLO3 and P5CS genes, both labeled with biotin (**b**) and with AAA-2 gene, labeled with biotin (**c**); Tyramide-FISH with MGM gene labeled with biotin (**d**) and MDAR gene labeled with biotin (**e**); Sequential multicolor Tyramide-FISH with digoxigenin labeled MLO2 gene and biotin labeled PAL and MGM genes (**f**). Centromere of the chromosome that contains the physically mapped gene(s) is indicated by an arrowhead. Bar = 10 μm

Table 3 Physical location of the target genes on *R. wichurana* pachytene chromosomes

Gene name	Chromosome number/ arm	Location on chromosome arm (%)[a]
MLO3	4/L	44.0 ± 1.5
AAA-2	4/L	8.0 ± 1.0
P5CS	4/L	30.0 ± 2.0
MLO2	7/L	44.8 ± 0.5
MDAR	7/L	52.0 ± 1.5
MGM	7/L	18.0 ± 1.5
PAL	7/L	46.6 ± 1.0
RIN-2	Multiple signals	-

[a]- Distance from telomere of the long arm to the signal × 100 % / length of whole arm

chromosome of *R. wichurana*. Based on the length of the pachytene bivalents carrying non-overlapped signals (41 ± 1.5 μm), the relative length of the mitotic chromosome 7 (10.0 ± 0.1 %; Kirov et al. [47]), the genome size for *R. wichurana* (562 Mb/1C, [52]) and 0.2 μm - resolution limit for light microscope, we may calculate the physical distance between the MLO2 and PAL genes which is about 270 kb. An overview of the genes located on chromosome 4 and chromosome 7 and their order is shown in Fig. 4.

Discussion

Chromosome preparation is a key factor for Tyramide-FISH success

To our knowledge, this is the first report on the application of FISH on pachytene chromosomes of a member of Rosaceae family. Pachytene chromosome preparation using the SteamDrop procedure has number of advantages [47]: 1) After cell suspensions are prepared they can be stored for months; 2) The chromosome preparation takes only 3–5 min from made cell suspensions; and 3) up to 20 slides can be prepared from one flower bud. Here we modified the SteamDrop protocol [47] for easy pachytene chromosome preparation of *Rosa wichurana*. One of the modifications is a final treatment of cells by acetic acid providing better chromosome spreading and

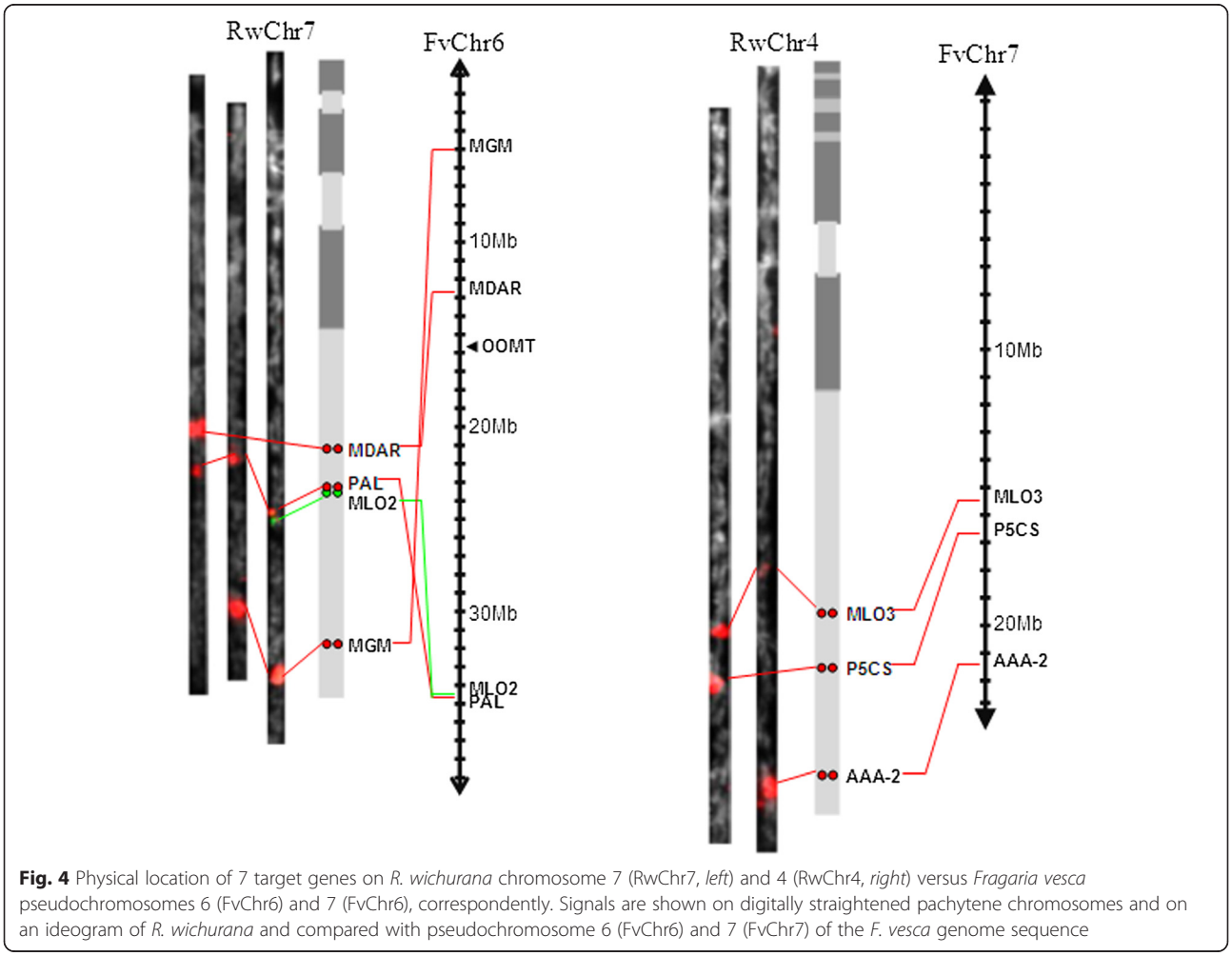

Fig. 4 Physical location of 7 target genes on *R. wichurana* chromosome 7 (RwChr7, *left*) and 4 (RwChr4, *right*) versus *Fragaria vesca* pseudochromosomes 6 (FvChr6) and 7 (FvChr6), correspondently. Signals are shown on digitally straightened pachytene chromosomes and on an ideogram of *R. wichurana* and compared with pseudochromosome 6 (FvChr6) and 7 (FvChr7) of the *F. vesca* genome sequence

Tyramide-FISH results. The time of treatment with acetic acid significantly influences the Tyramide-FISH results. Over-treatment as well as insufficient treatment resulted in a manifold reduction of signal frequency. PMCs require an optimal time of acetic acid treatment for reducing the amount of cytoplasm and the thickness of the organic layer covering the chromosomes [53]. Overtreatment of chromosomes by acetic acid results in histone elimination [54] and chromosome becomes flatten which reduces the chromatin accessibility [55] and the amount of electron-rich amino acids (e.g., tyrosine, tryptophan, phenylalanine) required for tyramide anchoring after HRP activation [56]. Therefore the chromosome preparation procedure is the most important step in high resolution physical mapping using Tyramide-FISH and should be optimized first to obtain satisfactory results.

Resolution of FISH on pachytene chromosomes of R. wichurana

Mitotic chromosomes of *Rosa wichurana* are very small, ranging from 2.2 to 3.7 μm in length [29]. Their small size leads to a lower resolution (5–5.5 Mb) when using FISH, which hampers the use of FISH for physical mapping in *R. wichurana*. In contrast, pachytene chromosomes of *R. wichurana* are up to 20 times longer than mitotic chromosomes, which is comparable with data obtained on banana [41], *Arabidopsis* [39], tomato [57], *Medicago truncatula* [32] and rice [58]. Based on the genome size for *R. wichurana* (562 Mb/1C, [52]), 0.2 μm - resolution limit for light microscope and total length of pachytene chromosomes (235–411 μm) we can conclude that the spatial resolution of FISH mapping on *R. wichurana* pachytene chromosomes is 270–500 kb. Pachytene stage and location of probe in euchromatin or heterochromatin region influence on FISH resolution [14]. Resolution of FISH is much higher for zygotene, leptotene and early pachytene. For example the order of partially overlapped BAC clones can be determined on early pachytene chromosomes [35]. DNA condensation varies significantly along a pachytene chromosome – it is highly condensed in heterochromatin regions and less condensed in euchromatin regions [30, 59]. For example, the FISH resolution in the euchromatic regions of tomato pachytene is 10 times higher than in heterochromatin regions [30]. For *Rosa wichurana*, clear pericentromeric heterochromatin was observed on all pachytene chromosomes but also a number of weak stained heterochromatin bands were identified in euchromatic parts. Therefore, further study is necessary in order to estimate the precise resolution of FISH in heterochromatin and euchromatin of pachytene chromosomes of *R. wichurana* to convert microscopic distance into base pairs.

Advantages and limitations of Tyramide-FISH for high-resolution physical mapping

Tyramide-FISH on pachytene chromosomes resulted in a higher signal frequency compared to mitotic chromosomes. We were able to visualize the signals on 70 % of the pachytene spreads. This is much higher than reported for Tyramide-FISH on mitotic chromosomes [29, 60]. Moreover, Tyramide-FISH allows physical mapping of short DNA fragments. Gene fragments with a length as small as 1.7Kb were successfully visualized on pachytene chromosomes of *R. wichurana*. Because most of the genes are free of repetitive DNA it is not necessary to use blocking DNA (e.g. $C_o t$ fraction) for physical mapping as is the case for physical mapping by BAC-FISH [61]. The efficiency of physical mapping using Tyramide-FISH is high. In this study 7 out of the 8 (87.5 %) isolated genes were successfully mapped on the pachytene chromosomes.

However, Tyramide-FISH have some disadvantages for physical mapping. Tyramide-FISH is highly dependent on the quality of the slide preparation. And another drawback of Tyramide-FISH is that sometimes gene fragments give multiple signals which cannot be reliably physically mapped, e.g., the RIN-2 probe in this study and [62]. In addition, multicolor Tyramide-FISH is a quite time-consuming process because each probe is detected sequentially.

Advantages of an orthology-based probe design

Different strategies can be used for single gene probe design for FISH mapping. One approach is the use of EST [29] or genomic sequences [59, 62] of genes for primer design and further cloning of PCR products. Another strategy is to isolate orthologous gene fragments of one genus based on the whole genome sequence of another closely related genus. This approach is useful for the isolation of genes of which no full-length sequences or mRNA sequences are available in databases. This latter approach allowed for *Rosa wichurana* the design of specific primers and amplification of gene fragments with the predicted size based on the genome of *Fragaria vesca*. Moreover, it allowed the development of probes containing exon-intron fragments of the genes. On the contrary, EST clones often are too short as FISH probes and can contain highly conserved exon sequences which cross hybridize with other members of the same gene family, resulting in multiple signals ditributed along all chromosomes [62].

Macro-synteny between R. wichurana chromosome 4 and 7 and Fragaria vesca pseudochromosomes

In our study, three genes, physically mapped on *R. wichurana* chromosome 4 (RwChr4), showed a perfect collinearity with *F. vesca* pseudochromosome 7 (FvChr7).

The collinearity between *R. wichurana* chromosome 7 and FvChr6, however, is not well established yet. Tyramide-FISH results on mitotic chromosomes showed that OOMT and PAL genes, belonging to the same pseudochromosome FvChr6 of *F.vesca,* were located on two different chromosomes (chromosome 1 and 7) in *R. wichurana* [29]. Previously Gar et al. [48] also found that genes located on FvChr6 are located on two different linkage groups of *R. wichurana*, suggesting an ancient translocation event. Here we physically mapped 3 additional genes located terminal (MLO2 and MGM) and proximal (MDAR) on FvChr6. Our Tyramide-FISH results show that these 3 genes are all located on chromosome 7 of *R. wichurana.* Gathering all results from physical mapping on mitotic [29] and pachytene chromosomes and genetic mapping [48], it can be hypothesized that FvChr6 has a complex evolutionary history since *Fragaria* and *Rosa* were diverged from a common ancestor.

Conclusion

Tyramide-FISH mapping of single-copy genes on pachytene chromosomes opens possibilities for the development of detailed physical maps of *R. wichurana* chromosomes. This approach will assist the integration of physical and genetic maps and will accelerate comparative genomic studies of genera in the Rosaceae family. For further experiments, cytogenetic marker development would be valuable for the identification of all pachytene chromosomes of *R. wichurana*. The application of larger numbers of single-copy gene probes covering all chromosomes of the karyotype of *Rosa* and the construction of the integrated genetic and physical maps for all chromosomes of *R. wichurana* is the scope of our future research.

Availability of supporting data

The partial sequences of the gene fragments used for Tyramide-FISH are available as Additional file 1.

Additional file

Additional file 1: The partial sequences of the clones used for Tyramide-FISH.

Competing interests

The authors declare that they have no competing interests

Authors' contributions

IK: designed and performed the experiments and wrote the paper; KVL and LK conceived of the study, and participated in its design and coordination and wrote the paper. All authors read and approved the final manuscript.

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The Chromosome Organization of Genes and Some Types of Extragenic DNA in *Allium*

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Abstract

We have studied the physical organization of genes and non-coding DNA in two *Allium* species, *A. cepa* and *A. fistulosum*. Expressed sequence tags (ESTs) clones and polymerase chain reaction (PCR) products of gene fragments obtained with primers designed for the gene sequences available in the public GenBank were physically mapped onto chromosomes using a Tyr-FISH technique. The allinase and lacrymatory factor synthase (LFS) genes that encoded enzymes operating in one metabolic way were cloned sequences and physically mapped onto *A. cepa* and *A. fistulosum* chromosomes. The alliinase gene probe (1100 bp) hybridized to the distal region of the long arm of chromosome 4 of *A. cepa.* The LFS gene probe (550 bp) hybridized to the proximal region of the long arm of chromosome 5 in both species. Inter-simple sequence repeats (ISSRs) that are located between SSR loci were used for chromosomal location of microsatellites in *A. fistulosum* using common FISH. The chromosomal organization of the Tyl-co*pia* group retrotransposons were investigated in *A. fistulosum*. Dispersed hybridization of the probe along the chromosome arms apart from telomeric ends was detected. Chromosomal distribution of DNA methylation pattern in *A. fistulosum* L. was studied using a specific antibodies against 5-methylcytosine (anti-5mC). Highly methylated distal regions in all chromosomes were found. Differences in the methylation level between corresponding regions of homologue chromosomes were shown. We describe recent progress in exploiting the ultrasensitive Tyr-FISH technique for development of visual gene maps for chromosomes of *A. cepa* and *A. fistulosum*. The results on the chromosomal location of individual genetic loci aided in assembling physical and genetic maps. We related the physical organization of expressed genes to the contrasting patterns of chiasma distribution and to the organization of repetitive DNA family and highly methylated DNA in these two *Allium* species.

INTRODUCTION

A half century ago we learned that higher eukaryotic organisms possess much more DNA in their genome than they likely need for genetic information (MacLean, 1973). In onion, the difference between gene fraction (3-5% of the genome) and extragenic DNA fraction (93-97%) is extremely high (Flavell et al., 1974; Stack and Comings, 1979). Onion has one of the largest genomes among monocots (16 415 Mbp per 1C nucleus). The extraordinary huge genome of onion delays the construction of genomic resources for this economically important crop. Onion is used in every home, daily. Onion is the second most valued vegetable crop (FAO, 2010). In Russia in 2010, onion was cultivated on 88,000 ha with total onion production mounted 1,536,300 tons (FAO, 2010).

The best way to get a comprehensive insight into chromosome organization of genes and extragenic DNA is the genome sequencing of species. With the development of second generation sequencing techniques that are capable of sequencing thousands of

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millions of nucleotide bases in each run and the introduction of third generation single-molecule sequencing methods, it is possible to sequence large and complex genome of crop plants. However, a BAC library of onion would require an enormous number of clones for sequencing. To overcome this problem some approaches were proposed: (i) sequencing of methyl-filtered genomic clones to enrich for genic regions in onion or (ii) sequencing of close relative species with smaller genome (Harvey et al., 2008). Japanese bunching onion (*A. fistulosum*) is the best candidate for sequencing because it has a 28% smaller genome (Ori et al., 1998) and may contain gene-rich euchromatic regions proximal to the centromeres (Khrustaleva et al., 2005). In other plant crops (*Arabidopsis*, rice, tomato) sequencing was successful by using the BAC-by-BAC approach. This was based on exploitation of the existing genetic maps (Arabidopsis Genome Initiative, 2000; International Rice Genome Sequencing Project, 2005; Muller et al., 2005). However, BAC contig building by using a genetic map cannot always be determined unequivocally, especially in genomic regions, in which, recombination is suppressed (Szinay et al., 2008). Knowledge of the chromosomal organization of genes and non-coding DNA may dramatically increase the speed and cost-effectiveness of genome sequencing for crop improvement.

Fluorescence in situ hybridization (FISH) is a powerful tool for structural, comparative and functional genomics that places plant cytogenetics in a unique position to complement, accelerate, or guide plant-genome research (Figueroa and Bass, 2010). FISH offers an effective method for sequence localization on physical chromosomes (Suzuki et al., 1991; Lamb et al., 2007) characterization of complicated regions such as centromeres (Nagaki et al., 2004; Jin et al., 2005) and integration of physical and recombination maps (Khrustaleva et al., 2005; Danilova and Birchler, 2008; Szinay et al., 2008).

In order to characterize the chromosome organization of genes and some types of extragenic DNA in *Allium* we mapped EST clones, PCR-products of gene fragments from the public GenBank, microsatellites, Ty1-*copia* group retrotransposable elements and methylated DNA onto physical chromosomes using molecular cytogenetic methods including FISH, Tyramide-FISH and immunochemical detection with a specific antibody against 5-methylcytosine.

RESULTS AND DISCUSSION

Feature of Onion Genome

The onion genome contains 32% GC nucleotides, which is the lowest amount among known angiosperms (Kirk et al., 1970), whereas *Allium* possess GC-rich unique telomeric repeats (Adams et al., 2001; Fajkus et al., 2005). However the content of GC nucleotides in onion ESTs (Expressed sequence tag) is considerably lower than the GC-content in coding regions of other monocots belonging to the Poales (Kuhl et al., 2004).

Gene Chromosomal Mapping

We physically mapped onto chromosomes *A. cepa* and *A. fistulosum* expressed sequence tags (ESTs) clones and PCR products of gene fragments obtained with primers designed on gene sequences available in the GenBank. To visualize a short DNA sequence on the physical plant chromosome we used the ultrasensitive Tyr-FISH method (Khrustaleva and Kik, 2001). The Tyr-FISH technique combines the advantage of an enzymatic procedure that provides signal amplification due to the deposition of many substrate molecules, and that of fluorescence-based detection, which is higher than absorbency used in enzymatic detection.

EST clones from the *A. cepa* bulb cDNA library were kindly provided by Prof. M. Havey (the University of Wisconsin-Madison, USA) and by Dr. A. Bovy (Plant Research International, Wageningen University and Research Centre, The Netherlands). The EST library was subdivided into 25 hybridization mixtures, consisting of 25 random EST clones and hybridized to *A. cepa* and *A. fistulosum* chromosomes. A total of

620 clones obtained from the onion bulb cDNA library were analyzed. 80.1% of EST hybridization sites in *A. cepa* were detected in distal and interstitial regions. In *A. fistulosum* 80.9% of ESTs were located in proximal and interstitial regions. Recent research on the organization and evolution of wheat genomes definitely proved that gene density and recombination rate correlate (Akhunov et al., 2003). In *A. cepa* recombinations occurred predominantly in interstitial and distal locations whereas in *A. fistulosum* they were tightly clustered around the centromere (Albini and Jones, 1988). The chromosomal location of ESTs coincided with our results on integration of recombination and chromosome maps (Khrustaleva et al., 2005). The integrated maps showed that in *Allium* 57.9% of *Pst* I/*Mse*I markers were located in close proximity to the centromeric region, suggesting the presence of genes in this region. It was experimentally confirmed that *Pst*I/*Mse*I markers were predominantly located in gene-rich regions (Burr et al., 1988; Michalek et al., 1999). However, on the basis of the number of EST clones analyzed (620 clones) we can only draw partial conclusions concerning the organization of expressed DNA in the *Allium* genome. Also we can only refer to in situ hybridization sites, because all of the fluorescent signals we found on metaphase chromosomes represented expressed genes. Some may be pseudogenes or other silent copies of expressed genes. A fluorescent probe does not distinguish between expressed or non-expressed sequences.

We obtained multiple-valued results with Tyr-FISH mapping of individual EST clones. Some clones produced one twin-signal on both homologous chromosomes while other clones did not reveal an expected unique hybridization site on the chromosome. EST clone API66 was mapped previously into linkage group assigned to chromosome 5 (Martin et al., 2005). Tyr-FISH mapping of API66 (Fig. 1A) showed 1-3 hybridization signals on each chromosome of onion complement. This points to the DNA sequence presence comprising an 80% homology to the probe (stringency of hybridization – 78% and stringency of washing – 80%) that are scattered through the onion genome. BLASTX analysis of API66 showed 66% identity to genes encoding sucrose transporter, 5'-partial [*Oryza sativa* Japonica Group]. The sucrose transporter belongs to a large enzyme family. Searching in the NCBI database showed that sucrose transportase genes were located in a number of loci almost on each chromosome of *Arabidopsis thaliana* and *Oriza sativa* – species with fully sequenced genomes (http://www.ncbi.nlm.nih.gov/mapview). API81 EST clone mapped on chromosome 6 of the genetic map (Martin et al., 2005) also revealed multi-signals in Tyr-FISH experiment (Fig. 1B). API81 possesses a transcript with high similarity to peroxidase 12 precursor. Analysis of the *A. thaliana* database (http://www.ncbi.nlm.nih.gov/mapview) showed a comparable pattern of peroxidase gene distribution on the chromosomes. The EST clone API59 hybridized to onion metaphase chromosomes (Fig. 1C) showed a strong twin-fluorescent signal on two homologues of chromosome 5. This corresponded to the position of API59 on the genetic map (Martin et al., 2005).

We used another strategy for chromosomal gene mapping. We cloned, sequenced and mapped the genomic DNA sequences of both allinase and lacrymatory factor synthase (LFS) genes. These encode enzymes operating in one metabolic way produced compounds responsible for onion's characteristic flavour.

The allinase gene primer set was designed on a region including two exons (3 and 4) and three introns (2, 3 and 4) of the genomic DNA clone (L48614.1 GenBank) and PCR was carried out with genomic DNA of both *A. cepa* and *A. fistulosum* (Kirov et al., 2011). The PCR product with expected size (1100 bp) was cloned and sequenced. BLASTN sequence analysis of clones possessing 1100 bp alliinase gene fragments from *A. cepa* showed identity to the nucleotide sequence of the bulb alliinase gene of *A. cepa* from GenBank (L48614. 1, Gilpin et al., 1995). BLASTN analysis of the All3(a,b,c) clones that possessed PCR products of *A. fistulosum* revealed 91% identity to the sequence of bulb alliinase of *A. cepa* (L48614. 1) and 73% identity to the cDNA clone of the allinase gene of *A. fistulosum* (AF409954. 1) that expressed in roots. Phylogenetic tree based on the multiple alignments of the conservative exon 3 sequences of alliinase gene

was constructed using neighbor-joining method. It was found that All3 was in the same cluster with the bulb alliinase of *A. cepa* (Fig. 2). Our knowledge about alliinase and gene encoded alliinase in *A. fistulosum* is scant. Until now in the GeneBank only one sequence of the *A. fistulosum* alliinase mRNA was present. Thus, our finding indicated that most probably we isolated the exon-intron fragment of a novel bulb alliinase gene in *A. fistulosum*.

Tyr-FISH mapping the 1100 bp allinase gene fragment of *A. cepa* showed strong hybridization signals in the distal region of chromosome 4 of the *A. cepa* mitotic metaphase (Fig. 1D). Previously a bulb alliinase gene was mapped in the linkage group A of a low-density genetic map (King et al., 1998). The authors revealed two loci of the alliinase markers located in a distance of 6.9 cM using a cDNA clone of the enzyme alliinase (cysteine sulphoxide lyase;EC 4.4.1.4) constructed by van Damme et al. (1992). Because van Damme et al. (1992) detected only one alliinase transcript in onion shoots the presence of two loci was explained by expression of an identical transcript in other tissues, or by existence of pseudogenes. Van Heusden et al. (2000) using a linkage map of *A. cepa* based on the AFLP linkage data from an interspecific cross between *A. cepa* and *A. roylei* mapped an alliinase gene in the AFLP linkage group assigned to the chromosome 4 of *A. cepa* via monosomic addition lines. Later Martin et al. (2005) reported construction of the onion intraspecific cDNA map where its linkage groups were assigned to chromosomes using alien addition lines of *A. fistulosum* L. carrying single onion chromosome. In the map two alliinase gene loci were located in an unassigned group. Analysis of the two genetic maps produced by King et al. (1998) and Martin et al. (2005) showed the presence of common markers (AOB77-E5-8.0, API61-E1-3.0, D03-0.7 and API55-E5-9.0/15.0) for linkage group A and chromosome 4. Thus, taking in account the result of the Tyr-FISH mapping of the alliinase clone we may assume that the unassigned linkage group possessing the alliinase loci belongs to chromosome 4. Future experiments on the Tyr-FISH mapping of other markers located on chromosome 4 may help to clarify the discussion.

The LFS gene primer set was designed on cDNA sequence (GenBank accession AB089203). Two specific primer sets were constructed: one primer set (LFS1) allowed to amplified LFS gene only in *A. cepa* and another one (LFS2) gave a PCR product in both species (Kirov et al., 2011). The PCR products were cloned and sequenced. A multiple alignment of LFS amplicons showed 15 single nucleotide substitutions within the *A. cepa* genome. This may be because DNA has been isolated from the mix of seedlings. 18 single nucleotide substitutions between *A. cepa* and *A. fistulosum* were found but some of them may represent PCR errors because the LFS sequence variants were supported by a single read. The LFS gene probe (570 bp) obtained with the LFS2 primer set was used in Tyramide-FISH experiments. A fluorescent signal arisen from the LFS hybridization site was revealed on the proximal region of the long arm of chromosome 5 in both *A. cepa* and *A. fistulosum*. The same results were obtained previously with the BAC probe bearing LFS genes using conventional FISH (Masamura et al., 2012).

Extragenic DNA Mapping

The importance of extragenic DNA is becoming increasingly apparent – chromosome folding, gene regulation and protein evolvability. Extragenic DNA plays an important role in the generation of the new patterns of variability and in speciation. A high level of polymorphism makes extragenic DNA a rich source for marker assisted selection.

Simple sequence repeats (SSRs) are major components of many plant genomes and could be good cytogenetic markers for individual chromosome identification. Six ISSR-PCR primers were used for amplification of inter simple sequence repeats in *A. fistulosum*. The most abundant PCR product was obtained with K10 $[AC]_8YG$. We examined its chromosomal distribution patterns by fluorescent in situ hybridization (FISH). The probe produced a scattering hybridization signal of different intensity along all chromosomes. In chromosome 2, a rich pattern in the proximal region of the short arm

was observed (Fig. 3). This strong fluorescent signal was found in almost all metaphases of both homologous chromosomes 2. The distinctive hybridization pattern showing characteristics specific to both homologous chromosomes might be applicable as markers for chromosome identification.

Retrotransposons of the Ty1-*copia* group are ubiquitous in plants (Flavell, 1992; Kumar, 1996; Heslop-Harrison et al., 1997). In the *A. cepa* genome there are 100,000-200,000 copies of Tyl-*copia* group retrotransposons (Pearce et al., 1996). Here data are presented from in situ hybridization of Ty1-*copia* group retrotransposable elements to *A. fistulosum* chromosomes. The probe DNA for the FISH experiment was obtained by PCR using primers for the Ty1-copia reverse transcriptase gene. Dispersed hybridization of the probe along the chromosome arms apart from the telomeric ends was detected (Fig. 3). This agrees with data obtained for *Vicia faba* whose chromosomes contain no Ty1-*copia* elements in telomeric heterochromatin (Fuchs et al., 1994). Pich and Schubert (1998) also reported that a cloned sequence of the highly conserved reverse transcriptase region of Ty1-*copia* elements hybridized rarely to the chromosome telomeric end of *A. cepa.* This research showed that cloned *En/Spm*-transposable element-like sequence frequently hybridized to telomeric ends of *A. cepa*. In contrast, in situ hybridization of Tyl-*copia* retrotransposon sequences isolated from *A. cepa* by PCR using degenerate primers corresponding to two conserved domains of the reverse transcriptase gene to *A. cepa* metaphase chromosomes revealed that Tyl-*copia* retrotransposons were distributed throughout the euchromatin of all chromosomes and were enriched in the terminal heterochromatic regions (Pearce et al., 1996). In our previous work we amplified a 100-bp fragment with PCR using the satellite primer and primer of the reverse transcriptase fragment of retrotransposon Ty1-*copia* (Fesenko et al., 2002)**.** This fragment was dispersed throughout the chromosome, with predominant location in the terminal heterochromatin. It was shown depletion of Ty1-*copia* group retrotransposable elements in regions with frequently large blocks of tandem repeats, for instance, at the centromeres and major rRNA gene loci in barley (Heslop-Harrison et al., 1997) or in sub-telomeric regions as in rye (Pearce et al., 1997). In contrast, in *Arabidopsis* the retroelements tend to be clustered in the centromeric DNA (Heslop-Harrison et al., 1997). All things taken together, it should be possible to learn more about the evolution and distribution mechanisms of Ty1-*copia* group retrotransponsons in *A. cepa* and *A. fistulosum*, both agronomically important close relative species.

DNA Methylation

Despite DNA methylation being an epigenetic process, and reflecting the structure and functional activity of chromosomes, analysis of the methylation site distribution in human and animal genomes showed the presence of stable methylation sites (Miller et al., 1974; Schnedl et al., 1976). It is known that plant DNA is highly methylated (Law and Jacobson, 2010). The level of DNA methylation in plants is 10 times higher than found in vertebrates (Doerfler, 1981; Erlich and Wang, 1981). The origin and function of such a high levels of methylation in plants are not clear. Plant genes may be methylated at both adenine and cytosine residues; specific adenine DNA-methyltransferase was described (Vanyushin, 2006). Chromosomal distribution of DNA methylation patterns in *A. fistulosum* L. was studied using a specific antibody against 5-methylcytosine (anti-5mC) and immunochemical detection with the FITC fluorochrome. Analysis of the frequencies and intensity of the fluorescent signal in distal, interstitial and proximal regions of each chromosome arms revealed highly methylated distal regions in all chromosomes (Fig. 4). A lower percentage of methylation was observed at the proximal and interstitial regions. Our results also revealed differences in the methylation level between corresponding regions of homologue chromosomes. Such regional differences between homologues regarding anti-5mC binding may reflect different transcriptional activities. In *A. cepa* metaphase chromosomes a preferential binding of anti-5mC was found on telomeric regions but was also evident in the proximal regions of almost all chromosome arms and in some intercalary bands (Castiglione et al., 1995). Recently Suzuki et al. (2010) using

immunodetection of 5mC and in situ nick-translation analysis showed that *A. cepa* chromosomes were highly methylated and the methylated CG dinucleotides were distributed on all chromosomes. It is worth noting that the information about the distribution of the methylated sites may be useful for understanding better of the relationships between these close *Allium* taxa. Moreover, the importance of DNA methylation in the intergeneric distant hybrids stabilization was recently reported by Li et al. (2010).

CONCLUSION

This study shows the successful mapping of genes on physical chromosome, including the alliinase and LFS genes that operating one metabolic pathway. Our major focus is now on the development of a high sensitive in situ hybridization method (HsTyr-FISH) for a reliable visualization of a small DNA sequence on plant chromosomes to map a number of genes and molecular markers and integration of chromosomal and genetic maps. We are also constructing a BAC library of *A. fistulosum* to aid in creation of cytogenetic markers. Study will continue of the distribution of repetitive DNAs along chromosomes, which are crucial elements for understanding the organization and evolution of plant chromosomes and *Allium* crop improvement.

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Figures

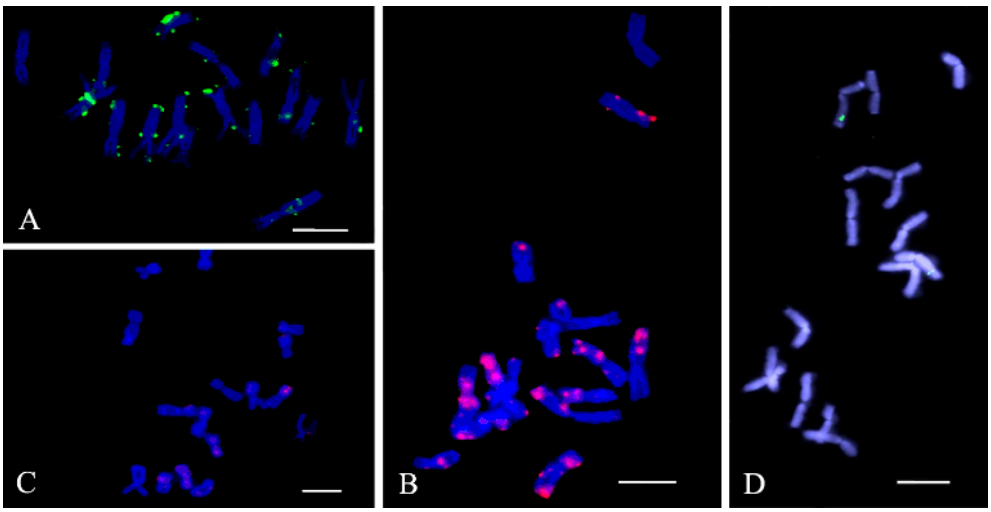

Fig. 1. Tyramide-FISH with ESTs and ALL3 clone on mitotic metaphase chromosomes of *A. cepa*: A. API66; B. API81; C. API59; D. ALL3 (probes were labelled with Biotin-16-dUTP or Dig-11-dUTP and detected with Tyramide-Cy3 (red) or Tyramide-FITC (green), respectively and contrastain with DAPI). Bar is 10 μm.

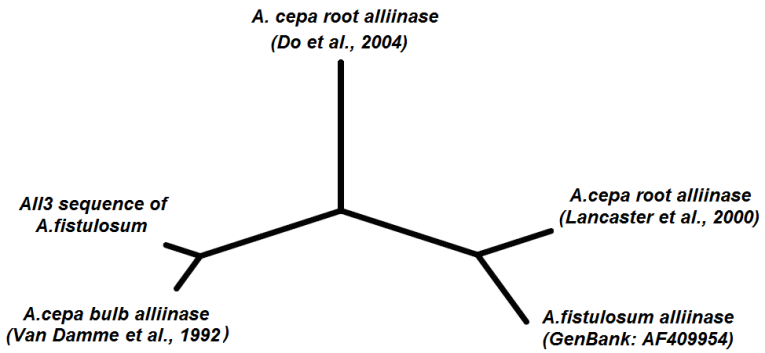

Fig. 2. Phylogenetic tree of the alliinase – multigene family constructed by multiple alignment of the exon 3 nucleotide sequences using CLUSTAL W program (http://www.genome.jp).

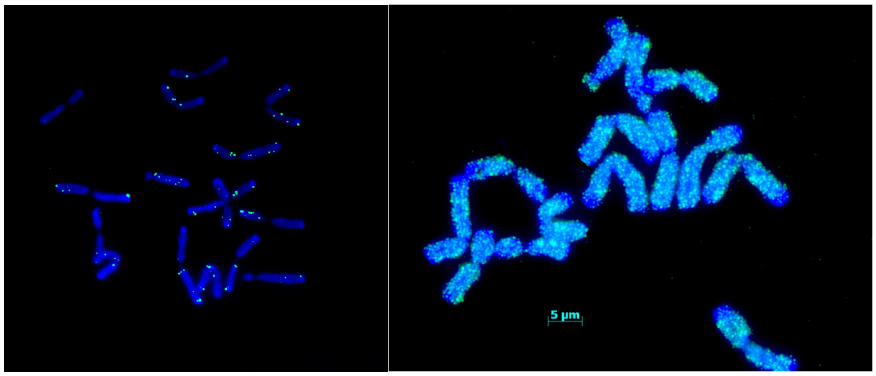

Fig. 3. FISH with the inter simple sequence repeat (primer [AC]8YG; left) and Ty1-copia retotransposons (right) on mitotic metaphase chromosomes of *A. fistulosum*. Green fluorescence – sites of the probes hybridization.

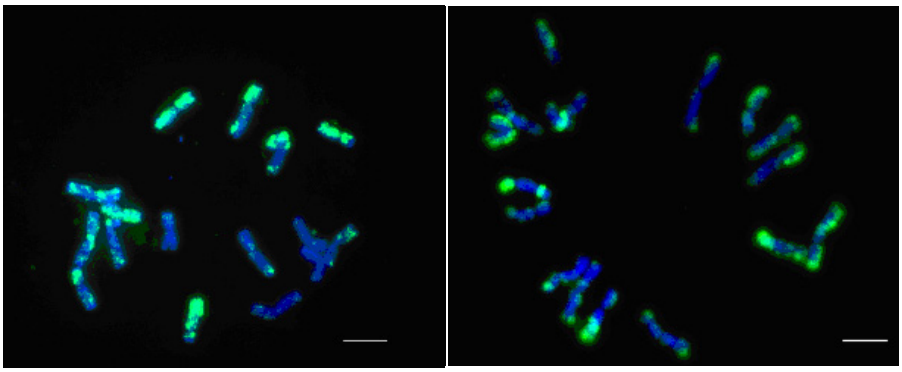

Fig. 4. Chromosomal distribution of the DNA methylation pattern in *A. fistulosum* (left) and *A. cepa* (right). Green fluorescence – 5mC rich region. Bar is 10 µm.

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PART III: GENERAL DISCUSSION AND FUTURE PERSPECTIVES

1. CHROMOSOME PREPARATION QUALITY AS THE KEY FACTOR IN TYRAMIDE-FISH REPRODUCIBILITY

Our experiments on rose pachytene chromosomes showed that chromosome preparation in general and the acetic acid treatment (concentration and duration) more specifically, significantly influences the results of Tyramide-FISH (paper 5). There are two reasons that can explain these results. First, chromosomes that are prepared using a low concentration of acetic acid (15 – 25%) or/and a shorter time of final acetic acid treatment (pachytene chromosomes) have less damaged chromosome structures. As shown by atomic force microscopy, the chromosome structure is significantly affected by acetic acid treatment (Sugiyama et al., 2004). In that study, the diameter of chromosomes was larger and the 3D structure of chromosomes was better preserved when a low concentration of acetic acid was used. Based on this, it can be assumed that the application of lower concentrations of acetic acid for chromosome preparation results in a better chromatin accessibility and, as a consequence, in higher Tyramide-FISH signal frequencies. Especially when short DNA sequences are used for FISH, chromatin accessibility is crucial (Jiang and Gill, 2006). Second, the nature of the tyramide-HRP reaction has an influence on the signal frequency across the chromosomes on the slide. After oxidation by HRP, the tyramide molecules bind with electron-rich molecules such as tyrosine, phenylalanine or tryptophan (Bobrow et al. 1991). The source of these molecules on the slides is not known. Our hypothesis is that the chromosomal associated proteins such as histones that contains these amino acids (Table 1) are the main source of electron-rich amino acids required for tyramide deposition after their reaction with HRP.

Table 1. The number of electron-rich amino acids (tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe)) in H2A, H2B, H3 and H4 histones.

	H2A	H2B	H3	H4
Tyr	2	3	3	4
Trp	-	-	-	-
Phe	1	4	4	2

It is shown that the acetic acid treatment reduces the amount of histones in the chromosomes (Dick and Johns, 1968; Sugiyama S. et al. 2004). If our hypothesis is correct, a prolongation of acetic acid treatment of the chromosomes or increasing the concentration of acetic acid during chromosome

preparation will result in a reduced Tyramide-FISH signal frequency. Indeed, the long (60 sec) treatment of *R. wichurana* pachytene chromosomes with acetic acid resulted in a reduction of the Tyramide-FISH signal frequency across chromosomes (Paper 5) by 4 times compared with the shorter duration of acetic acid treatment (30 sec). We also observed a significant decrease in the signal frequency value when chromosomes were prepared using high concentration of acetic acid in the spreading solution compared to a low concentration of acetic acid (15% vs 100% acetic acid in ethanol, data not shown). Thus these results are in concordance with our hypothesis. Surprisingly, too short treatment of the chromosomes by acetic acid (15 sec) also resulted in decreasing value of signal frequency (Paper 5). Probably, lowering the duration of acetic acid treatment results in an inappropriate digestion of cell wall debris and organic layer, which can cover the slide surface after the chromosome preparation procedure (Martin et al., 1994) and negatively influences the FISH results via increasing background signals and decreasing the chromatin accessibility (Martin et al., 1994). Based on our experiments carried out on *Rosa wichurana* and Allium chromosomes we propose a model in which all the observed effects of the acetic acid treatment on the Tyramide-FISH signal frequency are explained (Figure 2).

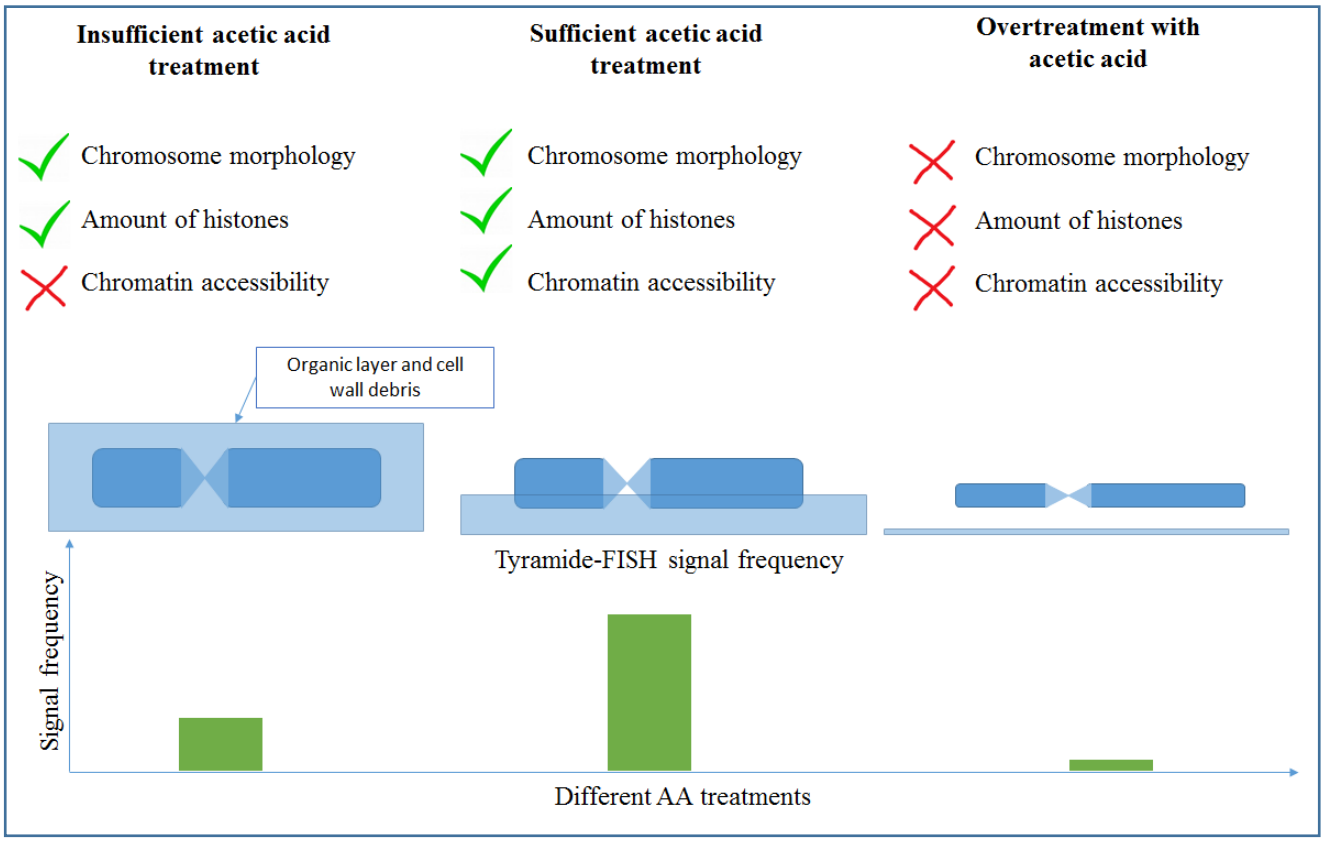

Figure 2. Theoretical model explaining the effect of acetic acid (AA) treatment on Tyramide-FISH results. Green bars show average signal frequencies in experiments with different AA treatments. In the top of the figure the observed (chromosome morphology) and hypothetical (amount of histones, chromatin accessibility) characteristics are specified under different acetic acid treatments.

According to this model, results of Tyramide-FISH (in terms of signal frequency) rely on the balance between chromatin accessibility and amount of histones as a source of electron-rich molecules. Finding the optimal concentration and duration of acetic acid treatment for chromosome preparation is a key factor in achieving the optimal balance. The "SteamDrop" protocol that we have developed, allows easy adjustment of acetic acid concentration and duration of acetic acid treatment during slide preparation.

In future research enrichment of the chromosome surface by an artificial coupling of the electron-rich molecules (e.g. by reactions of chromosomal proteins with 3-p-Hydroxyphenyl propionic acid N-hydroxysuccinimide ester) can be used to improve Tyramide-FISH sensitivity. Such reaction was already successfully applied for tyramide-HRP reaction to increase the sensitivity of Dot-ELISA (Bhattaharia et al. 1999)).

2. INCREASING SIGNAL FREQUENCY IS STILL THE BIGGEST CHALLENGE FOR TYRAMIDE-FISH

One of the serious limitations of Tyramide-FISH is the low signal frequency ((the number of chromosomes with signals x 100)/total number of chromosomes) across chromosomes. This value strongly depends on chromosome preparation and the probe length and decreases dramatically when the probe length is less than 1Kb (Table 2). According to Khrustaleva et al. (2016) signal frequency for probes of 5.5 Kb, 2.3 Kb and 800bp was 85%, 43% and 15%, respectively. In our research on rose mitotic chromosomes, a minimum signal frequency of 30% was obtained for a probe length of 1100bp (OOMT gene) (Paper 2). On average, the obtained Tyramide-FISH signal frequencies for *Allium*, rose and other species are comparable (Table 2).

Table 2. Comparison of signal frequencies after Tyramide-FISH with different types of probes on different plant genera

Species	Detection system	Tyramides used	The shortest probe length, bp	Signal frequency	Reference
Allium cepa	Direct	Tyramide-FITC	710	36.5%[1]	Khrustaleva and Kik, 2001;
Allium cepa	Direct	Tyramide-FITC	800	15%	Khrustaleva et al., 2016
Triticum aestivum	Indirect	Tyramide-biotin	2000	37.5%	Perez et al. 2009
Avena sativa	Direct	Tyramide-Cy3	2000	>37.5%	Sanz et al., 2011
Rosa wichurana (mitotic chromosomes)	Indirect	Tyramide-biotin	1100	>30%	Kirov et al. 2014 (paper 2)
Rosa wichurana (pachytene chromosomes)	Indirect	Tyramide-biotin	1100	60-70%	Kirov et al. 2015 (paper 5)

[1] – CCD camera was used to increase sensitivity (Khrustaleva and Kik, 2001)

Probes with a length around 1-2 Kb have an average signal frequency of 30-40%. Although these values are not high, they are sufficient for routine mapping of short probes using Tyramide-FISH. Our experiments performed on rose pachytene chromosomes demonstrated that a signal frequency of up to 60% can be obtained for probes of 1-2Kb on high quality pachytene chromosomes.

3. EFFICIENT PROBE DESIGN AND DETECTION SYSTEM ENHANCE TYRAMIDE-FISH EFFICIENCY

Our experiments on *Allium* and Rose chromosomes (Paper 5) demonstrate that not all gene-derived probes are suitable for Tyramide-FISH. On average, 70-80% of the probes that we used can be cytogenetically mapped by Tyramide-FISH. Other probes resulted in multiple signals spread over all the

chromosomes. Similar results were obtained when EST clones were used for mapping on *Allium cepa* chromosomes (Khrustaleva et al., 2012). Gene-derived probes may also contain repetitive DNA and/or conserved motifs for multiple genes resulting in cross-hybridization. As Tyramide-FISH is a very sensitive technique, the probe design has to be carried out using bioinformatic filters to avoid all potential sources for cross-hybridization. If the genome sequence is available a BLAST search with the target sequence can be used to identify multiple copy and repetitive sequences. If no genome sequence is availvable, a BLAST search for similarity across different databases (e.g. NCBI (www.ncbi.nlm.nih.gov), phytozome (phytozome.jgi.doe.gov/pz/portal.html) and repbase (www.girinst.org/repbase/index.html)) can be helpful to identify probe regions that may cause multiple Tyramide-FISH signals. Another filter that can be used are the k-mer based tools (e.g. Kmasker (Schmutzer et al., 2013)) for the identification of regions with a high probability of being repetitive. The results obtained on *Allium* chromosomes (Paper 5; Romanov et al., 2015) suggested that using gene fragments containing both exons and introns as a probe for Tyramide-FISH results in less cross-hybridization compared to using EST clones containing only exons of the target gene as probe. Efficient probe design strategy can make Tyramide-FISH more efficient for physical mapping.

To detect the probe hybridization both direct and indirect Tyramide-FISH detection systems have been used in plants (Table 2). We found that for *Allium* chromosomes both detection systems are applicable for gene mapping but the protocol for direct detection is shorter and requires less reagents. However, no clear signals have been obtained when the direct detection system was applied for *Rosa* chromosomes (Paper 2). Only the indirect detection system with increased concentration of streptavidin-Cy3 allowed to detect hybridization of unique single-copy probes on *R. wichurana* chromosomes. These results demonstrate that optimization of the detection system is required when Tyramide-FISH is applied for chromosomes of other species. The protocols for Tyramide-FISH detection designed in this thesis can be a valuable starting point for Tyramide-FISH application in other species.

4. INCREASED SPATIAL RESOLUTION (SR) OF PHYSICAL MAPPING BY TYRAMIDE-FISH

Previously Tyramide-FISH was only applied for mitotic chromosomes. Although mitotic chromosomes are easy to obtain they provide low spatial resolution (SR) due to high chromatin compaction resulting in 20 times less resolution (around 5–5.5 Mb (Paper 5)). Using pachytene chromosomes for Tyramide-

FISH increases spatial resolution (SR) of physical mapping (around 270Kb). Application of multicolor Tyramide-FISH on pachytene chromosomes further increases SR because even overlapping signals from two neighbour genes located closer than 270Kb from each other can still be distinguished using different colors. To achieve higher SR, less compact chromosomes can be used such as stretched pachytene chromosomes (Koo and Jiang, 2009). Recently this approach has been used to map the Ms (onion male sterility) locus on onion chromosomes (Khrustaleva et al., 2016). Extended DNA fibers allow further increase of SR up to a few Kb (de Jong et al., 1999; Jiang and Gill, 2006). However, Tyramide-FISH optimization and application on extended DNA fibers is probably useless because the sensitivity of fiber-FISH is already high enough (0.7Kb, de Jong et al., 1999). Combination of Tyramide-FISH and fiber-FISH can be a powerful approach to resolve the order of closely located signals and map them to the chromosomes.

5. GUIDELINES FOR TYRAMIDE-FISH OPTIMIZATION AND APPLICATION IN OTHER PLANT SPECIES

Our study shows that for efficient visualization of single-copy genes by Tyramide-FISH three key steps need optimization when starting an experiment in a new plant species or plant genus: chromosome preparation, detection system and probe design. Of them preparation of high quality chromosome slides has the most significant influence on the Tyramide-FISH signal frequency.

We propose following guidelines for different optimization steps in the Tyramide-FISH protocol, based on the observations during this PhD study:

(1) Chromosome preparation. Optimization of the concentration of acetic acid in the solutions (first drop and second drop) used for chromosome spreading (e.g. 6:1/3:1, 3:1/1:1) as well as duration of the last acetic acid treatment step (30 - 45 sec) is needed. For the first time, it is recommended to prepare several slides (e.g. 5 slides, Figure 3B) under different conditions and compare signal frequencies after Tyramide-FISH. In the first experiments, the 45S or 5S rDNA probe can be used as a positive control. The best slide preparation conditions are those resulting in the best signal-to-noise ratio.

(2) Detection system. As we have shown in this study, direct detection may result in high background signals making it very difficult to identify signals for some species. Therefore both direct and indirect

detection systems should be evaluated in the first experiments. For the next experiments, the detection system resulting in the best signal-to-noise ratio is chosen (Figure 3B).

(3) Probe design. Cloned DNA fragments (EST, genes) should be used as a probe for Tyramide-FISH. The length of the probe influences the signal frequency (Section 2). In this study, probes with a length between 1Kb and 2Kb were used and successfully mapped. Long probes (>3Kb) have higher chances to contain repetitive sequences and, as a consequence, can generate multiple signals. All possible filters should be applied to avoid repeats in the probe, multi-copy gene probes (e.g. R-genes) and probes with conserved domains that can hybridize over multiple loci on chromosomes. If no genome sequences are available for the species the following approaches can be used to identify the repetitive sequences:

a) BLAST nucleotide database of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi);
b) RepeatMasking by Censor (http://www.girinst.org/censor/index.php);
c) BLAST against genomes of closely related species (https://phytozome.jgi.doe.gov/pz/portal.html);

In Paper 5 we used an orthology-based approach for probe design. In this approach a target sequence of closely related species is identified and used as a query in BLAST versus any known short sequence of the studied species (short transcriptomic reads, ESTs etc.). Then the distance between mapped short sequences is determined and primers are designed on those short sequences which have a distance longer than 1Kb. PCR with designed primers is performed and optimized to obtain one major band. PCR products are then cloned, verified by sequencing and used for FISH (Figure 3A).

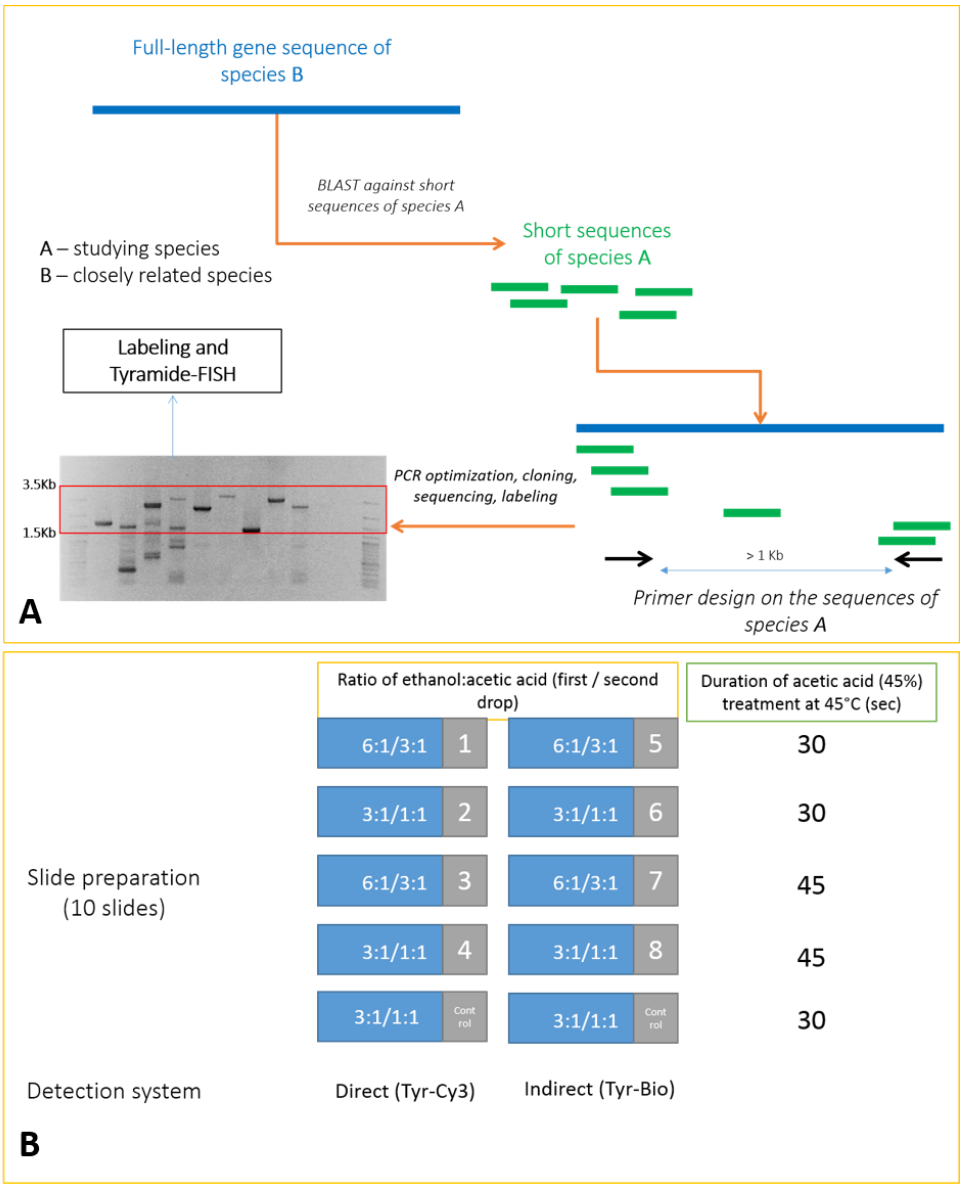

Figure 3. (A) A workflow for orthology-based approaches of probe design that can be used if long enough (>1Kb) sequences are not available for the studied species (species A, green lines). Available sequences of closely related species (species B, blue line) is used to identify the short sequences of species A which are located > 1Kb from each other according to BLAST with a reference sequence. (B) An example of a first Tyramide-FISH experiment for a new species. 10 slides are prepared: 5 slides for each detection system. Slides are prepared with different concentrations of acetic acid in the first and second drop of the spreading solution (ethanol:acetic acid) as well as with different times of final treatment of the chromosomes by 45% acetic acid (right).

6. LIMITATIONS OF TYRAMIDE-FISH

Although, improving the chromosome preparation method, revealing the main factors influencing the Tyramide-FISH mapping, optimization of detection system and increasing the spatial resolution, there are still some limitations of Tyramide-FISH for high throughput physical mapping of plant chromosomes.

Firstly**+,** the ability to visualize several target sequences on chromosomes at once is important for increasing the accuracy of physical mapping. Determing the location of target sequences on chromosomes regarding each other is becoming trivial if they can be visualized simultaneously. Current multicolor Tyramide-FISH protocols enable target-by-target detection sequentially. This strategy leads to increasing non-specific signals because each detection layer increases background signals on the slide. In addition, sequential detection is time-consuming because one target detection requires more than 1.5 hours.

Secondly, spatial resolution of Tyramide-FISH is now limited by the degree of compactization of pachytene chromosomes that were currently used for physical mapping (Paper 5, Khrustaleva et al., 2016). To further increase the spatial resolution of Tyramide-FISH more advanced technique should be applied such as Tyramide-FISH on fiber and extended DNA. Another strategy is to use super resolution microscopy (e.g. STED, SIM).

Finally, as it was mentioned above the signal frequency across chromosomes for Tyramide-FISH is 30-60%. It would be of benefit for physical mapping to increase this value. However, this is mainly unfluenced by chromosome preparation procedure. In this thesis, it was demonstrated that the chromosome preparation procedure has a strong impact on the results of Tyramide-FISH experiments. In our experience, it is difficult to prepare each slide with a similar quality. Therefore the results of Tyramide-FISH vary between slides, limiting the reproducibility of physical mapping by Tyramide-FISH.

7. TYRAMIDE-FISH IN THE POST-GENOMIC ERA

Phenotypic diversity between and within plant taxa can not always be explained by the existence of taxon-specific genes or gene families but rather by genome structural differences. Revealing chromosomal differences may shed light on the evolutionary processes leading to plant speciation. The most effective way to unravel such differences is to perform whole-genome assembly (WGA) of genomes of distantly related species, variaties, strains or ecotypes, followed by genome wide association

studies. High-quality assembly of *Arabidopsis* genomes triggered the number of comparative genomic studies in the Brassicaceae family allowing a better understanding of the crucifer evolution (Gan et al., 2016;). However, in contrast to NGS sequencing of DNA, *de novo* genome assembly from reads for larger plant genomes such as *Rosa* and *Allium* genomes (~4 times and > 100 bigger than *Arabidopsis thaliana* genome, respectively) is almost impossible only by NGS sequencing.

Although, the resolution of Tyramide-FISH is not comparable with *de novo* WGA approach, it can be applied to closely related species without prior knowledge of their genome sequences. Such studies have been applied on the Solanaceae and Brassicaceae family using a high resolution BAC-FISH approach (Gaiero el al., 2016; Fransz et al., 2016), showing many chromosomal differences between even closely related species (Solanaceae) and ecotypes (Ler and Col ecotypes of *A.thaliana*). Also for the Rosaceae family a set of conserved single-copy genes can be established using available genome sequences. These genes can further be used for probe design for Tyramide-FISH on pachytene chromosomes of modern rose cultivars and species involved in rose breeding and can shed more light on genomic rearrangements that occurred during rose domestication. The same set of probes can be used to better understand the genome constitution of *R. canina* showing a special type of meiosis. The chromosome level resolution established in Tyramide-FISH experiments with such set of conserved probes will provide unique information to estimate structural variation which can not be achieved by short-read-based genome-wide studies.

7.1. PHYSICAL MAPPING IN *ALLIUM*

Allium was the first plant for which Tyramide-FISH was applied to physically map short unique T-DNA sequences (Khrustaleva and Kik, 2001). Because of a huge genome and a high portion of dispersed repeats, physical mapping of the *Allium* genome is challenging by conventional FISH such as BAC-FISH (Suzuki et al., 2001). Therefore optimization and application of Tyramide-FISH for physical mapping of genes on *Allium* chromosomes is a very important task. In this thesis, we performed evaluation of indirect and direct Tyramide-FISH detection systems for gene mapping. In contrast to *Rosa*, both detection systems can be successfully used for physical mapping of *Allium* chromosomes. As *Allium* chromosomes are large, obtaining an optimal chromosome spreading and good chromosome slides

suitable for Tyramide-FISH is problematic. We tested "SteamDrop" protocol for few *Allium* species (Paper 1) and showed that even large *Allium* chromosomes can be efficiently spread under steam action. An other result of this PhD thesis for the physical mapping in *Allium* is the identification of new tandem repeats, HAT58 and CAT36, which we applied for cytogenetic mapping of *A. fistulosum* chromosomes (Paper 4). Using a direct detection system, we mapped (Paper 6) two important *Allium* genes, LFS and alliinase, involved in generation of Sulphur-organic molecules in *Allium* tissues (Imai et al., 2002). Two enzymes encoded by these genes are the main players in the metabolic pathway resulting in release of specific onion flavor when onion tissue is disrupted. We also used a mixture of EST clones for Tyramide-FISH to check the hypothesis about the differences in gene distribution between two *Allium* species, *A. cepa* and *A. fistulosum*. The protocols optimized during this PhD are useful tools for further physical mapping of *Allium* chromosomes that are currently ongoing (Romanov et al., 2015; Khrustaleva et al., 2016) .

7.1.1 NEW CYTOGENETIC MARKERS FOR *ALLIUM* CHROMOSOMES

Cytogenetic markers can significantly accelerate physical mapping of the *Allium* genome by simplifying chromosomal identification. Not all mitotic chromosomes of *Allium* can be distinguished by morphological parameters such as centromeric index and relative chromosome length. Therefore cytogenetic markers are required. In this thesis we discovered two new cytogenetic markers for *A. fistulosum*, HAT58 and CAT36 (Paper 4). These markers are tandemly organized repeats and allow identification of 4 of 8 *A. fistulosum* chromosomes. Of these, chromosome 5 contains a number of genes affecting desirable traits such as bulb dry matter content, pungency and storability, leaf waxiness, and resistance to abiotic factors (Romanov et al. 2015). The development of these cytogenetic markers is a very important milestone for detailed physical mapping of *Allium* pachytene chromosomes since they do not have enough chromosomal patterns to be distinguished (de Jong et al., 1999; Khrustaleva et al. 2016). For future research, more cytogenetic markers for *Allium* chromosomes are needed. Application of NGS data for other *Allium* species followed by RepeatExplorer clustering will help to isolate new repeats that are valuable as cytogenetic markers. Also centromeric tandem repeats, which can be chromosome or chromosome subset specific, are valuable sources for cytogenetic marker development (Gong et al., 2012). The *Allium* centromere has not been fully sequenced yet and detailed information about its full-length sequence is lacking (Nagaki et al., 2012; Kiseleva et al., 2014). By RepeatExplorer we could not identify the cluster corresponding to the *A. fistulosum* centromeric repeat. Increasing genome coverage will probably help to isolate centromeric repeats for *Allium.* These repeats can be used as

cytogenetic markers. Interestingly, our RepeatExplorer analysis showed several clusters for a subtelomeric repeat of *A. fistulosum.* These results indicate significant polymorphisms in subtelomeric tandem repeat sequences. If the variants of this subtelomeric repeat are located on different chromosomes their sequences can be used to design labeled oligo probes which can be further used as cytogenetic markers. Cytogenetic marker development and probe design will be easier when ongoing genome sequencing of *A.cepa* will be finished. It will allow to identify closely located single-copy genes. Pooling these genes in one hybridization mixture will facilitate their visualization by conventional FISH making them useful cytogenetic markers.

7.1.2 *ALLIUM* COMPARATIVE GENOMICS BASED ON SINGLE-COPY GENE PHYSICAL MAPPING

Comparative physical mapping of genes on chromosomes of *Allium* species can shed light on the synteny and collinearity between the *Allium* genomes. This information can be useful for effective interspecific breeding because genome collinearity is a strong prerequisite for homologous recombination and transfering desirable traits from donor species (Gaiero et al., 2016). The huge size of most of the *Allium* genomes and the absence of any reference genome sequence make comparative genomic studies in *Allium* difficult. In this study, we performed Tyramide-FISH with a pool of EST clones to gather knowledge about the genome organization differences between *A. cepa* and *A. fistulosum* (Paper 6). The results showed that these species may have different trends in gene distribution along chromosomes with higher gene density in proximal regions of *A. fistulosum* genome. These data support the early study demonstrating the differences in distribution of recombination nodes between chromosomes of *A. cepa* and *A. fistulosum* (Albini and Jones, 1988). However, hybridization with a mixture of EST clones may result in misinterpretation because observed signals can result from hybridization of only limited number of EST clones with few copies per genome while other EST hybridization sites are not detected. Therefore more efforts are required to check the differences in gene distribution between these important *Allium* species. Huge amounts of transcriptomic data has been accumulating for *A. cepa* and *A. fistulosum* the last few years. These data include raw reads and assembled transcripts (Baldwin et al., 2012; Duangjit et al., 2013; Kim et al., 2014; Khosa et a., 2016) providing information for efficient FISH probe design. Cloning of gene fragments by primer design on the existing assembled transcripts followed by sequence verification, labeling and Tyramide-FISH mapping on chromosomes of different species will facilitate *Allium* comparative genomics. Recently single-copy FISH maps have been established for two

plant species, wheat (Danilova et al., 2014) and barley (Aliyeva-Schnorr et al., 2016), with FISH being an indispensable tool for comparative cytogenetic studies between these species and other grasses.

Using repetitive DNA as FISH probes can be valuable for comparative genomic studies. 45S and 5S rDNA tandemly organized genes have been used for FISH mapping in different *Allium* species (Shibata and Hizume, 2002; Guetat et al., 2015). However, the information provided by these studies is limited because only few loci are occupied by rDNA. Therefore more efforts are needed to isolate other tandem repetitive DNA sequences and to perform cytogenetic mapping. As a first step in this, we used NGS data to isolate the *A. fistulosum* tandem repeat CAT36 and HAT58. This approach can be used in the future for other *Allium* species.

7.1.3 *ALLIUM* BREEDING CAN BENEFIT FROM PHYSICAL MAPPING

Marker assisted selection and plant breeding approaches have been widely used to improve onion yield, quality, and resistance against biotic and abiotic stresses (Khosa et al., 2016). However, to meet the growing demand of the world human population, onion breeding requires new tools to shorten the breeding process and to increase the breeding efficiency. Onion has a number of traits including its biennial life cycle, cross-pollinated nature, high inbreeding depression and large genome size, which make utilization and development of new molecular tools challenging (McCallum, 2007). The development of a physical map is one of the tools that can be applied in onion breeding. Physical maps can assist in marker development through the isolation of genes of interest or tightly linked markers. Application of Tyramide-FISH allowed to physically map Ms-linked (male sterility) markers and to show their pericentromeric location (Khrustaleva et al., 2016). Based on results of Tyramide-FISH mapping it was concluded that map-based cloning of the Ms-gene will be time- and cost-consuming. In addition, this work shows that current markers can be located far from the Ms-gene (Khrustaleva et al., 2016). Fungal diseases are the most widely distributed type of pathogens for onion causing significant damage. Downy mildew (*Peronospora destructor* Casp. (Berk.)) is a severe threat for *Allium* crops both during vegetation as well as during storage period. Brown spot disease is often accompanied by downy mildew. Both brown spot disease and downy mildew decreased onion seed production by 85% (Scholten et al. 2007). *Allium fistulosum* and *A. roylei* are valuable sources of resistance genes to brown spot disease and downy mildew. However, obtaining late generation interspecific hybrids between *A.cepa* and *A.roylei* being a homozygous genotype of resistance is complicated. In this case, cytogenetic mapping of

the homologous recombination points will help to obtain late generation hybrids between *A. cepa* and *A. roylei* and create a new onion variety (Scholten et al. 2007).

7.2 *ROSA WICHURANA* CAN BE A MODEL SPECIES FOR MOLECULAR CYTOGENETICS WITHIN THE *ROSA* GENUS

Parts of this chapter are published in:

Kirov, I. V., Khrustaleva, L. I., Van Laere, K., & Van Roy, N. (2015). Molecular Cytogenetics in the Genus Rosa: Current Status and Future Perspectives. Acta Horticulturae *1087*, pp. 41-48.

Before this PhD work, only few molecular cytogenetic studies in *Rosa* were performed. This is mainly because *Rosa* is a very difficult cytogenetic object due to the presence of a very small genome size (the diploid genome size is 0.83 to 1.30 pg/2C; Roberts et al., 2009) and small chromosomes (Paper 2). Also the occurrence of polyploidy in the *Rosa* genus (Vamosi and Dickinson, 2006) hampers cytogenetic studies. The ploidy of wild species range between 2n=2x=14 and 2n=8x=56 (Roberts et al., 2009), and recently even decaploidy was observed in the genus (Jian et al., 2010). Finally, also the low mitotic index in roots and shoots and the development of very weak and tiny to handle roots (Ma et al., 1996) are obstacles for cytogenetic research in roses. To accelerate the progress in molecular cytogenetic studies in the genus *Rosa*, we optimized the chromosome preparation protocol and developed the "SteamDrop" protocol (Paper 1) which we successfully used for the preparation of high quality mitotic and pachytene chromosome slides for *Rosa wichurana* (Paper 1, Paper 2, Paper 5). By this method many possibilities are open for cytogenetic studies in *Rosa* species.

Optimization of Tyramide-FISH for *R. wichurana* resulted in successful mapping of several gene fragments as small as 1.1kb on mitotic chromosomes (Paper 2). Moreover, we were able to anchor three linkage groups to the physical chromosomes of *Rosa wichurana*. However, the resolution of Tyramide-FISH on mitotic rose chromosomes is rather low: the physical location of two genes on *R. wichurana* mitotic chromosomes can only be distinguished if the distance between these genes is more than 5.6Mb (Paper 5). To overcome this problem, we used the less condensed pachytene chromosomes in Tyramide-FISH mapping in *Rosa* (Paper 5). This study demonstrated that Tyramide-FISH is a valuable tool for physical mapping of the *Rosa* genome. Our paper was the first to describe the application of Tyramide-FISH on plant pachytene chromosomes. By our established Tyramide-FISH protocol more (single-copy)

genes can now be mapped and their order can be determined with a high resolution (see further). As genes are very conserved between species, comparative cytogenetic mapping of genes on chromosomes from different *Rosa* species is feasible. Our pilot Tyramide-FISH experiments performed with a *R. wichurana* probe on *R. chinensis* pachytene chromosomes confirmed this possibility (data not shown). The development of a set of 14 conserved single copy gene based probes which can be mapped to all 14 chromosome arms of *R. wichurana,* and use them in Tyramide-FISH mapping on chromosomes of other *Rosa* species, would allow to trace large genomic rearrangements such as translocations and inversions. By this, more knowledge could be gathered about the importance of chromosome rearrangements in the speciation of the *Rosa* genus.

R. wichurana (subgenus Rosa, sect. Synstylae) turned out to be a good model species for molecular cytogenetic studies in the *Rosa* genus because of several reasons. *R. wichurana* is a diploid species (2n=2x=14; Figure 4B)) and has intensively growing apical meristems (Figure 4A) containing many dividing cells suitable for chromosome preparation.

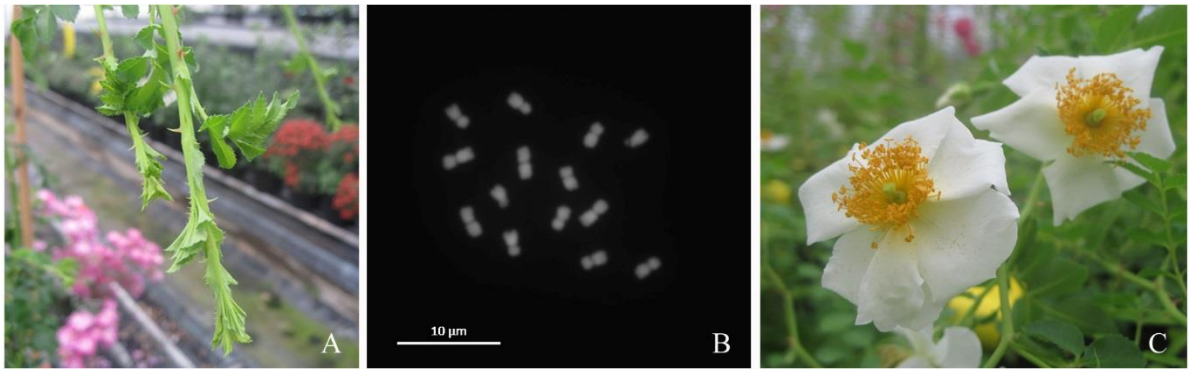

Figure 4. Features of *R. wichurana* valuable for cytogenetic studies: intensively growing apical meristems (A), diploid species (2n=2x=14) (B) and flowers containing tens of anthers (C).

Also the flowering period is long enough and the flowers have many anthers (flower with simple corolla; Figure 3C), providing sufficient start material for pollen mother cell (PMC) isolation and pachytene chromosome preparation. *R. wichurana* is one of the species involved in the origin of modern rose cultivars and is a source of resistance to powdery mildew (Dugo et al. 2005; Moghaddam et al. 2012; Leus et al. 2009). Obtained knowledge for *R. wichurana* can be transferrable to modern rose cultivars. In addition, *R. wichurana* is one of the parental species used in the construction of genetic maps for roses (Crespel et al. 2002; Dugo et al. 2005; Shupert et al. 2007; Spiller et al. 2011; Moghaddam et al.

2012). This will simplify the integration of physical and genetic maps. Also important to accelerate progress in cytogenetics for *Rosa* is our development of cytogenetic markers for 5 chromosome pairs of *R. wichurana* including 5S, 45S and Arabidopsis-telomeric repeats have been found (Paper 3).

Since rose chromosomes are so difficult to distinguish, cytogenetic markers are the only way for (FISH-based) chromosome identification. Tandemly organized repeats (TR) are a suitable source for the development of cytogenetic markers. TRs can be efficiently isolated using next generation sequencing (NGS) data obtained from genomic DNA (Novak et al. 2013). It was demonstrated that even low-coverage NGS data is sufficient for TR isolation (Novak et al. 2014). The development of cytogenetic markers and their application for FISH on rose chromosomes will be useful for (1) chromosome identification, (2) study of phylogenetic relationships in *Rosa*, and (3) exploration of the genomic relationships between modern rose cultivars and their ancestors.

7.3 AN INTEGRATED APPROACH FOR GENOME ASSEMBLY AND ITS APPLICATION FOR *R. WICHURANA* GENOME SEQUENCING

Current next generation sequencing technologies allow performing shotgun genome sequencing with high genome coverage and low price of sequencing. Up to now, more than 100 plant genomes have been sequenced. However, most of the plant genome sequencing projects result in a ‘‘draft’’ genome version presenting sets of contigs or scaffolds (Claros et al., 2012; Xie et al., 2015). Only a small number of the sequenced genomes is assembled into pseudochromosomes, thus providing an indispensable tool for biological and evolutionary studies using comparative genomics (Spannaglet al., 2016; Bauer et al., 2016; Zapata et al., 2016). The level of genome assembly (contigs, scaffolds, superscaffolds, pseudochromosomes) is mostly determined by the complexity of a genome (large genome size, repetitive elements, genome-wide genome duplications, (allo)polyploidy). In addition the objectives of the genome sequencing determines the level of genome assembly (Figure 5). For example, gene family study (e.g. He et al., 2013; Nystedt et al., 2013), orthologous gene analysis (e.g. Nystedt et al., 2013), SNP calling (e.g. Poecke et al., 2013) and genome-based transcriptome assembly and gene model prediction (e.g. Chang et al., 2014) are common objectives for plant genome sequencing projects and

require high genome completeness while knowledge about linear order of scaffolds is less important. For the latter case, high quality assembly of contigs and scaffolds is sufficient (Figure 5).

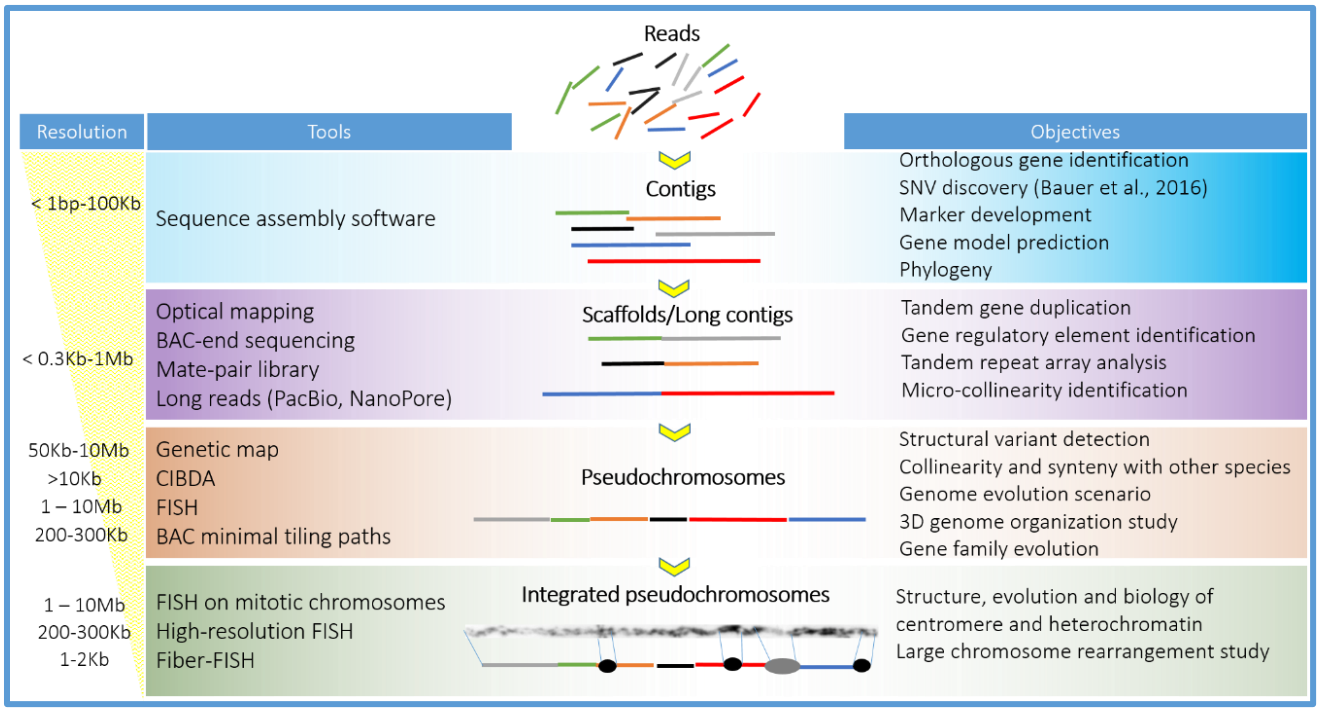

Figure 5. Main tools and objectives in plant genome sequencing projects.

In contrast, some biological questions can only be answered when contigs and scaffolds are arranged according to their physical location in the genome. Genes are not isolated units in a genome and their regulation depends on other genomic loci that can be located on the same (cis regulation) or different (trans regulation) chromosomes. Distinct genomic elements (protein-encoded genes, regulatory elements, genomic repeats) communicate with each other via direct (DNA:DNA interaction) or indirect (by RNA or protein molecules intermediates) interactions (Dekker and Mirny, 2016). In this communication process, the distance between the genomic loci and the genomic context where the loci are located (e.g. heterochromatin, euchromatin, A-compartment, B-compartment (Lieberman-Aiden et al., 2009)), play an essential role. To unravel this 3D genome organization and to better understand the gene regulation network, chromosome level assembly of the genome is required. In addition, a rapidly growing number of studies carried out on different plant species and humans demonstrated that microscopic structural variants (SV, variants longer than 3Mb) and submicroscopic structural variants (1Kb - 3Mb) have significant impact on biological diversity of organisms (Feuk et al. 2006; Chaney et al. 2016). SV break genome synteny and collinearity between organisms play a key role in plant speciation (Yeaman, 2013; Mandakova et al., 2015; Fransz et al., 2016; Zapata et al., 2016; Hou et al., 2016). SVs

are also important for plant breeding as they create variability among plant material for future selection. However, they may also negatively influence gene transfer between parental plants by recombination suppression leading to gene hitchhiking (Döring et al., 2011). Genome-wide identification of the structural variants also demands knowledge about the linear order of sequences in the genome. Many of the genome sequencing projects aim to build a physical map of the genome using high-density genetic maps to establish scaffold order and orientation. However, a significant part of the genome is located in recombination-poor regions such as pericentromeric heterochromatin leading to errors in the final genome assembly. Genetic map resolution in these regions is very low (few Mb per 1cM) resulting in long-range linkage disequilibrium (Fransz et al., 2016). Errors during pseudochromosome assembly are also caused by repetitive sequences and duplicated chromosomal regions. DNA repeat sequences may be collapsed by genome assemblers resulting in a high number of short contigs and genome sequence incompleteness (Veeckman et al., 2016). Indeed, FISH and optical mapping validation of tomato genome sequencing suggested that most of the incorrectly placed scaffolds are located in the heterochromatin and repeat-rich regions (Shearer et al. 2014; Pietrella and Giuliano, 2016).

Ordering of the sequences into pseudochromosomes requires an efficient assembly and validation strategy implicating recombination-independent tools. Tools for scaffolding, including optical mapping, mate-paired library with long insert size and long-read sequences obtained by PacBio or NanoPore sequencing (Van Bakel et al., 2011; English et al., 2012; Jia et al., 2013; Shearer et al., 2014; Zapata et al., 2016) allow generating long scaffolds and ordering them with a very high resolution (the minimum physical distance between two distinct sequences at which their order can be revealed) ranging from a few Kb (mate-paired library (Boetzer and Pirovano, 2012)) up to 1Mb (optical mapping (Levy-Sakin and Ebenstein, 2013)). Therefore, combining long and short read data is becoming a powerful approach for *de novo* genome assembly although it does not allow entire plant chromosome assembly and the length of the obtained sequences is much shorter than the length of the entire chromosome (Zapata et al., 2016). However, additional methods for efficient ordering of contigs/scaffolds into pseudochromosomes are required. A new approach called chromatin interaction-based *de novo* assembly (CIBDA), was proposed to fill in this gap (Burton et al., 2013; Dekker et al., 2013; Kaplan and Dekker, 2013). CIBDA is a recombination-independent method and relies on the probabilistic model of DNA interactions in 3D nuclear space. This model links the physical distance between two DNA sequences with the probability for them to be in contact in the nucleus. DNA:DNA contacts are retrieved from *in vivo* cross-linking (formaldehyde fixed tissue, Burton et al., 2013) or reestablished *in vitro* by

specific proteins (Putnam et al., 2016). CIBDA has been tested for human, mouse, *Drosophila*, alligator, fungus (*Trichoderma reesi*) and yeast genomes (Kaplan and Dekker, 2013; Burton et al., 2013; Marie-Nelly et al., 2014; Putnam et al., 2016) and was recently evaluated for *de novo* assembly of the small *Arabidopsis* genome (Xie et al., 2015). The authors showed that a CIBDA-based genome assembly is in concordance with the last version of the *Arabidopsis* genome. Moreover it was even possible to include short scaffolds (10Kb) in the obtained chromosome level genome sequence (Xie et al., 2015). Therefore CIBDA can be used to group and order DNA sequences into chromosomes. The minimum length of the sequence for CIBDA assembly depends on the number of interactions which can be detected by Hi-C data (sensitivity). Genome coverage by Hi-C reads, number of restriction sites and type of restriction enzyme used for Hi-C library preparation are limiting factors for sensitivity of a Hi-C map (Jin et al. 2013; Chang et al., 2016). The accuracy of both genetic maps and CIBDA based genome assembly is affected by highly repetitive regions (Xie et al., 2015). To overcome this problem, FISH guided genome assembly has recently been introduced for plants (Albert et al., 2013; Shearer et al., 2014; Cao et al., 2016; Pietrella and Giuliano, 2016). These studies demonstrated that FISH is indispensable to verify and correct genome assembly. However, FISH is not a high-throughput method and the resolution is rather low (>200Kb on pachytene chromosomes) to be able to order short contigs (10 - 50Kb). Therefore FISH is used in the final steps of genome assembly to further validate ((Tyramide-) FISH with single-copy probes) genome assembly, to map repetitive regions (FISH with repetitive DNA sequences) and to integrate genome assembly with chromosomal landmarks such as heterochromatin and (peri)centromeric regions (Figure 6). The latter step is important to understand evolution and function of the centromere, heterochromatin and repetitive elements (Zhao et al., 2016).

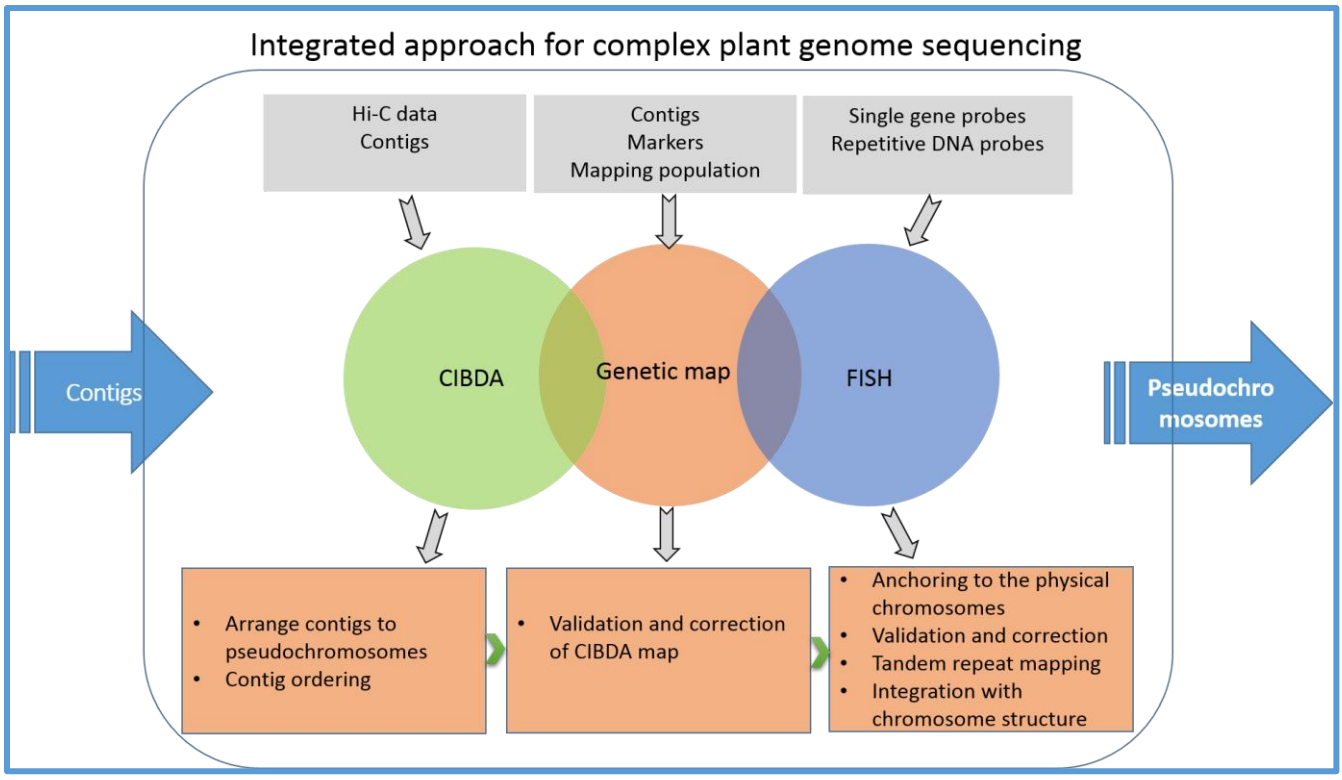

Figure 6. Integration of data from a CIBDA map, a genetic map and a FISH-based physical map to achieve high quality genome assembly into pseudochromosomes and their integration with chromosome structure.

Centromere length may range from 9 Mb (chromosome 1 of *Arabidopsis*, Hosouchi et al., 2002) to 124 kb (rice chromosome 4, Zhang et al., 2004). Centromeres consist of repetitive elements, such as centromeric retrotransposon and tandem repeats, and some genes (Zhao et al., 2016). Because of the repetitive nature and low recombination frequency of centromeric regions, only few plant centromeres have been fully assembled. These include rice chromosome 4 (Zhang et al., 2004) and maize chromosome 2, 5 and 10 (Wolfgruber et al., 2009, 2016). Hence only limited information about the centromere organization is available. The centromere is established epigenetically (Fukagawa and Earnshaw, 2014; reviewed by Ohzeki et al., 2015) and the role of centromeric DNA in this process is not clear. It was shown that centromeric RNA contains centromeric repeats which play an essential role in kinetochore assembly (Quénet and Dalal, 2014). Centromeric DNA sequencing and assembly can help to identify the functional motifs (such as CENP box) and to understand the evolutionary mode of centromeric DNA, neocentromere formation and high turnover of centromeric repeats. In addition, because centromeres occupy distinct, mostly silenced compartments of nuclear space, the regulation of transcription of the genes located in the centromere is an intriguing question. Sequencing of centromeres from other plant species will shed more light on centromere organization and evolution.

The application of genetic map, CIBDA and FISH-based physical map to complete genome sequencing and pseudochromosome assembly will provide a more robust and complete genome sequence. All three methods have different principals of sequence order reconstruction and resolution and therefore they complement each other.

In the future, this strategy can be used for *R. wichurana* genome sequencing. In this thesis, we established a Tyramide-FISH protocol for *R. wichurana* and showed that it can be used to physically map unique sequences with few hundred thousand bp resolution (200-300Kb) on pachytene chromosomes. To anchor assembled pseudochromosomes to each arm of the physical chromosomes and to determine their north-south orientation at least 14 probes should be used, 2 probes per pseudochromosome. During this PhD, we designed probes for 2 chromosomes (4 and 7) and these were already physically mapped (Paper 5; Figure 7). For 4 other chromosome pairs the design of at least 2 probes per chromosome and their physical mapping is still required. In addition, at least one more probe for chromosome 1 is needed since already one probe (OOMT) has been designed and mapped (Paper 2).

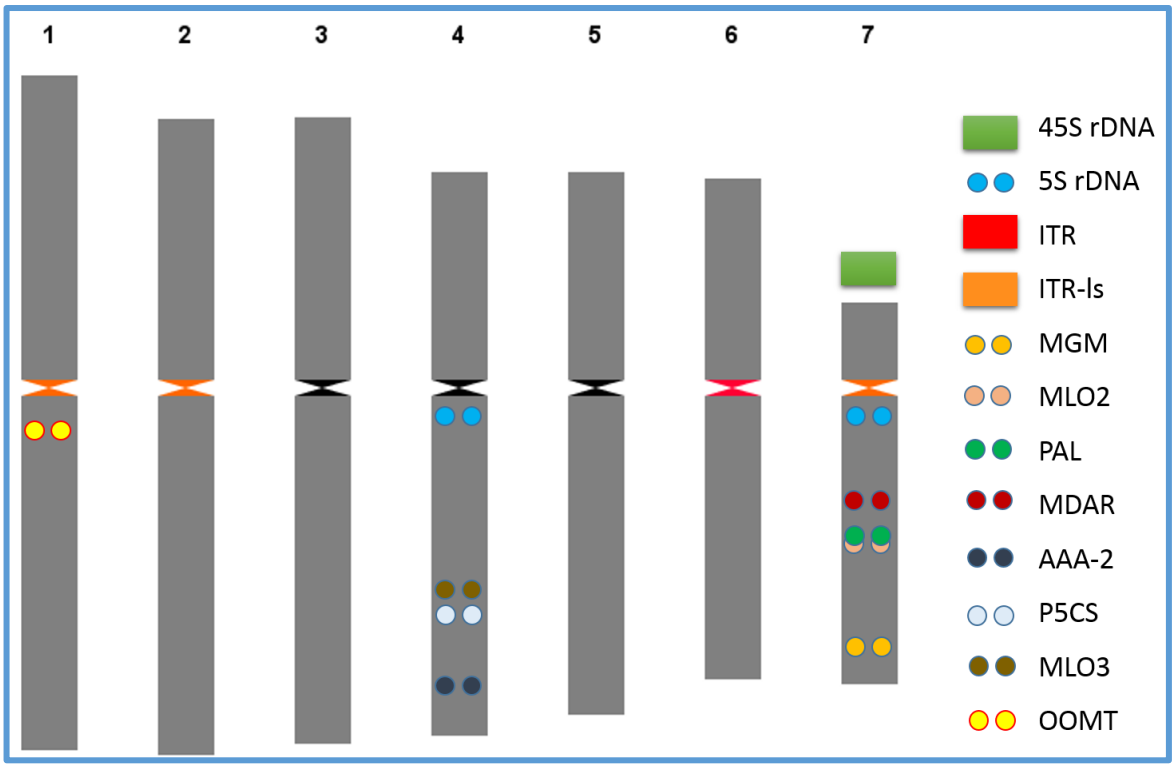

Figure 7. Cytogenetic map of repetitive (5S, 45S rDNA genes, *Arabidopsis*-type telomeric repeat) and unique (genes) sequences on *R. wichurana* chromosomes. The map was build based on data from paper 2 and paper 5. ITR and ITR-ls – Interstitial Telomeric Repeat signals that are visible under 37°C and room temperature (23 - 25°C), respectively.

However, to be able to use FISH for genome assembly validation and correction, the number of Tyramide-FISH anchor points has to be significantly increased. To anchor a scaffold to the chromosome a minimum of 2 probes are needed per scaffold. In addition, only those scaffolds that have a length exceeding the resolution (> 200 – 300Kb) of FISH can be used because only then their north-south orientation can be determined by high-resolution FISH. Therefore, the number of probes for Tyramide-FISH would depend on the number and length of the contigs/scaffolds/superscaffolds that is obtained after scaffolding and CIBDA. To verify CIBDA genome assembly, the FISH probes need to be distributed over the pseudochromosome length. For this probably 10 – 20 probes per chromosome need to be designed which would result in 4-8Mb of genome coverage by each FISH probe. It would also help to estimate the presence of large-scale errors in the CIBDA genome assembly and to perform corrections.

To determine the centromere location on pseudochromosomes and integration of pseudochromosome sequences with the chromosome structure, the isolation of centromeric and other heterochromatin-associated tandem repeat sequence(s) will be needed. These sequences can then be used for FISH probe design and mapping on physical chromosomes as well as for determining their locations on

pseudochromosomes by BLAST. In that way, data from chromosomal and pseudochromosomal locations of the repeats can be integrated and the length of the repeat arrays in the genome can be estimated. Identification and FISH mapping of these sequences on *R. wichurana* chromosomes will also be important for further cytogenetic marker development in *Rosa wichurana* and other *Rosa* species.

At the end, integration of data from CIBDA assembly, genetic mapping and FISH mapping with chromosome structure data will allow to perform *R. wichurana* genome assembly with high accuracy.

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Scientific activities

Scientific training:

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Grants:

1. “Molecular genetic and functional organization of genes and genomes of important crops”, the Ministry of Education (№ 02.740.11.0286, 2009-2011)
2. “Physical organization of genes and repetitive DNA in chromosomes of plants with huge genome (*Allium*)”, the Ministry of Education (№ P809, 2009-2011)
3. “Integration of genetic and physical maps: development of new ultrasensitive method for visualization of gene/markers on the chromosomes”, the Ministry of Education (№ 8112, 2012 - 2013)
4. “Cytogenetic study of satellite DNA of plant sex chromosomes: case study in Cannabaceae family”, RFBR (№13-04-01804, 2013-2014)

5. "Physical mapping of *Cannabis sativa* chromosomes using whole genome sequencing data", RFBR (№15-04-06244, 2014-2016)
6. "Integration of physical and genetic map of onion chromosome 5", RFBR (№ 14-04-31595, 2014 - 2016)
7. "Comparative analysis of Allium centromeme", RFBR (№16-34-00142, 2015-2016)

Achievements and Awards:

1. Scholarship holder of V. Potanin's charitable foundation (2008-2009, 2009-2010)
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3. Rector's stipendium (2011)
4. Grant for young scientists from OPTEK company (2012)

Publications:

Publication in scientific journals with peer review (A1 citation index)

1. Khrustaleva L., Karlov G, **Kirov I.**, Lapitskaya I., Romanov D., Budylin M., Kiseleva A. and I. Fesenko (2012) The Chromosome Organization of Genes and Some Types of Extragenic DNA in Alliums. Acta Horticulturae, 969
2. **Kirov, I.**, Divashuk, M., Van Laere, K., Soloviev, A., & Khrustaleva, L. (2014). An easy" SteamDrop" method for high quality plant chromosome preparation. *Molecular Cytogenetics (17558166)*, *7*(1)
3. **Ilya Kirov**, Katrijn Van Laere, Jan De Riek, Ellen De Keyser, Nadine Van Roy, and Ludmila Khrustaleva (2014) Anchoring linkage groups of the Rosa genetic map to physical chromosomes with Tyramide-FISH and EST-SNP markers. *PLoS ONE 9*(4), e95793.
4. Divashuk MG, Alexandrov OS, Razumova OV, **Kirov IV**, Karlov GI (2014) Molecular Cytogenetic Characterization of the Dioecious Cannabis sativa with an XY Chromosome Sex Determination System. PLoS ONE 9(1): e85118.

5. Kiseleva, A. V., I. V. Kirov, and L. I. Khrustaleva (2014) Chromosomal organization of centromeric Ty3/gypsy retrotransposons in Allium cepa L. and Allium fistulosum L. *Russian Journal of Genetics* 50.6: 586-592.

6. Kirov, I. V., Van Laere, K., & Khrustaleva, L. I. (2015). High resolution physical mapping of single gene fragments on pachytene chromosome 4 and 7 of Rosa. *BMC genetics*, *16*(1), 1.

7. Laskowska, D., Berbeć, A., Van Laere, K., Kirov, I., Czubacka, A., & Trojak-Goluch, A. (2015). Cytology and fertility of amphidiploid hybrids between Nicotiana wuttkei Clarkson et Symon and N. tabacum L. *Euphytica*, *206*(3), 597-608.

8. Kirov, I. V., Khrustaleva, L. I., Van Laere, K., & Van Roy, N. (2015). Molecular Cytogenetics in the Genus Rosa: Current Status and Future Perspectives. Acta Hort. 1087: 41-48

9. Divashuk, M. G., Khuat, T. M. L., Kroupin, P. Y., Kirov, I. V., Romanov, D. V., Kiseleva, A. V., ... & Razumova, O. V. (2016). Variation in Copy Number of Ty3/Gypsy Centromeric Retrotransposons in the Genomes of Thinopyrum intermedium and Its Diploid Progenitors. *PloS one*, *11*(4), e0154241.

Abstracts and participation on international conference

1. Kirov I.V., Romanov D.V., Fesenko I.A., Khristaleva L.I.: Physical mapping of alliinase and lachrymatory factor synthase genes on Allium cepa L. by Tyr-FISH, XI scientific conference for young scientists: «Biotechnology in plant growing, animal husbandry and veterinary medicine», p. 23, 2011
2. Kirov I.V., Romanov D.V., Fesenko I.A., Khristaleva L.I.: Cloning and Sequencing of the alliinase and lachrymatory factor synthase genes, XI scientific conference for young scientists: «Biotechnology in plant growing, animal husbandry and veterinary medicine», p. 23, 2011 (Oral presentation)
3. Kirov I.V.: Cytogenetic mapping of alliinase and lachrymatory factor synthase genes on Allium cepa chromosomes by multicolor Tyr-FISH, Proceeding of XVIII international scientific conference for student, PhD students and young scientists “Lomonosov”, pp. 90-91, 2011 (Oral presentation)
4. Romanov D.V., Kirov I.V., Fesenko I.A., Khristaleva L.I.: Cloning, Sequencing and Physical Mapping of the alliinase and lachrymatory factor synthase genes in genus Allium, Conference

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