DOI: 10.1007/s10535-015-0558-2 BIOLOGIA PLANTARUM 60 (1): 25-36, 2016

Precise karyotyping of carrot mitotic chromosomes using multicolour-FISH with repetitive DNA

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Abstract

Carrot (*Daucus carota* L.) chromosomes are small and uniform in shape and length. Here, mitotic chromosomes were subjected to multicolour fluorescence *in situ* hybridization (mFISH) with probes derived from conserved plant repetitive DNA (18-25S and 5S rDNA, telomeres), a carrot-specific centromeric repeat (Cent-Dc), carrot-specific repetitive elements (DCREs), and miniature inverted-repeat transposable elements (MITEs). A set of major chromosomal landmarks comprising rDNA and telomeric and centromeric sequences in combination with chromosomal measurements enabled discrimination of carrot chromosomes. In addition, reproducible and unique FISH patterns generated by three carrot genome-specific repeats (DCRE22, DCRE16, and DCRE9) and two transposon families (*DcSto* and *Krak*) in combination with telomeric and centromeric reference probes allowed identification of chromosome pairs and construction of detailed carrot karyotypes. Hybridization patterns for DCREs were observed as pericentromeric and interstitial dotted tracks (DCRE22), signals in pericentromeric regions (DCRE16), or scattered signals (DCRE9) along chromosomes similar to those observed for both MITE families.

Additional key words: *Daucus carota* repetitive elements, fluorescence *in situ* hybridization, miniature inverted-repeat transposable elements.

Introduction

Carrot (*Daucus carota* L., 2n = 2x = 18) is the most economically important member of the *Apiaceae* family, and it ranks among the top ten vegetable crops. The species is a major source of provitamin A carotenoids (Arscott and Tanumihardjo 2010). The haploid genome size of carrot has been estimated at 473 Mbp (Arumuganathan and Earle 1991). Carrot somatic chromosomes are small and uniform in shape and length (Iovene *et al.* 2008, Nowicka *et al.* 2012), therefore they are a difficult object for cytogenetic research. However, progress observed in molecular cytogenetic studies created new opportunities to study plant species with that type of chromosomes. Multicolour fluorescence *in situ* hybridization (mFISH) allows simultaneous application of several DNA probes and their localization on chromosomes. The pattern of hybridization signals greatly facilitates chromosome identification and accurate karyotype analysis (Kato *et al.* 2004, Paesold *et al.* 2012, Wolny *et al.* 2013). Furthermore, comparative studies of plant repetitive sequences are useful for the investigation of evolutionary relationships among plant species (Menzel *et al.* 2008). Various combinations of repetitive sequences, both those occurring in tandem and forming large clusters, such as ribosomal genes, telomeric repeats, and satellite DNAs, including centromere-specific repeats, and those having a dispersed distribution typical for transposable elements have been cytogenetically mapped by FISH (Heslop-Harrison 2000, Schwarzacher 2003). For instance, simultaneous double FISH with 5S and 18S-25S rDNA probes enable discrimination of a

Submitted 21 April 2015, *last revision* 8 June 2015, *accepted* 18 June 2015.
Abbreviations: BAC - bacterial artificial chromosome; DAPI - 4′,6-diamidino-2-phenylindole; DCRE - *Daucus carota* repetitive element; FISH - fluorescence *in situ* hybridization; FITC - fluorescein isothiocyanate; mFISH - multicolour fluorescence *in situ* hybridization; MITE - miniature inverted-repeat transposable element; NOR - nucleolar organizer region; SSC - saline-sodium citrate; TE - transposable element; TIR - terminal inverted repeat; TRS - telomeric repeat sequence; TSD - target site duplications.
Acknowledgments: This research was financed by the Ministry of Science and Higher Education of the Republic of Poland.
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substantial number of chromosomes of the complement of all diploid and tetraploid *Brassica* species (Hasterok *et al.* 2001). A combination of ribosomal genes and telomeric repeats as probes effectively distinguishes chromosomes and allows karyotyping in species belonging to the genera *Pinus* (Hizume *et al.* 2002) and *Centaurea* (Dydak *et al.* 2009), in cucumber cultivars (Hoshi *et al.* 2011), and recently in grass *Phleum echinatum* (Grabowska-Joachimiak *et al.* 2015). Due to the application of conserved plant repetitive sequences and various satellite repeats in one hybridization experiment, chromosomes are identified for *Vicia* species (Navratilova *et al.* 2003), a model plant *Medicago truncatula* (Kulikova *et al.* 2004), several lines of maize (Kato *et al.* 2004), and sugar beet (Paesold *et al.* 2012). High-resolution chromosome mapping of large-insert genomic DNA clones, such as BACs, can be performed using mFISH (Szinay *et al.* 2008, Findley *et al.* 2010, Wolny *et al*. 2013, Idziak *et al.* 2014). Additionally, the mFISH technique has been useful to reveal a chromosome structure by physical mapping of various transposable elements (TEs) (Altinkut *et al.* 2006, Yu *et al.* 2007).

In the case of cultivated carrot and other *Apiaceae* species, the first utilization of the double colour FISH included application of rRNA genes as probes (Iovene *et al.* 2008). In another experiment, Iovene *et al.* (2011) described the pachytene karyotype of carrot based on the FISH with a probe cocktail comprising chromosome-specific carrot BACs. In addition, they identified a tandem satellite-repeat localized in centromeric regions of all carrot chromosomes. In turn, more detailed carrot mitotic karyotype studies were carried out by using arbitrarily amplified DNA fragments as probes for the triple colour FISH (Nowicka *et al*. 2012).

In order to extend carrot cytogenetic research, novel carrot repetitive elements may be a good source of probes for the FISH. The sequences were identified by Cavagnaro *et al.* (2009) following the analysis of BAC-ends of the carrot genome library and named *Daucus carota* repeat elements (DCREs). Despite their abundance in the carrot genome, they do not show any similarity to known repetitive sequences occurring in plant genomes. Also, they do not show any coding capacity. One of the DCRE sequences, DCRE20, was subsequently shown to constitute carrot centromeric repeats (Iovene *et al*. 2011). In total, the other 11 DCREs were estimated to account for ~8.4 % of the carrot genome (Cavagnaro *et al.* 2009).

Miniature inverted-repeat transposable elements (MITEs) are the most abundant group of DNA transposons characterized by a small size (< 600 bp). Like other DNA transposons, MITEs carry terminal inverted repeats (TIRs) and develop target site duplications (TSDs) upon insertion but encode no transposase (Wicker *et al.* 2007). *Tourists* and *Stowaways* are two major groups of plant MITEs (Jiang *et al*. 2004). Whereas *Tourist*-like MITEs are related to and activated by PIF/*Harbinger* transposons, *Stowaway*-like elements are derivatives of *Tc1/mariner* transposons (Turcotte *et al.* 2001, Feschotte *et al.* 2003, Menzel *et al.* 2006). Two groups of miniature inverted-repeat transposable elements, *i.e*., *DcSto* and *Krak*, were identified in carrot. The *DcSto* elements belong to the *Stowaway*-like group, their length is approximately 300 bp and the copy number in the genome of carrot has been estimated at 5 000 (Macko-Podgorni *et al.* 2013). The *Krak* elements belong to the *Tourist*-like MITEs, are less than 400 bp long, and the number of copies in the carrot genome is *ca.* 3 600 (Grzebelus *et al.* 2007, Grzebelus and Simon 2009).

The present study focused on the localization of different types of repetitive probes on somatic chromosomes using the multicolour-FISH. Among the probes were: *1*) conserved repeats, *i.e.*, rDNA and telomeric probes; *2*) carrot-specific centromeric probes described by Iovene *et al*. (2011); *3*) novel carrot repetitive elements identified by Cavagnaro *et al.* (2009); and *4*) two MITE families occurring in the carrot genome (*Stowaway*-like *DcSto* and *Tourist*-like *Krak*). The main objectives of the study were: *1*) to develop an efficient and precise system for the identification of somatic chromosomes in carrot based on repetitive sequences; *2*) to analyze the morphometric features of the carrot karyotype; *3*) to investigate whether the distribution patterns of DCRE and MITE repeats are useful in chromosome identification; and *4*) to investigate the distribution of repetitive sequences on carrot chromosomes.

Materials and methods

Chromosome preparations: Roots of seven-d-old seedlings of *Daucus carota* L. subsp. *sativus* cv. Dolanka grown on a filter paper moistened with distilled water at 24 °C in the dark were used. In order to inactivate the mitotic spindle, meristems were treated with 2 mM 8-hydroxy-quinoline at room temperature for 3 - 4 h, then fixed in a freshly prepared methanol:glacial acetic acid (3:1, v/v) solution for at least 48 h, and stored in the same solution at -20 °C until required. A portion of roots was not treated with 8-hydroxyquinoline and fixed directly after harvest. Slides were prepared according to Nowicka *et al.* (2012) at 37 °C for 45 min using an enzyme mixture consisting of 4 % (m/v) cellulase *Onozuka R10* (*Serva,* Heidelberg, Germany) and 2 % (m/v) pectolyase *Y23* (*Duchefa*, Haarlem*,* The Netherlands) in distilled water, pH 4.8. Chromosomes of good quality were selected using a phase-contrast microscope (*AxioImager M2*, *Zeiss,* Oberkochen, Germany). Before a hybridization step, the slides were pretreated with pepsin (10 μg cm^{-3} in 10 mM Tris-HCl) at 37 °C for 10 min, then rinsed in 2× saline-sodium citrate (SSC) (3 × 5 min), dehydrated in 70, 90, and 100 % (v/v) ethanol (3 min each), and air-dried.

DNA probes and labelling: Three combinations of the following probes were used in the triple colour FISH experiments:
1) Ribosomal probes (the probes of rRNA genes). The rDNA probes pTa71 and pTa794 originally isolated from wheat (Gerlach and Bedbrook 1979, Gerlach and Dyer 1980) were used for the FISH. These probes were kindly provided by M. Iovene (the CNR-Institute of Biosciences and Bioresouces, Bari, Italy). The probe pTa71 contains 18S-25S rDNA genes and spacer regions. The probe pTa794 consists of a 400-bp *Bam*HI fragment including a 120-bp coding sequence for the 5S rDNA gene. The 18S-25S rDNA probe was labelled with digoxigenin-11-dUTP using a nick translation kit (*Roche*, Mannheim, Germany) according to the manufacturer's instructions. The 5S rDNA probe was also labelled with digoxigenin-11-dUTP (*Roche*) by PCR with universal T7 and SP6 primers under the following conditions: 94 °C (30 s); 30 cycles of 94 °C (30 s), 55 °C (30 s), 68 °C (1 min), and a final extension step of 68 °C (5 min). *Quick Spin G-50 Sephadex* columns (*Roche*) were used for purification of labelled DNA.
2) A *Daucus carota* centromeric probe (Cent-Dc). A synthetic 35 nucleotide-long probe 5′-ACTCGTTTG AAGTTGGAAACAACTTGTAGCTTCATT-3′ labelled at the 5′ end with Cy5 (*Metabion,* Martinsried, Germany), named Cent-Dc following a nomenclature proposed by Iovene *et al.* (2011), was used to identify carrot centromeres. The probe was a consensus sequence of the basic monomer constituting the previously described carrot centromeric repeat DCRE20 (Cavagnaro *et al.* 2009).
3) A telomeric probe (TRS). A synthetic oligonucleotide $(5'\text{-TTTAGGG-}3')_5$ of an *Arabidopsis*-type telomeric repeat sequence (TRS) labelled at the 5′ end with Cy3 (*Metabion*) was used as telomeric probe.
4) Probes derived from *Daucus carota* repeat elements (DCREs). Eleven DCRE-specific probes with sizes ranging from 388 to 1 242 bp (Cavagnaro *et al*. 2009) were used. 'Forward' and 'reverse' primers for amplification of DCREs (Table 1 Suppl.) were designed with *Primer3 v. 0.4.0* (Rozen and Skaletsky 2000). Total genomic DNA was extracted from freeze-dried leaf tissue of plants representing cultivated carrots of different origin using a modified protocol of Chomczynski and Sacchi (1987). Approximately 40 ng of genomic DNA was used as template. The PCR was set up in 20 mm^3 with 0.25 mM dNTPs, 1 mM each primer, 1 U of Taq DNA polymerase (*Thermo Scientific*, Waltham, MA, USA), and a 1× Taq buffer. For 10 out of 11 DCREs the following thermal conditions were applied: an initial denaturation at 94 °C (1 min), 30 cycles of 94 °C (30 s), 58 °C (30 s), 68 °C (1 min), and a final extension step of 68 °C (5 min). For DCRE14 the annealing temperature was 53 °C. The products were separated in a 1 % (m/v) agarose gel stained with ethidium bromide, purified with *Wizard SV Gel and PCR Clean-Up* (*Promega*), ligated into *pGEM-T* (*Promega*) and cloned into the *Escherichia coli* strain DH10B. Subsequently, the plasmids were extracted using a *Wizard SV Miniprep* kit (*Promega*), and the target DCRE products were sequenced. Sequencing reactions were performed with *DTSC Premix* (*Beckman Coulter*, Fulerton, CA, USA) as described by the manufacturer and run on a *CEQ8000* automated DNA sequencer (*Beckman Coulter*). The sequences were queried against the GenBank database using *BLASTN* (Altschul *et al.* 1997). All DCREs were labelled with digoxigenin-11-dUTP (*Roche*). In the case of fragments > 500 bp, labelling was carried out by nick translation, and for the products < 500 bp, it was carried out by PCR with primers T7 and SP6 as described above for 5S rDNA. *Quick Spin G-50 Sephadex* columns (*Roche*) were used for purification of the labelled DCRE probes.
5) Probes of miniature inverted-repeat transposable elements (MITEs). Full-length *DcSto1* (Macko-Podgórni *et al*. 2013) and *Krak* elements (Grzebelus *et al*. 2006) were amplified from genomic DNA of the three cultivated carrots. The PCR reactions were prepared in 20 mm^3 and contained 40 ng of genomic DNA, a 1 mM DcS-TIR or Krak-TIR primer (Table 1 Suppl.), 0.25 mM dNTPs (*Thermo Scientific*), 1 U of Taq DNA polymerase (*Thermo Scientific*), and a 1× Taq buffer. The reactions were carried out as follows: 94 °C (1 min), 30 cycles of 94 °C (30 s), 50 °C (40 s), 68 °C (1 min) and 68 °C (4 min). The PCR products were separated in a 1 % (m/v) agarose gel, excised, purified, cloned, and sequenced as described for the DCREs. Both the transposon-specific probes were labelled with digoxigenine-11-dUTP (*Roche*) by PCR as described for the 5S rDNA probe.

Multicolour fluorescent *in situ* hybridization (mFISH): An *in situ* hybridization procedure was carried out according to Nowicka *et al.* (2012) with minor modifications. A hybridization mixture contained a cocktail of three or four probes at a final concentration of each probe of 2 - 3 ng mm^{-3}, 1 μg mm^{-3} sheared herring sperm DNA, 50 % (m/v) deionized formamide, 10 % (v/v) dextran sulphate, and 20× SSC. Digoxygenin labelled probes were immuno-detected with 2 μg cm^{-3} fluorescein isothiocyanate an FITC-conjugated anti-digoxygenin antibody (*Roche*), whereas Cy3- and Cy5-labelled probes were visualized directly. Chromosomes were counterstained with 1 μg cm^{-3} 4′,6-diamidino-2-phenylindole (DAPI) in a *Prolong Gold* antifade solution (*Invitrogen*, Carlsbad, CA, USA).

All images were captured using a *BV MV* camera (*Applied Spectral Imaging*, Edingen-Neckarhausen, Germany) attached to an *AxioImager M2* (*Zeiss*) epifluorescence microscope. The images were captured separately for each fluorochrome using appropriate excitation and emission filters. The monochromatic images were pseudocolored and merged using *Case Data Manager 4.0* (*Applied Spectral Imaging*). Final image adjustments were done with *Adobe Photoshop 5.0* (*Adobe Systems*, San Jose, CA, USA). Measurements of chromosomes were made on the digital images using *Case Data Manager 4.0*.

Chromosome measurements and construction of karyotypes: For karyotype description, 12 well-spread, complete metaphases were measured. For each chromosome, the short and long arms were measured and the total length was calculated. Additionally, a distance of 5S rDNA locus to the centromere was measured. Satellite and nucleolar organizer regions (NOR) were not measured because chromatin condensation at secondary constrictions was highly variable. The chromosome arm ratio (AR = a length of the long arm/a length of the short arm) was used to classify a chromosome type as recognized by Levan *et al.* (1964). The mean length of the karyotype and the relative length of each chromosome expressed as the percentage of the total karyotype length were calculated. Karyotype symmetry was defined according to Stebbins (1971).

To construct karyotypes, chromosomes were paired according to chromosome length, position of rRNA genes, centromeres and telomeres, presence/position of hybridization signals for DCRE and MITE probes, and localization of a prominent satellite. In order to construct an idiogram, chromosome measurements including accurate positions of centromeres, lengths of chromosome arms, a position of the 5S rDNA locus, as well as an approximate position of other hybridization signals were considered.

Results

A set of four probes was used to develop a molecular-cytogenetic karyotype of carrot mitotic chromosomes. Six slide preparations were used in this experiment and more than 50 metaphases were analyzed. The triple colour FISH experiment produced clear hybridization signals (Fig. 1). Both 18S-25S and 5S rDNAs were located as single loci on two different chromosome pairs. The 18S-25S rDNA probe hybridized to a large region of the chromosome, from the distal end of the short chromosome arm across the secondary constriction to the end of the satellite, giving rise to dumbbell-shaped signals. The chromosome pair bearing the 18S-25S rDNA was subtelocentric. A single 5S rDNA locus was located in an intercalary position on the long arm of a submetacentric chromosome pair. Telomeric repeats were detected with the TRS probe at all chromosome ends and not

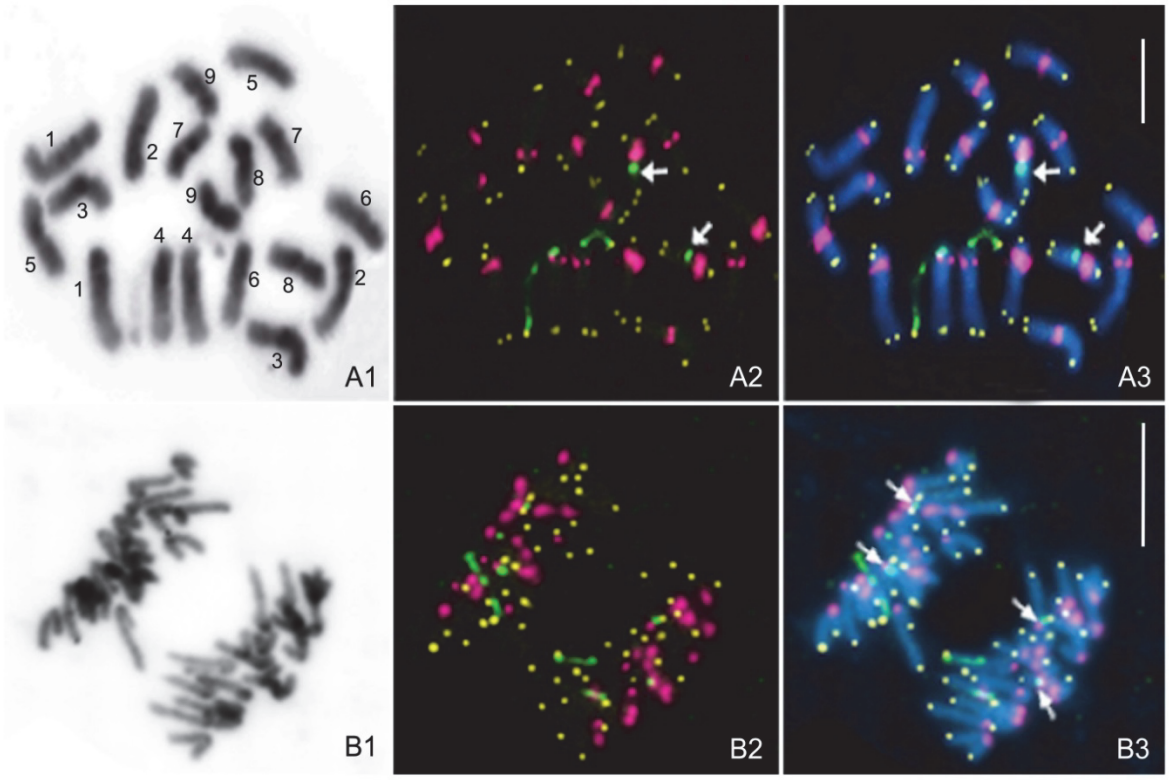

Fig. 1. Localization of rDNA (18S-25S and 5S), centromeric (Cent-Dc), and telomeric (TRS) repeat sequences on carrot metaphase (*A1* - *A3*) and V-shape anaphase (*B1* - *B3*) chromosomes using multicolour-fluorescence *in situ* hybridization. *A1* and *B1* - DAPI-stained chromosomes converted to a black-and-white image. *A2* and *B2* - distribution of 18S-25S and 5S rDNA probes (*green*), both detected with a fluorescein isithiocyanate-conjugated antidigoxigenin antibody, a Cent-Dc probe (*magenta* fluorescence of Cy5), and the TRS probe (*yellow*; a red signal of Cy3 was changed to yellow). *A3* and *B3* - hybridization signals overlapped on chromosomes contour-stained with 4′,6-diamidino-2-phenylindole (*blue*). The *arrows* point to 5S rDNA. Numbers (1 to 9) indicate pairs of homologous chromosomes. *Bar* = 5 μm.

elsewhere. For the pair with the secondary constriction, hybridization signals occurred at the end of the prominent satellite. The intensity of the hybridization signals slightly differed. The carrot-specific centromeric probe Cent-Dc was the fourth component of the hybridization mixture. It hybridized to centromeric regions of all nine pairs of carrot chromosomes. To test whether a synthetic oligonucleotide had an affinity to carrot centromeres

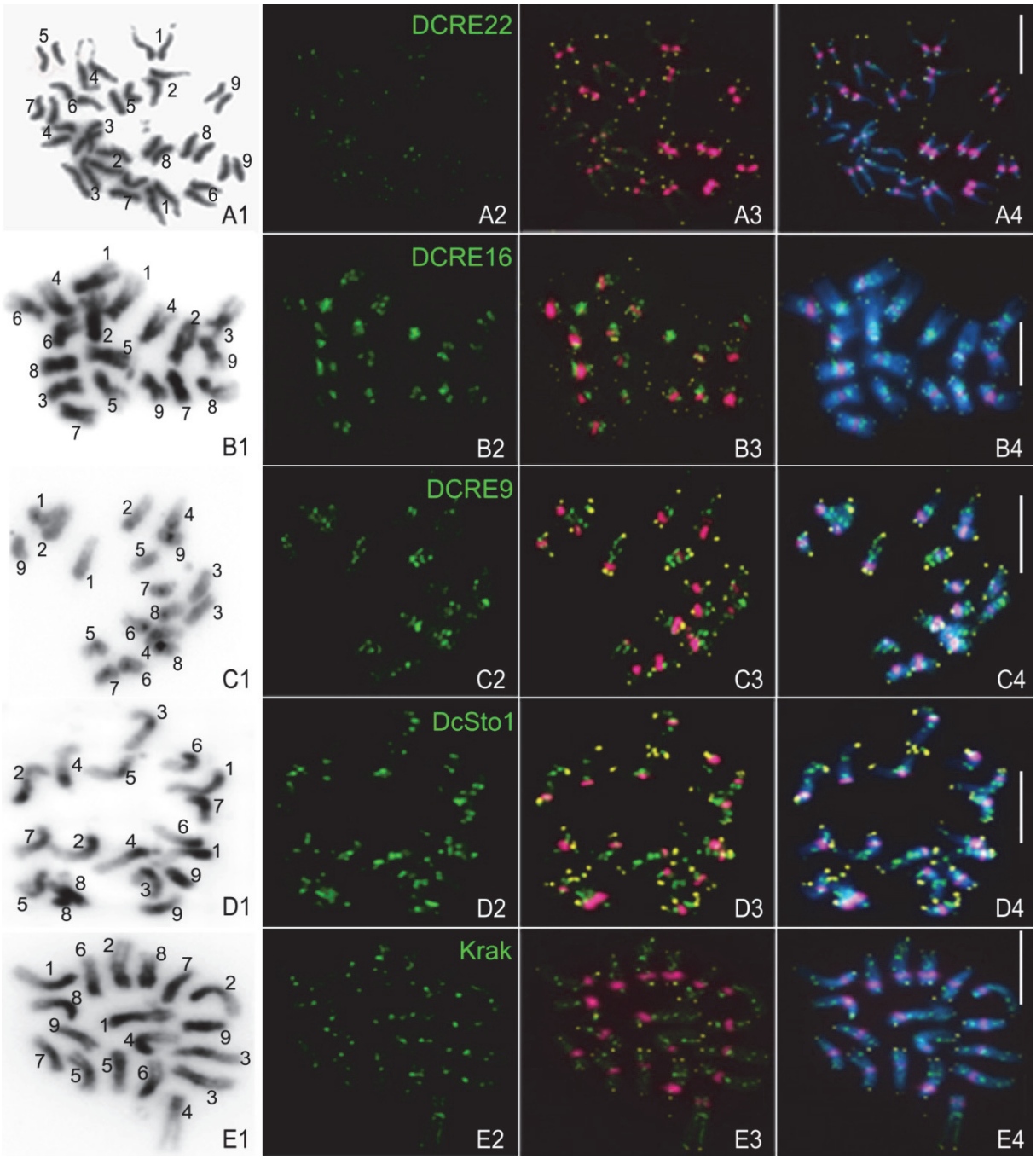

Fig. 2. Localization of *Daucus carota* repetitive element (DCRE) or miniature inverted-repeat transposable element (MITE) probes together with centromeric (Cent-Dc) and telomeric (TRS) repeat sequences on carrot metaphase chromosomes using multicolour-fluorescence *in situ* hybridization. *A1* - *A4*: DCRE22, *B1* - *B4*: DCRE16, *C1* - *C4*: DCRE9, *D1* - *D4*: *DcSto1*, *E1* - *E4*: *Krak*. *A1* - *E1*: DAPI-stained chromosomes converted to a black-and-white image; *A2* - *E2*: distribution of the DCRE or MITE probes (*green*) detected with a fluorescein isithiocyanate-conjugated antidigoxigenin antibody; *A3* - *E3*: distribution of the DCRE or MITE probes (*green*), with the Cent-Dc probe (*magenta* fluorescence of Cy5) and the TRS probe (*yellow*; a red signal of Cy3 was changed to yellow); *A4* - *E4*: hybridization signals overlapped on chromosomes contour-stained with 4′,6-diamidino-2-phenylindole (*blue*). Numbers (1 to 9) indicate pairs of homologous chromosomes. *Bar* = 5 μm.

and not to pericentromeric regions, we analyzed the distribution of hybridization signals on V-shape anaphase chromosomes. The Cent-Dc signals were located in the regions where chromosomes were attached to the mitotic spindle (Fig. 1). The intensity of FISH signals for Cent-Dc differed among chromosomes and was highest for chromosome 8 carrying the 5S locus and for the smallest subtelocentric chromosome 6. The weakest FISH signals were observed for the chromosome pair with the satellite (chromosome 4).

Four slide preparations were used for hybridization with each DCRE probe, and 40 to 72 metaphases were analyzed per one combination. Hybridization signals were detected along the carrot chromosomes for 3 out of 11 examined DCREs, *i.e*., DCRE22, DCRE16, and DCRE9 (Fig. 2*A,B,C*). Both patterns of hybridization signals for these probes and intensities of signals varied significantly. The hybridization signals for DCRE22 were observed as dotted tracks on chromosomes. Single hybridization signals occurred in the pericentromeric region on chromosomes 2, 5, 6, and 9. Dotted signals surrounding the centromeres appeared on chromosomes 1, 4, 7, and 8. Three signals of DCRE22, two pericentromeric and interstitial, were observed on chromosome 3. The chromosome pair bearing the 18S-25S rRNA site produced the strongest signal with DCRE22. For DCRE16, hybridization signals were located in the pericentromeric region of all chromosomes producing clear signals of varying intensities. For chromosomes 2 and 4, signals were localized in the centromeric region. A strong hybridization with DCRE16 was observed for chromosomes 1, 2, 3, 4, and 7, whereas a moderate signal intensity occurred for the remaining four chromosomes. The chromosome pair bearing the 18S-25S rRNA site was characterized by the most pronounced DCRE16 signal. In the case of DCRE9, intensities of hybridization signals differed considerably among chromosomes. However, signals were mostly detectable as doublets indicating the hybridization on both chromatids and resulting in a banding-like pattern. The most abundant DCRE9 signals occurred on chromosome 5, whereas the weakest signals were on chromosomes 7 and 9. The pattern of hybridization signals was similar for chromosomes 1 and 4 as well as for chromosomes 6 and 8. For chromosome 2, a large cluster in an intercalary localization on the long chromosome arm was observed. On chromosome 3, differences in localization of signals between homologous chromosomes were noted.

The *DcSto* and *Krak* FISH probes in a mixture with the centromeric and telomeric probes were used to investigate their localization on the carrot chromosomes (Fig. 2*D,E*). Four slide preparations were used for hybridization with the *DcSto* or *Krak* probes, and 68 and 57 metaphases were analyzed, respectively. The FISH with *DcSto* and *Krak* resulted in a pattern of signals widely dispersed along all chromosome arms including intercalary and pericentromeric localizations. Hybridization signals for both MITEs were absent in the centromeric, telomeric, and nucleolar organizer regions. Fluorescent signals for the *DcSto* probe were much more pronounced and produced a banding-like pattern as compared to *Krak*. Moreover, the intensity of the *DcSto* signals differed considerably among chromosomes. The weakest signals occurred on chromosome 6. The hybridization pattern of *Krak* was more uniform throughout chromosomes.

The simultaneous localization of the carrot-specific centromeric and telomeric probes indicated the major chromosomal landmarks and enabled accurate measurements of the chromosomes. On average, the karyotype length of the somatic metaphase chromosomes of *D. carota* subsp. *sativus* cv. Dolanka was 65.02 ± 10.42 μm. The chromosome length ranged from 2.75 to 4.70 μm. Thus, the ratio between the longest and shortest chromosome was 1.70. The karyotype had a relatively high symmetry and was classified to type 2A according to Stebbins (1971). The arm ratios of the carrot metaphase chromosomes ranged from 1.19 (chromosome 7) to 5.38 (chromosome 4). Based on the position of the

Table 1. The morphometric karyotype analysis in *Daucus carota* subsp. *sativus* cv. Dolanka. S - length of the short arm, L - length of the long arm, TL - total chromosome length, RL - relative length of a chromosome (where total karyotype length = 65.02 ± 10.42), AR - arm ratio (the ratio of the long arm length to the short arm length of a chromosome). Means ± SDs, *n* = 12. For chromosome 8, the distance between the centromere and the 5S rRNA locus was measured for seven metaphase plates and was estimated to be 0.86 ± 0.19 μm.

Chromosome	S [μm]	L [μm]	TL [μm]	RL [%]	AR	Morphological type
1	1.01 ± 0.30	3.69 ± 0.69	4.70 ± 0.84	14.45	3.84 ± 0.90	subtelocentric
2	0.71 ± 0.18	3.30 ± 0.60	4.00 ± 0.70	12.30	4.75 ± 0.92	subtelocentric
3	1.61 ± 0.36	2.34 ± 0.46	3.95 ± 0.66	12.15	1.47 ± 0.39	metacentric
4	0.62 ± 0.19	3.31 ± 0.68	3.93 ± 0.73	12.07	5.38 ± 1.10	subtelocentric
5	1.55 ± 0.33	1.98 ± 0.45	3.52 ± 0.67	10.83	1.32 ± 0.18	metacentric
6	0.79 ± 0.18	2.61 ± 0.54	3.39 ± 0.61	10.44	3.40 ± 0.93	subtelocentric
7	1.45 ± 0.26	1.72 ± 0.33	3.17 ± 0.57	9.74	1.19 ± 0.21	metacentric
8	0.83 ± 0.22	2.27 ± 0.44	3.10 ± 0.57	9.54	2.81 ± 0.50	submetacentric
9	1.26 ± 0.30	1.49 ± 0.31	2.75 ± 0.57	8.47	1.20 ± 0.23	metacentric

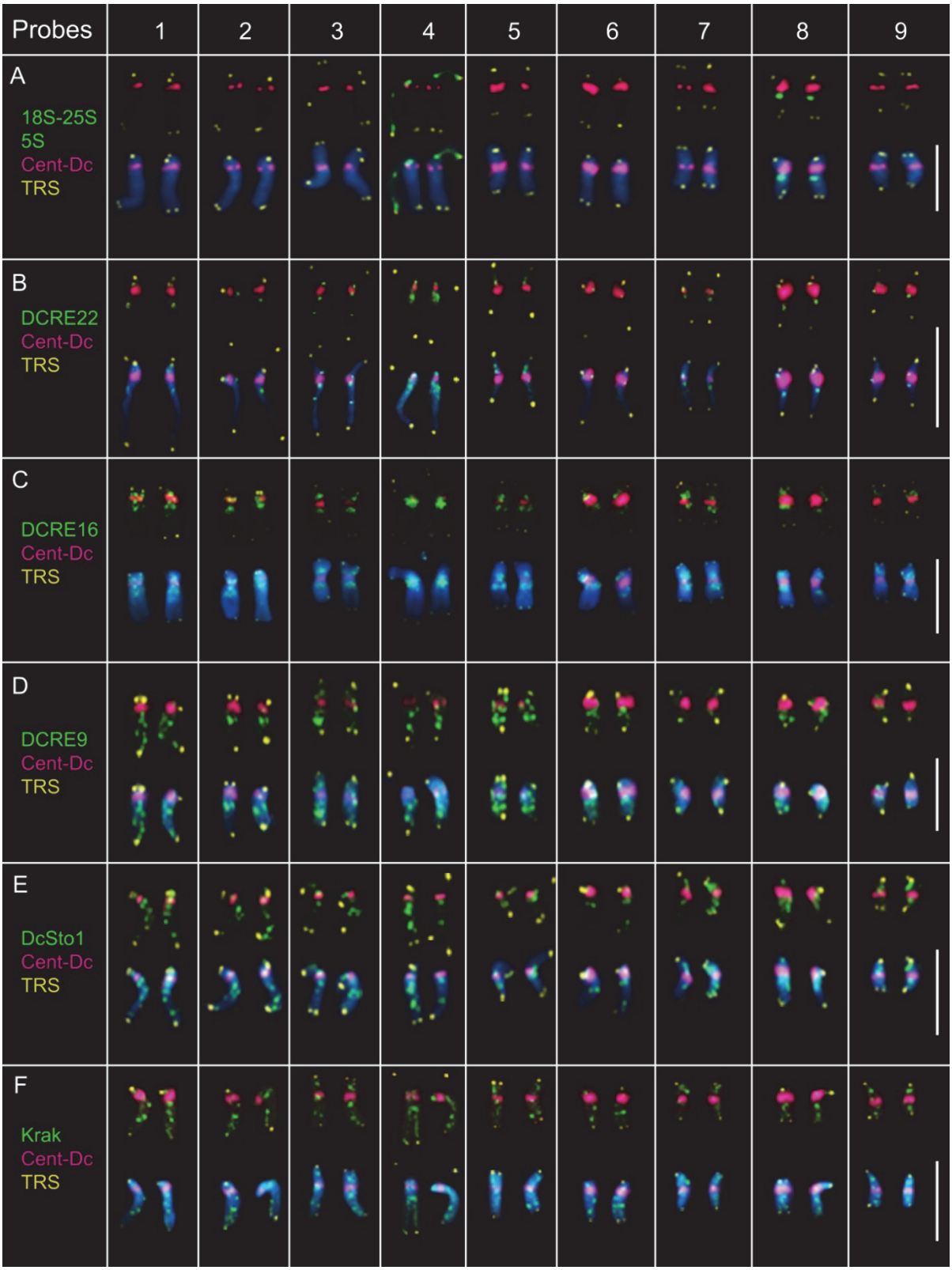

Fig. 3. The carrot karyotyping following hybridization with 18S-25S and 5S rDNA, and *Daucus carota* repetitive element and miniature inverted-repeat transposable element probes, together with centromeric (Cent-Dc) and telomeric repeat sequence probes. *Top rows* in each section show fluorescence *in situ* hybridization signals and *bottom row* their superimposition on 4′,6-diamidino-2-phenylindole contour-stained chromosomes. Homologous chromosomes were paired according to their hybridization pattern and size. The chromosome pairs are ordered from 1 to 9 according to their descending length. *Bar* = 5 μm.

centromere, the carrot mitotic chromosomes were classified as subtelocentric (chromosomes 1, 2, 4, and 6), submetacentric (chromosome 8), and metacentric (chromosomes 3, 5, 7, and 9), which produced a karyotype formula 2n = 18 = 8st + 2sm + 8m. The distance between the centromere and the 5S rDNA locus on chromosome 8 was 0.86 ± 0.19 μm. Characteristics of individual chromosomes, relative lengths, arm ratios and morphological types are summarized in Table 1.

The FISH experiments with rDNA, TRS, and Cent-Dc repeats, and DCREs were sufficient for precise chromosome measurements, unambiguous chromosome pairing, and construction of the carrot karyotype where chromosome pairs were ordered from 1 to 9 according to their descending length (Fig. 3). Hybridization signals for DCRE22, observed as dotted tracks, were most useful to distinguish chromosomes. The signals for 18S-25S, 5S rDNA, TRS, Cent-Dc, DCRE22, and DCRE16 were also used to plot an idiogram (Fig. 4).

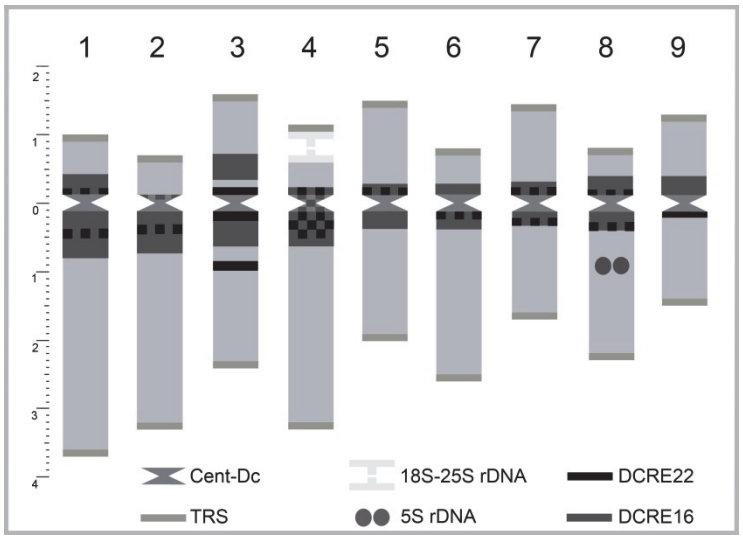

Fig. 4. An idiogram showing distribution of centromeric, telomeric, rDNA repeats, as well as *Daucus carota* repetitive elements (DCRE) 22 and 16. The length of each chromosome and the arm ratio correspond to the measurements of the chromosomes presented in Table 1. Satellite and nucleolar organizer regions were not measured as chromatin condensation at the secondary constrictions was highly variable. Scale in micrometers.

Discussion

Chromosome identification is critical for the construction of cytogenetic maps and further cytogenetic research. Until recently reports on carrot karyotyping were rare, as compared to other economically important crops with small chromosomes like sugar beet or *Brassica* species, and described condensed metaphase chromosomes which do not show significant differences in their chromosome length (Kumar and Widholm 1984, Essad 1985). Schrader *et al*. (2003) attempted to discriminate carrot chromosomes by using the Giemsa C-banding method. However, Giemsa C-bands were localized around the primary constrictions and generated defined centromeric banding patterns not applicable for routine karyotype analysis of carrot. A significant progress in carrot chromosome studies occurred after application of the FISH technique. The number of identified chromosomes ranged from two by using rDNA probes (Schrader *et al.* 2003, Iovene *et al.* 2008) to nine by application of chromosome-specific BAC probes (Iovene *et al.* 2011) or by utilization of repetitive sequences obtained on the basis of clones derived from arbitrary PCR amplification (Nowicka *et al.* 2012). Here, we used several combinations of repetitive sequences in the mFISH experiments in order to develop an accurate and reliable system for identification of somatic chromosomes. The crucial point in mFISH projects is appropriate labelling probes and optimization of the hybridization mixture (Hasterok *et al.* 2002). Therefore, we tried to use as many probes in one mixture as possible, which was enabled by application of directly labelled synthetic oligonucleotide probes. The approach is routinely employed in mammalian research (Boyle *et al.* 2011), and in plants it was previously reported for soybean (Findley *et al.* 2010). The advantage of directly labelled probes is not only simplification of the FISH procedure but also a significant reduction of costs. We used a cocktail of four probes corresponding to the major chromosomal landmarks, *i.e.*, 18S-25S and 5S rDNAs, telomeres, and centromeres; the combination has never been employed previously in carrot chromosome studies. Simultaneous application of these probes together with chromosomal measurements enabled precise chromosome discrimination. Differences in the fluorescent signals of Cent-Dc among chromosome pairs greatly facilitated pairing. Various intensities of signals

for the carrot centromere-specific probe were noted previously for both mitotic chromosomes and meiotic chromosomes (Iovene *et al.* 2011, Nowicka *et al.* 2012). Systematic differences in the intensity of fluorescent signals for centromere-specific probes were reported in other plant species, *e.g.*, rice (Cheng *et al.* 2002), maize (Jin *et al.* 2004), sugar beet (Menzel *et al.* 2008), and soybean (Findley *et al.* 2010). They might result from a different organization of centromere regions of individual chromosomes. The oligo Cent-Dc probe containing a fragment of the carrot-specific centromeric repeat proved to be as useful as the BAC-derived Cent-Dc probe applied by Iovene *et al.* (2011) to anchor the centromere position. The localization of the probe in the primary constriction region was clearly observed on V-shape anaphase chromosomes.

Fuchs *et al.* (1995) used a synthetic telomeric probe to localize telomeres on chromosomes of numerous angiosperms including carrot. However, we observed much clearer signals on chromosome ends. In addition, we noted that the telomeric probe also revealed slight differences in the intensity of hybridization signals. It may indicate different numbers of repeats at the telomeres, but the differences were too subtle to be used as a landmark for chromosome identification, by contrast to those observed for the centromeric probe. Similar variation was observed in *Lupinus angustifolius* (Hajdera *et al.* 2003), *Centaurea* species (Dydak *et al.* 2009) and *Cucumis sativus* (Hoshi *et al.* 2011).

The simultaneous localization of the carrot-specific centromeric and telomeric probes indicated the major chromosomal landmarks and enabled precise measurements of the chromosomes. We propose to use these two probes as references in further cytogenetic carrot studies. Schrader *et al.* (2003) described five carrot chromosome pairs as submetacentric of which one carries a satellite and other four were classified as metacentric (2n = 18 = 10sm + 8m). Iovene *et al.* (2008) marked out two subtelocentric pairs, one of which was a SAT-chromosome pair, three submetacentric, and four meta-centric chromosome pairs (2n = 18 = 4st + 6sm + 8m). Subsequently, Iovene *et al.* (2011) applied mapped chromosome-specific BACs on pachytene chromosomes, which enabled identification of all carrot chromosomes, and as result, a new karyotype formula, 2n = 18 = 4t + 6st + 8m, was proposed. Our measurements differ from those reported previously, however, it is close to that reported by Iovene *et al.* (2008) for somatic chromosomes. We argue that our measurements resulting in a modified karyotype formula 2n = 18 = 8st + 2sm + 8m, reclassifying two chromosome pairs from submetacentric (Iovene *et al.* 2008) to subtelocentric were more precise, as we were able to visualize accurate chromosome boundaries with telomere- and centromere-specific probes. The other morphological parameter important for karyotype studies is the total chromosome length. The number (65.02 μm) we obtained is somewhere between measurements provided in previous studies for mitotic chromosomes (Schrader *et al.* 2003, Iovene *et al.* 2008) and is over three times shorter than the total length of pachytene chromosomes (Iovene *et al.* 2011).

Cavagnaro *et al.* (2009) separated DCREs from other repetitive elements identified from BAC-end sequencing, as they cannot be attributed to any defined group of repeats present in plant genomes. They suggested that a FISH experiment may reveal interesting genomic distribution patterns of DCREs. Although we attempted to better characterize the DCREs by querying them against RepBase (Jurka *et al*. 2005) and the *NCBI* protein database (*BLASTX* search), we failed to find any significant similarity to known families of repetitive elements and coding capacity (data not shown). Until now, genome-specific sequences were identified in numerous plants, such as *Beta* species (Kubis *et al.* 1998, Schmidt *et al.* 1998, Dechyeva *et al.* 2003, Menzel *et al.* 2008), *Pisum sativum* (Neumann *et al.* 2001), *Medicago truncatula* (Kulikova *et al.* 2001, 2004), *Chenopodium quinoa* (Kolano *et al.* 2008, 2011) and mapped on chromosomes by the FISH. It is known that the size of a target sequence and the number of tandem copies are critical factors for the optimal detection of hybridization sites (Weber *et al.* 2010). In the case of DCREs, we observed hybridization signals only for three of eleven examined sequences. Visualization of these probes shows that they were present in clusters comprising many copies. No detectable hybridization signal for the remaining DCREs suggests that they were dispersed or their copy number in carrot cv. Dolanka was too low.

The hybridization patterns for DCRE22, DCRE16, and DCRE9 were different, however, the abundance and intensity of signals correlated with their estimated copy numbers of 2 990, 4 621, and 7 340, respectively (Cavagnaro *et al.* 2009). We conclude that DCRE22 was most useful for carrot chromosome identification as the hybridization signals for the probe were observed in the form of pericentromeric and/or interstitial dotted tracks. Similar results were observed for genome-specific sequences *MtR1* in *Medicago truncatula* (Kulikova *et al.* 2001, 2004) and *PisTR-B* in *Pisum sativum* (Neumann *et al.* 2001). In the case of DCRE16, which was also accumulated in the pericentromeric regions of carrot chromosomes, the hybridization profile was comparable to that observed for *pBV1* in *Beta vulgaris* (Kubis *et al.* 1998), *MtR2* in *Medicago truncatula* (Kulikova *et al.* 2004), and for 12-13P in *Chenopodium quinoa* and related species (Kolano *et al.* 2011). As compared to previous studies on carrot, the hybridization patterns for DCRE22 and DCRE16 were alike those of four repetitive FISH probes obtained by an arbitrary amplification (Nowicka *et al.* 2012). For DCRE9, the hybridization signals were dispersed along the chromosomes in a band-like manner. Dispersed repetitive sequences represent a large proportion of plant genomes, and among others include numerous copies of retrotransposons (Heslop-Harrison *et al.* 1997, Kumar and Bennetzen 1999). Such dispersed repetitive families have been observed in barley (Hueros *et al.* 1993), rice (Kiefer-Meyer *et al.* 1996), tobacco (Horakova and Fajkus 2000), pea (Neumann

et al. 2001), beet (Schmidt *et al.* 1998, Dechyeva *et al.* 2003, Menzel *et al.* 2008) and quinoa (Kolano *et al.* 2008). Dechyeva *et al.* (2003) noted that dispersed repeats *pAp4* and *pAp22* from *Beta procumbens* are likely derived from *Ty1-copia* or *env*-like retrotransposons. Similarly, Kolano *et al.* (2008) suggested that a dispersed pattern of *pTaq10* in quinoa results its relation to a *Ty3-gypsy* retrotransposon. Thus, we suppose that DCREs might be derived from mobile elements.

To date, only three types of TEs were identified in the carrot genome, *i.e.*, *Tdc1* of the CACTA superfamily (Ozeki *et al.* 1997, Itoh *et al.* 2003), *DcMaster* of the PIF/*Harbinger* superfamily (Grzebelus *et al.* 2007) with a related family of *Tourist*-like MITEs named *Krak* (Grzebelus and Simon 2009), and recently *Stowaway*-like MITEs named *DcSto* (Macko-Podgorni *et al.* 2013). Both groups of MITEs were shown to be abundant and diverse in the carrot genome. In the present study, we observed a scattered distribution of the *DcSto* and *Krak* elements mostly in the interstitial and pericentromeric regions of all the chromosomes. Nevertheless, the frequent occurrence of strong FISH signals suggests that both families of MITEs were predominantly organized in clusters.

To summarize, we demonstrated novel tools for differentiating the individual carrot chromosomes. They could have been distinguished in a simple and reliable way by using two reference probes, *i.e.*, centromeric and telomeric landmarks combined with one or two additional repetitive probes. Well-defined chromosome boundaries resulted in precise measurements and accurate karyotyping of the carrot genome. Besides, we visualized the chromosome distribution of several repetitive sequences along the carrot chromosomes.

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