

High-resolution tyramide-FISH mapping of markers tightly linked to the male-fertility restoration (*Ms*) locus of onion

Ludmila Khrustaleva¹ · Jiming Jiang² · Michael J. Havey³

Received: 17 July 2015 / Accepted: 30 November 2015 / Published online: 24 December 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract

Key message Tyramide FISH was used to locate relatively small genomic amplicons from molecular markers linked to *Ms* locus onto onion chromosome 2 near the centromere, a region of relatively low recombination.

Abstract Fluorescence in situ hybridization (FISH) has not been readily exploited for physical mapping of molecular markers in plants due to the technical challenge of visualizing small single-copy probes. Signal amplification using tyramide (tyr) FISH can increase sensitivity up to 100-fold. We used tyr-FISH to physically locate molecular markers tightly linked to the nuclear male-fertility (*Ms*) restoration locus of onion onto mitotic metaphase, pachytene, and super-stretched pachytene chromosomes. Relatively short genomic amplicons (846–2251 bp) and a cDNA clone (666 bp) were visualized in 9–42 % of observed cells. The markers were assigned to proximal locations close to the centromere on the long arm of chromosome 2, a region of lower recombination, revealing that tightly linked markers may be physically distant from *Ms*. This result explains why several labs have identified molecular markers tightly linked to the *Ms* locus after screening relatively few DNA

clones or primers and segregating progenies. Although these markers are still useful for marker-aided selection, our results indicate that map-based cloning of *Ms* will likely be difficult due to reduced recombination near this gene.

Introduction

Hybrid onion (*Allium cepa* L.) seed is commonly produced using cytoplasmic male sterility (CMS). For the most commonly used source of onion CMS, male sterility is conditioned by the interaction of the cytoplasm and a nuclear male-fertility-restoration (*Ms*) locus (Jones and Clarke 1943). Male-sterile plants possess male-sterile (S) cytoplasm and are homozygous recessive at *Ms*. Male-fertile plants may possess S cytoplasm and a dominant allele at *Ms*, or normal (N) male-fertile cytoplasm with any genotype at *Ms*. Seed propagation of male-sterile plants (S *msms*) is possible by crossing with maintainer (N *msms*) plants (Jones and Davis 1944), and selection of superior maintainer lines is a primary focus of hybrid onion breeding programs.

The identification of individual plants or inbred lines that maintain CMS requires the classification of the cytoplasm as well as genotypes at the nuclear *Ms* locus. S cytoplasm is likely an alien cytoplasm introduced in antiquity into onion populations (Havey 1993) and as a result, many polymorphisms have been identified in the organellar DNAs that distinguish N and S cytoplasm of onion (Courcel et al. 1989; Holford et al. 1991; Havey 1993, 1995; Satoh et al. 1993; Sato 1998; Lilly and Havey 2001; Engelke et al. 2003; Cho et al. 2005, 2006; Kim et al. 2009; von Kohn et al. 2013). The use of classical crossing to establish genotypes of the *Ms* locus is time-consuming

Communicated by R. G.F. Visser.

✉ Ludmila Khrustaleva
ludmila.khrustaleva19@gmail.com

¹ Center of Molecular Biotechnology, Department of Genetics and Biotechnology, Russian State Agrarian University-Timiryazev Agricultural Academy, 49, Timiryazevskaya Str., 127550 Moscow, Russia

² Department of Horticulture, University of Wisconsin, Madison, WI 53706, USA

³ USDA-ARS and Department of Horticulture, University of Wisconsin, Madison, WI 53706, USA

Table 1 Summary of molecular markers linked to the nuclear male-fertility restoration locus (*Ms*) of onion

Marker	Origin ^z	Type ^y	Family or population	References
AOB272	cDNA	RFLP and SNP	F ₂ family and open-pollinated plants	Gökçe et al. (2002) and Gökçe and Havey (2002)
WHR240	cDNA	SRAP	BC ₁ family	Huo et al. (2012)
DNF-566 RNS-357	Genomic	AFLP and SCAR	BC ₁ family	Yang et al. (2013)
ACms.1100	Genomic	RAPD and CAP	F ₂ family	Bang et al. (2013)
Isotigs 29186, 34671, 30856	cDNA	SNPs	F ₂ family and open-pollinated plants	Havey (2013)
Jnurf05 jnurf17	Genomic	RAPD and CAP	F ₂ and F ₃ families	Park et al. (2013)
Jurf13	Genomic	SCAR	F ₂ family	Kim (2014)

^z Markers identified using genomic or complementary (c) DNAs

^y AFLP amplified fragment length polymorphism, RFLP restriction fragment length polymorphism, RAPD Randomly amplified polymorphic DNA, CAP Cleaved amplified polymorphism, SNP Single nucleotide polymorphism, SCAR Sequence characterized amplified region, SRAP cDNA sequence-related amplified polymorphism

and expensive, requiring testcrosses of an individual male-fertile plant to a male-sterile (*S msms*) plant and scoring the male-fertility of the testcross progenies. Due to the biennial generation time of onion, this process can take four or more years. To more quickly determine genotypes at *Ms*, several research groups have identified molecular markers from the genome or transcriptome showing linkage to *Ms* (Table 1). Molecular markers in linkage disequilibrium with *Ms* allow for more efficient development of maintainer lines to facilitate hybrid development. Ideally the markers should be very tightly linked to confidently predict genotypes at *Ms* across diverse germplasm. Most researchers have used F₂ or F₃ families (Table 1) at or near maximum linkage disequilibrium to assess linkage to *Ms*; however Gökçe et al. (2002), and Havey (2013) assessed disequilibrium between markers and *Ms* using plants randomly selected from open-pollinated populations, presumably at or near linkage equilibrium. Given the enormous size of the onion nuclear genome [16.3 megabases per 1C nucleus (Bennett and Leitch 2012)], we were intrigued as to why so many markers linked to *Ms* have been identified (Table 1), even though in some cases relatively few clones or primers were screened. If the *Ms* locus was located in a chromosome region experiencing relatively few recombination events, markers showing tight genetic linkage to *Ms* could be physically quite distant and affect the likelihood of eventual map-based cloning of *Ms*.

Fluorescence in situ hybridization (FISH) is a tool to physically map DNA sequences onto chromosomes (reviewed by de Jong 2003; Jiang and Gill 2006; Figueroa and Bass 2010), as well as integrate physical and recombination maps (Cheng et al. 2001; Khrustaleva et al. 2005; Danilova and Birchler 2008; Iovene et al. 2008, Szinay et al. 2008). A disadvantage of FISH is limited sensitivity and usually only sequences greater than 10 kilobases

(kb) can be visualized on metaphase chromosomes. Still, researchers have successfully used FISH to map single-copy sequences from 3 to 7 kb in maize (Lamb et al. 2007; Danilova and Birchler 2008), barley (Karafiátová et al. 2013) and wheat (Danilova et al. 2012, 2014). These authors reported the development of cytogenetic markers for particular chromosomal regions by selecting from numerous DNA clones those that produced distinguishable FISH signals. However, the ability to use FISH to place any marker onto chromosomes, as opposed to selecting only those few clones that show clear signals, would be highly beneficial.

Tyramide (tyr) FISH is an approach to increase the sensitivity of FISH (Raap et al. 1995) and has been successfully applied to plants (Khrustaleva and Kik 2001; Stephans et al. 2004; Perez et al. 2009; Sanz et al. 2012; Kirov et al. 2014b; Romanov et al. 2015). Tyr-FISH takes advantage of an enzymatic reaction to deposit multiple copies of reactive tyramide conjugates at specific positions on chromosomes, resulting in up to 100-fold amplification of the fluorescent signal (Van Gijlswijk et al. 1997; Speel et al. 1999). However, the technique has not become routine because of inconsistent results for some probes, low detection frequency and low repeatability among different laboratories (Jiang and Gill 2006). Tyr-FISH is highly dependent on the quality of slide preparation, and often a cDNA probe can produce multiple signals because exon sequences can be conserved across a large number of genes (Kirov et al. 2015; Romanov et al. 2015). In our previous research, we used genomic fragments possessing both exons and introns as probes to enlarge the target DNA sizes and increase hybridization specificity (Kirov et al. 2014b, 2015; Romanov et al. 2015), and developed a robust and routine method for high-quality chromosome preparations for plants (Kirov et al. 2014a).

Table 2 Sequences of primers used to produce genomic amplicons for Tyramide-FISH analyses

Marker	Direction ^z	Primer sequences (5' to 3')	Size ^y
Isotig 29186 genomic	F	AAAACCACCTCTGACGTTGC	800
	R	ACAGGCACCATGTTTTTGGT	
Isotig 34671 genomic	F	TTTTGACAAGAAAAAGGATTGGA	2251
	R	GATAATGGCGAGGGATTCAA	
Isotig 30856 genomic	F	AGGGACGTTGTTTCATGGAG	1200
	R	AATGCGAACTTTTCATCTGTTT	
API21 cDNA	F	GTAAAACGACGGCCAGTG	666
	R	GGAAACAGCTATGACCATG	
AOB272 genomic	F	TTTGCATTGGCTTTTGTGTTGT	1200
	R	CAAAGATTACATTACAACGCATCA	

^z F forward, R reverse

^y Amplicon sizes in basepairs

In this research, we used tyr-FISH to physically locate markers linked to the *Ms* locus onto onion chromosomes with different degrees of compaction (mitotic metaphase, pachytene, and super-stretched pachytene). We demonstrate that *Ms* is located on the long arm of onion chromosome 2 close to the centromere, where recombination occurs less frequently.

Materials and methods

Isolation of genomic clones corresponding to markers linked to *Ms*

Five onion cDNAs linked to *Ms* were used. API21 and AOB272 (Genbank accessions AA451546 and AA451592) reveal restriction fragment length polymorphisms (RFLP) mapping 63 and 0.9 cM, respectively, from *Ms* (Gokce et al. 2002; Martin et al. 2005). Single nucleotide polymorphisms (SNPs) in cDNA isotigs 29186, 34671, and 30856 showed linkage disequilibrium with *Ms* among plants randomly selected from open-pollinated populations of onion (Havey 2013). Primers (Table 2) were designed to produce genomic amplicons, which were cloned after TA tailing into the pGEM-T Easy vector using the manufacturer's protocol (Promega, Madison WI USA), Sanger sequenced (Sambrook et al. 1989), and aligned using Sequencer version 5.1 (Gene Codes, Ann Arbor MI USA) to confirm identities with the original cDNA. Introns in genomic amplicons were identified after alignment with the corresponding cDNA sequences. Genomic sequences were evaluated for repetitive elements using CENSOR (Jurka et al. 2005).

Target material

Spreads of mitotic metaphase chromosomes were prepared from meristematic tips of young roots from onion

seedlings. Root tips were submerged in a saturated aqueous (50 %) solution of α -bromonaphthalene overnight at 4 °C, and subsequently fixed in fresh ethanol/acetic acid (3:1) for 1 h at room temperature (RT). Enzyme digestion and slide preparation were performed as described by Kirov et al. (2014a) with slight modification that after digestion 600 μ l of ice-cold TE buffer was added to cell suspension and centrifuged at 300 \times g for 45 s. Slide pretreatment was performed in 4 % buffered paraformaldehyde in 1 \times PBS (0.13 M NaCl, 7 mM Na₂HPO₄, and 3 mM NaH₂PO₄, pH 7.5) at RT for 10 min with subsequent dehydration in 70, 90 and 100 % ethanol.

Anthers were sampled and fixed in fresh ethanol/acetic acid (3:1) for 1 h at RT. Enzyme digestion and slide preparation of pachytene chromosomes were performed as described by Kirov et al. (2014a) with the modification described above. For super-stretched pachytene chromosomes, suspensions of PMCs at appropriate meiotic stage were prepared according to Kirov et al. (2014a) as described above. The cell suspension was placed on poly-L-lysine-coated slides and air-dried until a granule-like surface appeared, and then was stretched by pressing and rolling in 60 % acetic acid with Triton X-100 as described by Koo and Jiang (2009).

Tyr-FISH

DNA probes were labeled with digoxigenin-11-dUTP or biotin-16-dUTP by nick translation with plasmid DNA using DIG or Biotin-Nick Translation Mix (Roche, Mannheim, Germany). The hybridization mixture contained 50 % (v/v) deionized formamide, 10 % (w/v) dextran sulfate, 2 \times SSC, 0.25 % sodium dodecyl sulfate, and 100–200 ng of probe DNA per slide. The hybridization mix was denatured at 75 °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. Stringent washing at 82 % used 2× SSCT (2× SSC + 0.05 % Tween 20) at 42 °C for 15 min, 2× SSC at 42 °C for 5 min, 0.01× SSC at 55 °C twice for 7 min, and finally 2× SSC at RT for 5 min. The digoxigenin-labeled probe was detected with anti-digoxigenin-HRP (anti-DIG-POD, Roche, Mannheim, Germany) diluted 1:100 in TNB buffer (100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 % blocking reagent, Perkin Elmer, Waltham, Massachusetts, USA) and the biotin-labeled probe was detected with streptavidin-HRP diluted 1:1000 in TNB buffer according to the protocol of the TSA-direct kit (PerkinElmer, Waltham, Massachusetts, USA). The tyramide detection solution was prepared by thoroughly mixing 1:50 tyramide-CY3 or tyramide-fluorescein. Incubation times with the tyramide solution varied from 5 to 9 min. In sequential tyr-FISH before second round of detection, remaining HRP activity was deactivated by incubating slides in 3 % H₂O₂ in TN buffer (100 mM Tris–HCl pH 7.5, 150 mM NaCl) for 20 min. Chromosomes were counterstained in 1.5 µg/ml DAPI in Vectashield anti-fade (Vector Laboratories, <http://www.vectorlabs.com>). Slides were examined under a Zeiss AxioImager M1 microscope (<http://www.zeiss.com/>) or Olympus BX60 microscope (<http://www.olympusmicro.com/>) equipped with epifluorescence illumination, and small band filter sets for DAPI, FITC, and Cy3 fluorescence. Selected images were captured using a digital AxioCam camera (<http://www.zeiss.com/>) or a SenSys charge-coupled device camera (Photometrics, Tucson, AZ). Image processing and thresholding were performed using META IMAGING SERIES 7.5 (<http://meta.moleculardevices.com>) or AxioVisionv.4.6 image analysis software. Final image optimization was performed using Adobe PHOTOSHOP (<http://www.adobe.com>).

The captured images of the chromosomes and position of tyr-FISH signals were measured using the program MicroMeasure version 3.2 (Reeves and Tear 2000). Only non-overlapping chromosomes 2 were used for measurements of positions of tyr-FISH signals. The relative position of hybridization sites on chromosomes (RPHC) was calculated as the ratio of the distance between the site of hybridization and the centromere to the length of the chromosome arm. Statistical analysis was performed using one-way ANOVA tests with arcsine square root transformation of proportional data of the relative positions of tyr-FISH signals.

Results

RFLP marker AOB272 and SNPs in isotigs 29186, 30856, and 34671 are tightly linked to the *Ms* locus on chromosome 2 of onion (Martin et al. 2005; Havey 2013). An

RFLP revealed by cDNA clone API21 has been mapped at 63 cM from *Ms* (Martin et al. 2005). Genomic amplicons were produced for the markers tightly linked to *Ms*. These five markers were used as probes for tyr-FISH (Table 2). Sequence alignments of genomic amplicons and the corresponding cDNAs revealed presence of introns with a total length of 962 bp for isotig 34671, 159 bp for AOB272 g (genomic), and no introns for isotigs 29186 and 30856. CENSOR (Jurka et al. 2005) was used to reveal repetitive elements in the genomic amplicons. We did not find sequence similarity to any repeats in the database within sequences of AOB272 g and isotigs 29186 and 34671. The genomic amplicon of isotig 30856 carried a 168-bp region with homology to DNA transposon MuDR and a second 102-bp region with homology to the non-LTR retrotransposon L1.

Tyr-FISH of the largest genomic amplicon (isotig 34671 at 2251 bp) revealed paired signals on the long arm of chromosome 2. A relative position of the hybridization sites on chromosome (RPHC) from the centromere was 0.09 ± 0.02 (Fig. 1a, a'; Table 3). The size and sub-metacentric nature of this signal-bearing chromosome allowed us to identify it as chromosome 2 (length 14.4 ± 0.3 and centromeric index 33.9 ± 1.2 ; de Vries 1990), consistent with genetic mapping of *Ms* to chromosome 2 (Martin et al. 2005). The fluorescence signal appeared as double spots on both chromatids and at least on one homolog of chromosome 2 in 43 % of the observed mitotic metaphase cells (Table 3). Most of the cells revealed double fluorescent signals on only one homolog of chromosome 2. The different frequencies of FISH signal detection between two homologs using unique probes may result from the differences in chromatin accessibility between homologous metaphase chromosomes (Khan et al. 2014, 2015).

Tyr-FISH mapping of genomic amplicons of isotig 34671 and API21 revealed that API21 is located on the short arm of chromosome 2 (Fig. 1b, b', b'') distant from the centromere at a RPHC of 0.75 ± 0.08 (Table 3), consistent with its relatively loose linkage to *Ms* (Fig. 4).

Isotig 30856 was located on the long arm of chromosome 2 at a RPHC of 0.1 ± 0.02 from the centromere (Fig. 1c; Table 3). Although the presence of the repetitive elements in this genomic amplicon produced some background fluorescence, the hybridization signal on both chromatids from the target sequence can be distinguished from the background (Fig. 1c'). The efficiency of signal detection was 18 %, less than half of value observed for the larger genomic amplicon of isotig 34671 (Table 3).

Isotig 29186 was placed on the long arm of chromosome 2 at a RPHC of 0.1 ± 0.03 from the centromere (Fig. 1d, d'; Table 3). The target sequence for isotig

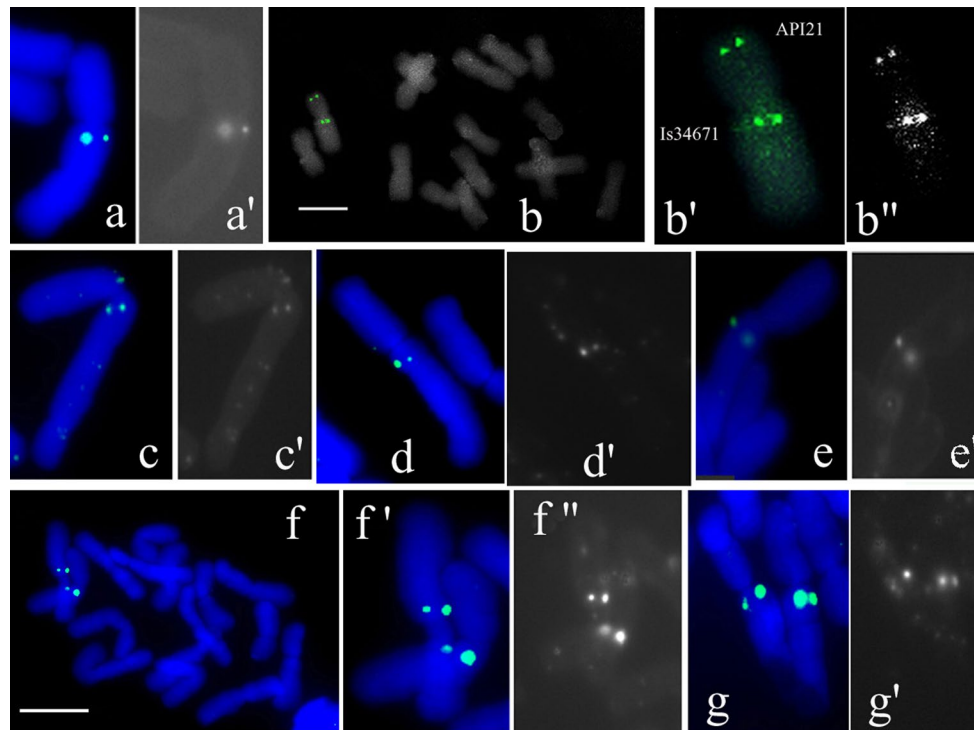


Fig. 1 Tyramide (tyr) FISH mapping of molecular markers linked to the *Ms* locus onto mitotic metaphase chromosome 2 of onion. Genomic amplicons of four (AOB 272 and isotigs 34671, 30856 and 29186 markers were labeled with Dig-11dUTP and detected with Tyramide-FITC: **a** probing with isotig 34671; **b** probing in one hybridization mix with both isotig 34671 and API21, mapped 63 cM from *Ms*, fluorescent signals were detected on only one homolog

of chromosome 2 due to the difference in the probe accessibility between the two homologs; **c** probing with isotig 30856; **d** probing with isotig 29186; **e** probing with AOB 272g; **f**, **g** probing with four amplicons in one hybridization mix (extracted two homologous chromosome 2 from two metaphase cells); **a**, **b**, **b'**, **c**, **d**, **e**, **f**, **f'**, and **g** merged DAPI and FITC filter images; **a'**, **b''**, **c'**, **d'**, **e'**, **f''**, and **g'** FITC filter images). *Cen* centromere. Scale bars = 10 μ m

Table 3 Relative positions and detection frequencies of tyr-FISH signals from molecular markers on mitotic metaphase chromosome 2 of onion

Probe	Relative position ^z		% Detection		
	Mean \pm SD	<i>n</i> ^y	Frequency	<i>n</i> ^x	
Isotig 34671	0.09 \pm 0.02 Long arm	12	43	56	
Isotig 30856	0.10 \pm 0.02 Long arm	8	18	45	
Isotig 29181	0.10 \pm 0.03 Long arm	6	15	43	
AOB272 g (genomic)	0.17 \pm 0.02 Long arm	6	19	41	
All four probes (isotigs 29181, 30856, and 34671 and AOB272 g) in one hybridization mix	Proximal 0.09 \pm 0.02	10	85	20	
	Distal 0.20 \pm 0.02				
API21	Long arm				
	0.75 \pm 0.03 Short arm	5	9	55	

^z The relative positions of hybridization sites on chromosomes were calculated as the ratio of the distance between the site of hybridization and the centromere to the length of the chromosome arm

^y *n* number of non-overlapping the analyzed chromosomes 2 used to measure positions of hybridization signals

^x *n* number of mitotic metaphase cells analyzed

29186 was 846 bp and the fluorescence signal was detected in 15 % of observed mitotic metaphase cells (Table 3).

The genomic amplicon AOB 272g (1198 bp) revealed a more distal location at a RPHC of 0.17 ± 0.02 from the centromere of chromosome 2 and 19 % of the efficiency

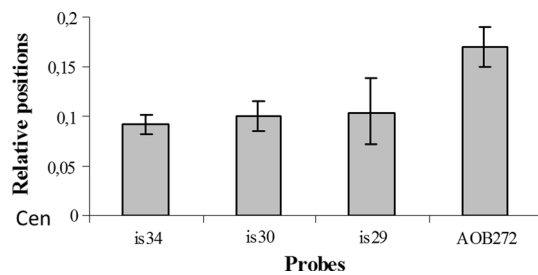


Fig. 2 Diagram of the relative positions of hybridization sites of AOB 272g and isotigs 34671 (is34), 30856 (is30); and 29181 (is29) on mitotic metaphase of onion chromosome 2. *Cen* centromere. The bars denote 95 % confidence intervals

of the signal detection (Fig. 1e, e'; Table 3). Statistically significant differences in RPHC were found between AOB 272g and the isotigs (one-way ANOVA $F = 18.52$, $P < 0.001$; Fig. 2). When amplicons of the four markers tightly linked to *Ms* (AOB 272g and isotigs 29186, 34671 and 30856) were pooled into one labeling reaction and hybridized to mitotic metaphase chromosomes, a single position was revealed near the centromere of chromosome 2 (Fig. 1f, f', f'', g, g'). The fluorescence signal was more intense as compared to the signals of the individual probes and the position of the proximal border of the signal was 0.09 ± 0.02 and the distal border was 0.2 ± 0.02 (Table 3). As an explanation of this result may be the mechanism of tyr-FISH detection based on the enzyme catalyzed deposition of many tyramide molecules. The advantage of

tyr-FISH is a highly increased sensitivity and the disadvantage lies in lowering of resolution. The tyramide deposition reaction runs very quickly and minor differences in amplification reaction time may lead to variations in the signal intensities. In an attempt to better resolve fluorescence signals, the amplification step with tyramide-FITC was reduced from 9 min to 5 min; however, this resulted in substantial decrease in the detection sensitivity and efficiency and did not improve resolution. The fluorescence signals arising from the pooled sequences were detected in 85 % of observed mitotic metaphase cells and 47 % of those showed signals on both homologs.

A single position was also revealed when these pooled amplicons were hybridized to less condensed pachytene chromosomes (Fig. 3a). Comparing the lengths of chromosome 2 at mitotic metaphase (14.4 μm) with pachytene (112.6 μm mean synaptonemal complex length of chromosome 2; Albini and Jones 1988), a 7.8-fold length difference was found. For comparison, this difference was 15-fold for tomato, >20-fold for *Arabidopsis*, and 40-fold for rice (de Jong et al. 1999).

To overcome the limit of spatial resolution, stretching of pachytene chromosomes (Koo and Jiang 2009) was applied to onion meiotic chromosomes. When isotig 34671 (digoxigenin-labeled and detected with tyramide-FITC) and AOB 272g (biotin-labeled and detected with tyramide-Cy3) were simultaneously hybridized to stretched pachytene chromosomes, distinct green and red fluorescent signals with partial overlapping were revealed. Isotig 34671 (green signal,

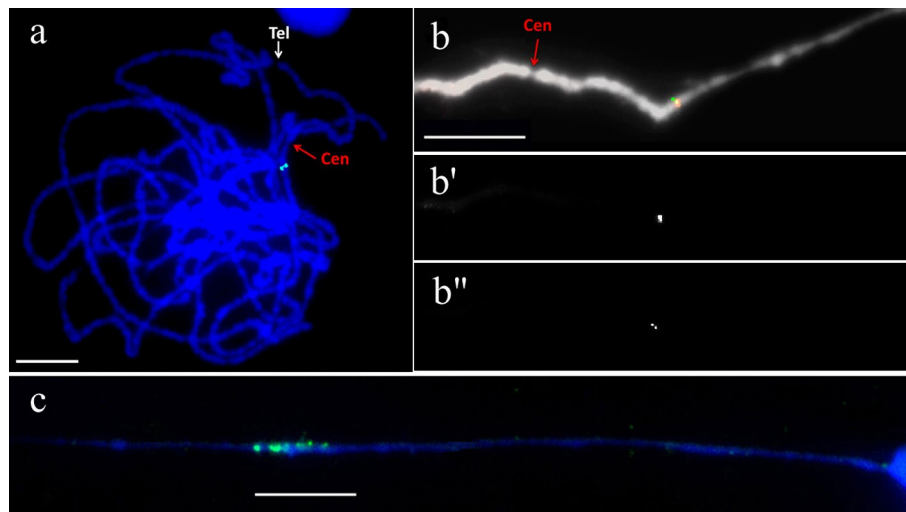


Fig. 3 Tyramide (tyr) FISH mapping of markers tightly linked to *Ms* locus on pachytene chromosomes and super-stretched pachytene chromosome: **a** four genomic amplicons (AOB 272g, and isotigs 34671, 30856 and 29186) labeled with Dig-11dUTP and detected with Tyramide-FITC on pachytene chromosomes; **b** AOB 272g labeled with Biotin-16dUTP and detected with Tyramide-Cy3 (red; **b'** Cy3 filter image) and isotig 34671 labeled with Dig-11dUTP

and detected with Tyramide-FITC (green; **b''** FITC filter image) on stretched pachytene; **c** four amplicons (AOB 272g, and isotigs 34671, 30856 and 29186) labeled with Dig-11dUTP and detected with Tyramide-FITC on super-stretched pachytene. *Tel* telomeric end of the short arm of chromosome 2, *Cen* putative position of centromere. Scale bars = 10 μm (color figure online)

Fig. 4 Alignment of the genetic and cytogenetic maps of onion chromosome 2. The genetic map (*left figure*) is the center part of the entire linkage group described by Martin et al. (2005) distances in centiMorgans are shown on the left of linkage group. The physical positions (*right figure*) of five mapped markers are expressed as percentage of the fractional length (distance from the end of the short arm to the signals divided by the length of the entire chromosome). Corresponding positions on the genetic map are indicated with *lines*

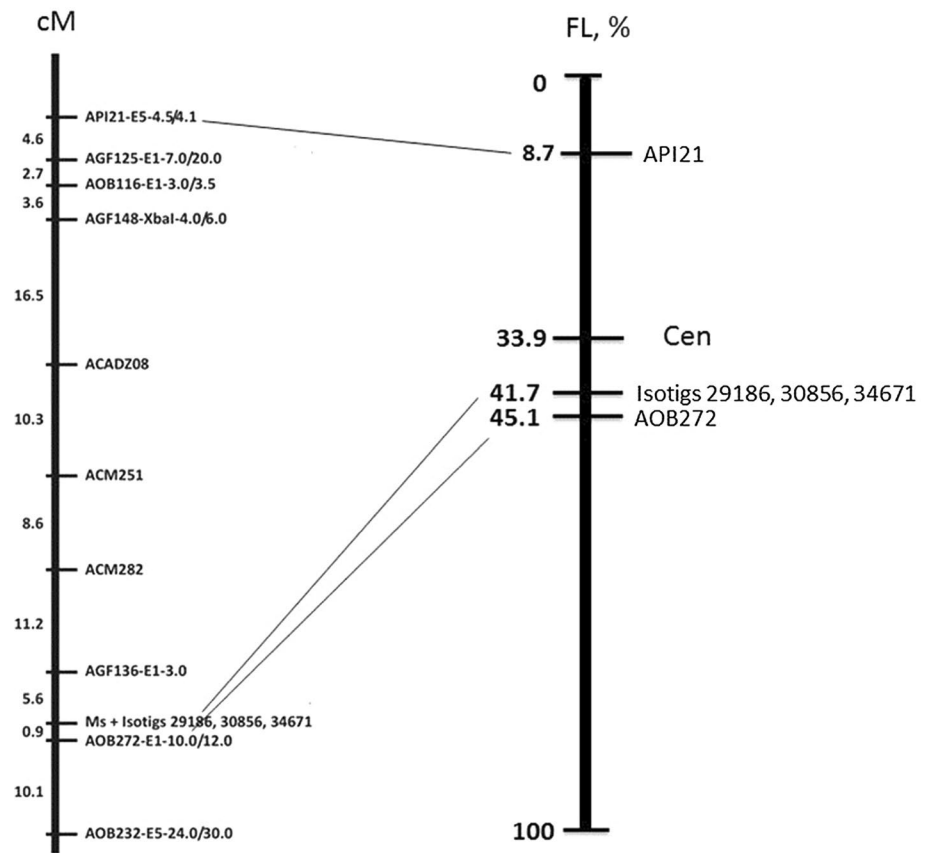


Fig. 3b, b'') was located more proximal relative to AOB 272g (red signal, Fig. 3 b, b'). On super-stretched pachytene chromosomes probed with a cocktail of four monochromic-labeled amplicons closely linked to *Ms*, tyr-FISH signals were readily separated from each other (Fig. 3c). The distance between signals varied depending on the degree of stretching; the track of the signals for the highest degree of stretched pachytene had a total length of $7.4 \pm 0.6 \mu\text{m}$.

The tyr-FISH map generated in this research was compared with the genetic linkage map (Martin et al. 2005). The physical positions of five mapped markers were expressed as a percentage of the fractional length from the end of the short arm of the chromosome 2, and corresponded with their positions in the genetic map (Fig. 4).

Discussion

Genes may be located in chromosome regions experiencing relatively low recombination, and markers showing tight genetic linkage may be quite physically distant from each other. Knowledge about the chromosomal locations of genetically linked traits and markers is important towards eventual cloning of target genes. Genomic in situ hybridization (GISH) has been used to assign a pollen fertility gene

to specific chromosome regions in interspecific hybrids of *Allium* (Yamashita et al. 2005) and *Helianthus* (Liu et al. 2013). Direct mapping of a small single-copy DNA fragment (less than 1 kb) on a physical plant chromosome remains a challenge because of the limit of FISH sensitivity. We exploited the increased sensitivity of tyr-FISH (Van Gijlswijk et al. 1997) to physically assign relatively short genomic amplicons (846–2251 bp) and a cDNA clone (666 bp) to one onion chromosome. The efficiency and sensitivity of tyr-FISH depend on ability of the probe to access the target DNA, as well as quality of the probe and slide preparation. Probes for tyr-FISH in our study did not carry repetitive motifs, except for isotig 30856 which possessed short repetitive sequences that did not mask signals. Slide preparations of mitotic metaphase and pachytene chromosomes were obtained from cell suspensions using the “SteamDrop” method (Kirov et al. 2014a) and a relatively low concentration (25 %) of acetic acid to produce high signal-to-noise ratios. The more common usage of 45 % acetic acid significantly diminished the height of chromosomes as revealed by atomic force microscopy of barley chromosomes (Sugiyama et al. 2004). Flattening of chromosomes over the glass slides may also impede access to target sites by the labeled probe. The efficiency of tyr-FISH to reveal the location of 5.5 kb (the total length of all four markers

genetically linked to *Ms*) was 85 % and signals on both homologues were observed in 47 % of cells (Fig. 1f, f', f', g, g'; Table 3). When the target sequence was reduced from 5.5 kb to 2.3 kb, the percentage of chromosomes showing signals decreased from 85 to 43 %. Further reducing the target length to 0.8 kb (isotig 29181) decreased detection efficiency to 15 %. The lowest efficiency (9 %) of tyr-FISH detection was for a target site of 0.7 kb (API21). However, detection efficiency might also depend on the level of condensation along chromosomes. Tyr-FISH detection of a 0.7 kb T-DNA insertion in transgenic shallot (*Allium cepa*) was 37 % (Khrustaleva and Kik 2001). The T-DNA insert was distally located on the long arm of chromosome 1 in a transcriptionally active and less condensed euchromatic region. The influence of chromosome condensation on the efficiency of tyr-FISH to detect single-copy sequences has also been demonstrated in wheat (Perez et al. 2009). Our results indicate that 2 kb is the minimum probe size for reliable detection when observing only a few metaphase cells with tyr-FISH under our experimental conditions. Probe sizes less than 2 kb can be located on chromosomes, but more cells need to be analyzed because detection efficiency is significantly decreased. Nevertheless, tyr-FISH successfully placed single-copy sequences from RFLP or SNP markers onto onion chromosomes, accelerating alignment of recombinant and cytogenetic maps as an aid for map-based cloning of important genes.

Spatial resolution is the shortest physical distance between adjacent sequences that can be resolved using fluorescence microscopy (de Jong et al. 1999; Cheng et al. 2002). A minimum separation of two to 10 Mb is necessary to resolve FISH signals from two adjacent sequences on metaphase chromosomes of numerous plants (Pedersen and Linde-Laursen 1995; de Jong et al. 1999; Cheng et al. 2002; Jiang and Gill 2006; Lamb et al. 2007). In this research, four probes were co-localized in a centromeric region that could not be resolved with tyr-FISH on both mitotic metaphase and pachytene chromosomes (Figs. 1f, f', f', g, g', 2a). The mean condensation of an onion mitotic metaphase chromosome has been estimated at 249.6 Mb/ μ m, assuming uniform condensation along the entire chromosome (Khrustaleva and Kik 2001). This is much greater than the condensation of tomato metaphase chromosomes at 40.6 Mb/ μ m (Anderson et al. 1985) and on average one onion chromosome possesses the same amount of DNA as the entire diploid genome of tomato. Assuming that a maximum distance of 0.2 μ m can be resolved using fluorescence microscopy, we estimate that the spatial resolution on onion metaphase chromosomes may be up to 50 Mb. Less condensed pachytene chromosomes have been used for mapping of single-copy sequences in plants with relatively small chromosomes such as tomato (Szinay et al. 2008), potato (Iovene et al. 2008), Arabidopsis (Fransz

et al. 2000), and rice (Cheng et al. 2001). Pachytene chromosomes of onion are only 7.8 times less condensed than metaphase chromosomes, and identification of individual onion chromosomes during pachytene is difficult (de Jong et al. 1999).

Super-stretching of pachytene chromosomes can increase lengths by 20 times their regular sizes. Strikingly, maize pachytene chromosomes can be stretched up to 1000 μ m (Koo and Jiang 2009). We applied this technique to onion pachytene chromosomes and were able to reveal the location of marker AOB 272g relative to a SNP marker (isotig 34671) and the centromere (Fig. 2b, b', b''). On super-stretched pachytene chromosomes, four markers (AOB 272g and isotigs 29186, 30856, and 34671) were visualized as a linear string of fluorescent signals measuring $7.4 \pm 0.6 \mu$ m (Fig. 2c). If the lengths of super-stretched pachytene chromosome are assumed to be 20 times longer than regular pachytene chromosomes and correspond to 1.5 Mb/ μ m (Koo and Jiang 2009), the markers would be located across a 10-Mb region. However, precise estimation of distances between these markers would require sequencing of a large genomic clone or contig from this region. The RFLP and SNP markers linked to *Ms* were used to screen a BAC library of onion (Suzuki et al. 2001), but were not able to isolate BACs carrying these markers likely due to its relatively low (0.3 \times) coverage.

CMS has been widely utilized for large-scale production of hybrid seed of many crops (Havey 2004), and hybrid development can be aided by genetic markers distinguishing cytoplasm and closely tagging nuclear male-fertility restoration loci (Gökçe et al. 2002). We used tyr-FISH to successfully assign molecular markers tightly linked to the *Ms* locus to a region adjacent to centromere on long arm of onion chromosome 2 (Fig. 1). The more loosely linked AOB 272g marker was distally located relative to the more closely linked SNP markers (Martin et al. 2005; Havey 2013). Albini and Jones (1988) assessed the locations of chiasmata along onion chromosomes and observed that regions near the centromeres experience significantly less recombination relative to the middle and distal regions, consistent with observations from other plants (Tankley et al. 1992; Werner et al. 1992; Sherman and Stack 1995; Künzel et al. 2000; Haupt et al. 2001; Anderson et al. 2003). Less recombination near the centromere of onion chromosome 2 indicates that tightly linked markers (Table 1) in this region may be physically distant from each other and *Ms*. This does not diminish the usefulness of these molecular markers to predict genotypes at *Ms*; however, it does indicate that eventual map-based cloning of *Ms* may be arduous. As the cost of DNA sequencing continues to decline, the nuclear genome of onion will eventually be sequenced and assembled. Nevertheless, identification of candidates for the *Ms* locus may be difficult because

flanking markers may not locate onto a single contig. A more fruitful approach to clone *Ms* may be transcriptome sequencing of well-characterized genetic stocks (such as S MsMs, N MsMs, and N msms), evaluating for gene-expression differences among these male-fertile onions.

Author contribution statement LK, JJ, MJH designed the experiments; LK performed the experiments; LK, JJ, MJH analyzed the results and wrote the paper.

Acknowledgments We gratefully acknowledge the support of a research fellowship to LK (IIE Grantee ID 68130146) from the Fulbright-Hayes Program.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Disclaimer Names are necessary to report factually on available data; however, the U.S. Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

References

- Albini SM, Jones GH (1988) Synaptonemal complex spreading in *Allium cepa* and *Allium fistulosum*. II. Pachytene observations: the SC karyotype and the correspondence of late recombination nodules and chiasmata. *Genome* 30:399–410. doi:10.1139/g88-069
- Anderson LK, Stack SM, Fox MH, Zhang C (1985) The relationship between genome size and synaptonemal complex length in higher plants. *Expl Cell Res* 156:367–377. doi:10.1016/0014-4827(85)90544-0
- Anderson LK, Doyle GG, Brigham B, Carter J, Hooker KD, Lai A, Rice M, Stack SM (2003) High-resolution crossover maps for each bivalent of *Zea mays* using recombination nodules. *Genetics* 165:849–865
- Bang H, Kim S, Park SO, Yoo KS, Patil BS (2013) Development of a codominant CAPS marker linked to the *Ms* locus controlling fertility restoration in onion (*Allium cepa* L.). *Sci Hortic* 153:42–49. doi:10.1016/j.scienta.2013.01.020
- Bennett MD, Leitch IJ (2012) Plant DNA C-values database (release 6.0, Dec. 2012) <http://www.kew.org/cvalues/>
- Cheng Z, Buell CR, Wing RA, Gu M, Jiang J (2001) Toward a cytological characterization of the rice genome. *Genome Res* 11:2133–2141. doi:10.1101/gr.194601
- Cheng ZK, Buell CR, Wing RA, Jiang JM (2002) Resolution of fluorescence in situ hybridization mapping on rice mitotic prometaphase chromosomes, meiotic pachytene chromosomes and extended DNA fibers. *Chrom Res* 10:379–387. doi:10.1023/A:1016849618707
- Cho KS, Hong SY, Kwon YS, Woo JG, Moon JY, Ryu SY, Park HG (2005) Selection of maintainer line in open-pollinated onion (*Allium cepa* L. cv. “Manchuhwang”) using SCAR marker linked to cytoplasmic male sterile factor. *Korean J Breed* 37:133–137. doi:10.1590/S0103-90162010000200015
- Cho KS, Yang TJ, Hong SY, Kwon YS, Woo JG, Park HG (2006) Determination of cytoplasmic male sterile factors in onion (*Allium cepa* L.) using PCR-RFLP and SNP markers. *Mol Cells* 21:411–417
- Danilova T, Birchler J (2008) Integrated cytogenetic map of mitotic metaphase chromosome 9 of maize: resolution, sensitivity and banding paint development. *Chromosoma* 117:345–356. doi:10.1007/s00412-008-0151-y
- Danilova TV, Friebe B, Gill BS (2012) Single-copy gene fluorescence in situ hybridization and genome analysis: *Acc-2* loci mark evolutionary chromosomal rearrangements in wheat. *Chromosoma* 121:597–611. doi:10.1007/s00412-012-0384-7
- Danilova TV, Friebe B, Gill BS (2014) Development of a wheat single gene FISH map for analyzing homoeologous relationship and chromosomal rearrangements within the Triticeae. *Theor Appl Genet* 127:715–730. doi:10.1007/s00122-013-2253-z
- de Courcel A, Veder F, Boussac J (1989) DNA polymorphism in *Allium cepa* cytoplasm and its implications concerning the origin of onions. *Theor Appl Genet* 77:793–798. doi:10.1007/bf00268328
- de Jong H (2003) Visualizing DNA domains and sequences by microscopy: a fifty-year history of molecular cytogenetics. *Genome* 46:943–946. doi:10.1139/g03-107
- de Jong H, Fransz P, Zabel P (1999) High resolution FISH in plants—technique and application. *Trend Plant Sci* 4:258–263. doi:10.1016/S1360-1385(99)01436-3
- de Vries JN (1990) Onion chromosome nomenclature and homoeology relationships—workshop report. *Euphytica* 49:1–3. doi:10.1007/bf00024124
- Engelke T, Terefe D, Tatlioglu T (2003) A PCR-based marker system monitoring CMS-(S), CMS-(T) and (N)-cytoplasm in the onion (*Allium cepa* L.). *Theor Appl Genet* 107:162–167. doi:10.1007/s00122-003-1230-3
- Figueroa DM, Bass HW (2010) A historical and modern perspective on plant cytogenetics. *Brief Funct Genom* 9:95–102. doi:10.1093/bfgp/elp058
- Fransz PF, Armstrong S, de Jong JH, Parnell LD, van Drunen C, Dean C, Zabel P, Bisseling T, Jones GH (2000) Integrated cytogenetic map of chromosome arm 4S of *A.thaliana*: structural organization of heterochromatic knob and centromere region. *Cell* 100:367–376. doi:10.1016/s1369-5266(00)80013-8
- Gökçe AF, Havey MJ (2002) Linkage equilibrium among tightly linked RFLPs and the *Ms* locus in open-pollinated onion populations. *J Amer Soc Hort Sci* 127:944–946
- Gökçe AF, McCallum J, Sato Y, Havey MJ (2002) Molecular tagging of the *Ms* locus in onion. *J Amer Soc Hort Sci* 127:576–582
- Haupt W, Fischer TC, Winderl S, Fransz P, Torres-Ruiz RA (2001) The centromere1 (CEN1) region of *Arabidopsis thaliana*: architecture and functional impact of chromatin. *Plant J* 27:285–296. doi:10.1046/j.1365-3113x.2001.01087.x
- Havey MJ (1993) A putative donor of S-cytoplasm and its distribution among open-pollinated populations of onion. *Theor Appl Genet* 86:128–134. doi:10.1007/bf00223817
- Havey MJ (1995) Identification of cytoplasmic maintainers using the polymerase chain reaction to aid in the extraction of maintainer lines from open-pollinated populations of onion. *Theor Appl Genet* 90:263–268. doi:10.1007/bf00222212
- Havey MJ (2004) The use of cytoplasmic male sterility for hybrid seed production. In: Daniel H, Chase C (eds) *Molecular biology and biotechnology of plant organelles*. Kluwer Academic Publishers, The Netherlands, pp 623–634
- Havey MJ (2013) Single nucleotide polymorphisms in linkage disequilibrium with the male-fertility restoration (*Ms*) locus of onion. *J Amer Soc Hort Sci* 138:306–309
- Holford P, Croft J, Newbury H (1991) Differences between and possible origins of the cytoplasmic male sterile and male-sterile onions (*Allium cepa* L.). *Theor Appl Genet* 82:737–744. doi:10.1007/bf00227319

- Hou Y, Miao J, Liu B, Yang Y, Zhang Y, Tahara Y, Meng Q, He Q, Kitano H, Wu X (2012) The expression of pectin methyl-esterase in onion flower buds is associated with the dominant male-fertility restoration allele. *Plant Breed* 131:211–216. doi:10.1111/j.1439-0523.2011.01907.x
- Iovene M, Wielgus SM, Simon PW, Buell CR, Jiang JM (2008) Chromatin structure and physical mapping of chromosome 6 of potato and comparative analyses with tomato. *Genetics* 180:1307–1317. doi:10.1534/genetics.108.093179
- Jiang J, Gill BS (2006) Current status and the future of fluorescence in situ hybridization (FISH) in plant genome research. *Genome* 49:1057–1068. doi:10.1139/g06-076
- Jones A, Clarke A (1943) Inheritance of male sterility in the onion and the production of hybrid seed. *Proc Amer Soc Hort Sci* 43:189–194
- Jones HA, Davis GN (1944) Inbreeding and heterosis and their relation to the development of new varieties of onions. *US Dept Agr Tech Bul No 874*, Washington, p 28
- Jurka J, Kapitonov VV, Pavlicek A, Klonowski P, Kohany O, Walichiewicz J (2005) Repbase update, a database of eukaryotic repetitive elements. *Cytogenet Genome Res* 110:462–467. doi:10.1159/000084979
- Karafiátová M, Bartoš J, Kopecký D, Ma L, Sato K, Houben A, Stein N, Doležel J (2013) Mapping nonrecombining regions in barley using multicolor FISH. *Chrom Res* 21:9380. doi:10.1007/s10577-013-9380-x
- Khan WA, Rogan PK, Knoll JHM (2014) Localized, non-random differences in chromatin accessibility between homologous metaphase chromosomes. *Mol Cytogenet* 7:70. doi:10.1186/s13039-014-0070-y
- Khan WA, Rogan PK, Knoll JHM (2015) Reversing chromatin accessibility differences that distinguish homologous mitotic metaphase chromosomes. *Mol Cytogenet* 8:65. doi:10.1186/s13039-015-0159-y
- Khrustaleva LI, Kik C (2001) Localization of single copy T-DNA insertion in transgenic shallots (*Allium cepa* L.) by using ultra-sensitive FISH with tyramide signal amplification. *Plant J* 25:699–707. doi:10.1046/j.1365-313x.2001.00995.x
- Khrustaleva LI, de Melo PE, van Heusden AW, Kik C (2005) The integration of recombination and physical maps in a large-genome monocot using haploid genome analysis in a trihybrid *Allium* population. *Genetics* 169:1673–1685. doi:10.1534/genetics.104.038687
- Kim S (2014) A codominant molecular marker in linkage disequilibrium with a restorer-of-fertility gene (*Ms*) and its application in reevaluation of inheritance of fertility restoration in onions. *Mol Breed* 34:769–778. doi:10.1007/s11032-014-0073-8
- Kim S, Lee E, Cho DY, Han T, Bang H, Patil BS, Ahn YK, Yoon M (2009) Identification of a novel chimeric gene, orf725, and its use in development of a molecular marker for distinguishing among three cytoplasm types in onion (*Allium cepa* L.). *Theor Appl Genet* 118:433–441. doi:10.1007/s00122-008-0909-x
- Kirov I, Divashuk M, Van Laere K, Soloviev A, Khrustaleva L (2014a) An easy “SteamDrop” method for high quality plant chromosome preparation. *Mol Cytogenet* 7:21. doi:10.1186/1755-8166-7-21
- Kirov I, Van Laere K, De Riek J, De Keyser E, Van Roy N, Khrustaleva L (2014b) Anchoring linkage groups of the Rosa genetic map to physical chromosomes with tyramide-FISH and EST-SNP markers. *PLoS One* 9(4):e95793. doi:10.1371/journal.pone.0095793
- Kirov IV, Van Laere K, Khrustaleva LI (2015) High resolution physical mapping of single gene fragments on pachytene chromosome 4 and 7 of Rosa. *BMC Genet* 16:74. doi:10.1186/s12863-015-0233-9
- Koo D-H, Jiang J (2009) Supper-stretched pachytene chromosomes for fluorescence in situ hybridization mapping and immunodetection of DNA methylation. *Plant J* 59:509–516. doi:10.1111/j.1365-313x.2009.03881.x
- Künzel G, Korzun L, Meister A (2000) Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation break points. *Genetics* 154:397–412
- Lamb JC, Danilova T, Bauer MJ, Meyer JM, Holland JJ, Jensen MD, Birchler JA (2007) Single-gene detection and karyotyping using small-target fluorescence in situ hybridization on maize somatic chromosomes. *Genetics* 175:1047–1058. doi:10.1534/genetics.106.065573
- Lilly JW, Havey MJ (2001) Sequence analysis of a chloroplast intergenic spacer for phylogenetic estimates and a PCR-based polymorphism detecting mixtures of male-fertile and male-sterile cytoplasmic onion. *Theor Appl Genet* 102:78–82. doi:10.1007/s001220051620
- Liu Z, Wang D, Feng J, Seiler GJ, Cai X, Jan CC (2013) Diversifying sunflower germplasm by integration and mapping of a novel male fertility restoration gene. *Genetics* 193:727–737. doi:10.1534/genetics.112.146092
- Martin W, McCallum J, Shigyo M, Jakse J, Kuhl JC, Yamane N, Sink KC, Town CD, Havey MJ (2005) Genetic mapping of expressed sequences in onion and in silico comparisons show scant colinearity with rice. *Mol Genet Genom* 274:197–204. doi:10.1007/s00438-005-0007-6
- Park J, Bang H, Cho DY, Yoon MK, Patil BS, Kim S (2013) Construction of high-resolution linkage map of the *Ms* locus, a restorer-of-fertility gene in onion (*Allium cepa* L.). *Euphytica* 192:267–278. doi:10.1007/s10681-012-0851-5
- Pedersen C, Linde-Laursen I (1995) The relationship between physical and genetic distances at the *Hor1* and *Hor2* loci of barley estimated by two-colour fluorescence in situ hybridization. *Theor Appl Genet* 91:941–946. doi:10.1007/bf00223904
- Perez R, de Bustos A, Jouve N, Cuadrado A (2009) Localization of Rad50, a single-copy gene, on group 5 chromosomes of wheat, using a FISH protocol employing Tyramide for signal amplification (Tyr-FISH). *Cytogenet Genome Res* 125:321–328. doi:10.1159/000235938
- Raap A, van de Corput M, Vervenne R, van Gijswijk R, Tanke H, Wiegant J (1995) Ultra-sensitive FISH using peroxidase-mediated deposition of biotin- or fluorochrome-tyramides. *Hum Mol Genet* 4:529–534. doi:10.1093/hmg/4.4.529
- Reeves A, Tear J (2000) MicroMeasure for Windows. Version 3.3. <http://www.colostate.edu/Depts/Biology/MicroMeasure>. Accessed 20 Feb 2015
- Romanov D, Divashuk M, Havey MJ, Khrustaleva L (2015) Tyramide-FISH mapping of single genes for development of an integrated recombination and cytogenetic map of chromosome 5 of *Allium cepa* L. *Genome*. doi:10.1139/gen-2015-0019
- Sambrook J, Russell DW (1989) *Molecular cloning: A laboratory manual*, vol 1. Cold Spring Harbor Press, New York, pp 33–162
- Sanz MJ, Loarce Y, Ferrer E, Fominaya A (2012) Use of tyramide-fluorescence in situ hybridization and chromosome microdissection for ascertaining homology relationships and chromosome linkage group associations in oats. *Cytogenet Genome Res* 136:145–156. doi:10.1159/000335641
- Sato Y (1998) PCR amplification of CMS-specific mitochondrial nucleotide sequences to identify cytoplasmic genotypes of onion (*Allium cepa* L.). *Theor Appl Genet* 96:367–370. doi:10.1007/s001220050750
- Satoh Y, Nagai M, Mikami M, Kinoshita T (1993) The use of mitochondrial DNA polymorphism in the classification of individual plants by cytoplasmic genotypes. *Theor Appl Genet* 86:345–348. doi:10.1007/bf00222100

- Sherman JD, Stack SM (1995) Two-dimensional spreads of synaptonemal complexes from Solanaceous plants. VI. High resolution recombination nodule map for tomato (*Lycopersicon esculentum*). *Genetics* 141:683–708
- Speel EJM, Hopman AHN, Komminoth P (1999) Amplification methods to increase the sensitivity of in situ hybridization: play CARD(S). *J Histochem Cytochem* 47:281–288. doi:10.1177/002215549904700302
- Stephens JL, Brown SE, Lapitan NLV, Knudson DL (2004) Physical mapping of barley genes using an ultrasensitive fluorescence in situ hybridization technique. *Genome* 47:179–189. doi:10.1139/g03-084
- Sugiyama S, Yoshino T, Kanahara H, Shichiri M, Fukushi D, Ohtani T (2004) Effects of acetic acid treatment on plant chromosome structures analyzed by atomic force microscopy. *Anal Biochem* 324:39–44. doi:10.1016/j.ab.2003.09.026
- Suzuki G, Ura A, Saito N, Do GS, Seo BB, Yamamoto M, Mukai Y (2001) BAC FISH analysis in *Allium cepa*. *Genes Genet Syst* 76:251–255. doi:10.1266/ggs.76.251
- Szinay D, Chang SB, Khrustaleva L, Peters S, Schijlen E, Bai Y, Stiekema WJ, van Ham RC, de Jong H, Klein Lankhorst RM (2008) High-resolution chromosome mapping of BACs using multi-colour FISH and pooled-BAC FISH as a backbone for sequencing tomato chromosome 6. *Plant J* 56:627–637. doi:10.1111/j.1365-313x.2008.03626.x
- Tanksley SD, Ganai MW, Prince JP, de Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB (1992) High-density molecular linkage maps of the tomato and potato genomes. *Genetics* 132:1141–1160
- van Gijlswijk RPM, Zijlmans HJMA, Wiegant J, Bobrow MN, Erickson TJ, Adler KE, Tanke HJ, Raap AK (1997) Fluorochrome-labeled Tyramides: use in immunocytochemistry and fluorescence in situ hybridization. *J Histochem Cytochem* 45:375–382. doi:10.1177/002215549704500305
- von Kohn C, Kielkowska A, Havey MJ (2013) Sequencing and annotation of the chloroplast DNAs of normal (N) male-fertile and male-sterile (S) cytoplasms of onion and single nucleotide polymorphisms distinguishing these cytoplasms. *Genome* 56:737–742. doi:10.1139/gen-2013-0182
- Werner JE, Endo TR, Gill BS (1992) Toward a cytogenetically based physical map of the wheat genome. *Proc Natl Acad Sci USA* 89:11307–11311. doi:10.1073/pnas.89.23.11307
- Yamashita K-I, Takatori Y, Tashiro Y (2005) Chromosomal location of a pollen fertility-restoring gene, Rf, for CMS in Japanese bunching onion (*Allium fistulosum* L.) possessing the cytoplasm of *A. galanthum* Kar. Et Kir. revealed by genomic in situ hybridization. *Theor Appl Genet* 111:15–22. doi:10.1007/s00122-005-1941-8
- Yang YY, Huo YM, Miao J, Liu BJ, Kong SP, Gao LM, Liu C, Wang ZB, Tahara Y, Kitano H, Wu X (2013) Identification of two SCAR markers co-segregated with the dominant Ms and recessive ms alleles in onion (*Allium cepa* L.). *Euphytica* 190:267–277. doi:10.1007/s10681-012-0842-6