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7 Size and location of radish chromosome regions carrying the fertility restorer

8 *Rfk1* gene in spring turnip rape

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23 **Keywords** turnip rape (*Brassica rapa*), radish (*Raphanus sativus*), fertility restoration gene, *Rfk1*, *Rfo*,

24 GISH, BAC-FISH

25 **Abbreviations**

26 BAC-FISH bacterial artificial chromosome – fluorescence *in situ* hybridization

27 CMS cytoplasmic male sterility

28 GISH genomic *in situ* hybridization

29 Rf restore fertility

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1 **Running title** Size and location of *Rfk1* gene

2

3 **Abstract**

4 In spring turnip rape (*Brassica rapa* L. spp. *oleifera*) the most promising F1 hybrid system would be
5 the Ogu-INRA CMS/Rf system. A Kosena fertility restorer gene *Rfk1*, homologue of the Ogura restorer
6 gene *Rfo*, was successfully transferred from oilseed rape into turnip rape and that restored the fertility
7 in female lines carrying Ogura cms. The trait was, however, unstable in subsequent generations. The
8 physical localization of the radish chromosomal region carrying the *Rfk1* gene was investigated using
9 GISH (genomic *in situ* hybridization) and BAC-FISH (bacterial artificial chromosome – fluorescence
10 *in situ* hybridization) methods. The metaphase chromosomes were hybridized using radish DNA as the
11 genomic probe and BAC64 probe, which is linked with *Rfo* gene. Both probes showed a signal in the
12 chromosome spreads of the restorer line 4021-2 Rfk of turnip rape but not in the negative control line
13 4021B. The GISH analyses clearly showed that the turnip rape restorer plants were either monosomic
14 ($2n=2x=20+1R$) or disomic ($2n=2x=20+2R$) addition lines with one or two copies of a single alien
15 chromosome region originating from radish. In the BAC-FISH analysis, double dot signals were
16 detected in sub-terminal parts of the radish chromosome arms showing that the fertility restorer gene
17 *Rfk1* was located in this additional radish chromosome. Detected disomic addition lines were found to
18 be unstable for turnip rape hybrid production. Using the BAC-FISH analysis, weak signals were
19 sometimes visible in two chromosomes of turnip rape and a homologous region of *Rfk1* in chromosome
20 9 of the *B. rapa* A genome was verified with BLAST analysis. In the future this homologous area in A
21 genome could be substituted with radish chromosome area carrying the *Rfk1* gene.

22

23

1 **Introduction**

2 Spring turnip rape (*Brassica rapa* L. spp. *oleifera*, $2n=2x=20$, genome constitution AA) is the major
3 oilseed crop cultivated for the production of vegetable oil and animal feed protein in many northern
4 areas including Finland, parts of Canada and Northern India. Compared to oilseed rape (*Brassica napus*
5 L., $2n=4x=38$), which has a higher yield potential, turnip rape is early maturing and therefore shows
6 better yield stability in Northern climates. Most of the cultivated varieties of turnip rape are open-
7 pollinated, despite the potential of hybrid breeding exploiting heterosis effects, which could increase
8 the seed yield (Niemelä et al. 2006). One of the main systems for F1 seed production in the genus
9 *Brassica* is based on utilization of cytoplasmic male sterility (CMS) and fertility restoring genes (Rf).
10 The CMS/Rf type of hybrid system is used commercially in many important agricultural crops,
11 dominating production in maize (*Zea mays*) and sunflower (*Helianthus annuus*), with more limited and
12 special uses in rice (*Oryza sativa*), cotton (*Gossypium hirsutum*) and oilseed rape (*B. napus*). In turnip
13 rape there is no fully functional CMS/Rf hybrid system in commercial use. However, one of the most
14 promising of the known hybrid systems is the Ogu-INRA CMS/Rf (Niemelä et al. 2010).

15 The Ogu-INRA CMS/Rf system was originally transferred from Japanese radish (*Raphanus*
16 *sativus*; $2n=2x=18$, genome RR) to oilseed rape (*Brassica napus*; genome AACC) (Bannerot et al.
17 1974; Bannerot et al. 1977; Heyn 1976). Nowadays it is one of the most commonly used systems for
18 F1 hybrid production in oilseed rape breeding programmes. The Ogura cms-associated gene, *orf138*,
19 induces abnormal flower development that prevents the production of functional pollen (Bonhomme et
20 al. 1992; Krishnasamy and Makarof 1994). This male sterile line is called A-line in the hybrid seed
21 production and the character is maintained using B-lines that have the normal cytoplasm without the
22 sterility inducing traits. Those lines which restore fertility trait are called R-lines. In oilseed rape
23 hybrids with the Ogura system, restorer lines carry one dominant nuclear gene, *Rfo* (Bellaoui et al.

1 1999). In oilseed rape, the *Rfo* restorer gene has been optimized to not contain flanking chromosomal
2 sequences from the original radish introgression (SOFIPROTEOL European Patent No EP1382612 and
3 PCT No WO02088179 (1). European Patent No EP1556495 and PCT No WO04039988 (2) – retrieved
4 from <http://www.pbltechnology.com/cms.php?pageid=322> 20.10.2011).

5 Previously, Ogura cms was transferred from oilseed rape into turnip rape (Sovero 1987;
6 Delourme et al. 1994) resulting in production of stable male sterile lines for use in turnip rape hybrid
7 production. The transfer of the fertility restorer gene (*Rfo*) from oilseed rape into turnip rape has
8 however been unsuccessful (F. Stoenescu, personal communication, Zeneca Seeds, Winnipeg, Canada,
9 1995). In recent studies, it was confirmed that the *Rfo* gene is introgressed into the C genome in oilseed
10 rape (Hu et al. 2008; Feng et al. 2009). This location of the *Rfo* gene in the C genome may be one of
11 the reasons its transfer into the turnip rape A genome has not yet succeeded. Therefore, to establish a
12 specific hybrid system for turnip rape, the Kosena fertility restorer gene (*Rfk1*), originating from radish
13 and homologue of the Ogura fertility restorer gene (*Rfo*) (Brown et al. 2003), was transferred from
14 oilseed rape into turnip rape through interspecific crosses followed by traditional backcrossing
15 (Niemelä et al. 2010). In contrast to the *Rfo*, the *Rfk1* gene was supposedly not integrated into the C
16 genome of the oilseed rape breeding lines, selected for our turnip rape hybrid breeding program.
17 During the course of the breeding program, it was observed that the *Rfk1* gene was able to restore the
18 fertility of turnip rape with Ogura cms, but the trait was unstable in the turnip rape genome (Niemelä et
19 al. 2010). After subsequent selection and interpollination of homozygous plants, the progeny also had
20 some heterozygous and male sterile plants. To advance the lines for improvement of the fertility-
21 restoring turnip rape male lines, it would be helpful to detect and measure the amount of introgressed
22 radish genome and its location in the turnip rape A genome.

1 The physical localization of the radish introgression carrying the *Rfk1* gene in turnip rape
2 genome can be investigated by fluorescence *in situ* hybridization (FISH) and genomic *in situ*
3 hybridization (GISH). This approach has successfully been used to detect different genomes in
4 interspecific hybrids as well as for locating introgressed genomes, alien chromosomes, or chromosomal
5 segments in another genomic background (Schwarzacher et al. 1992). The recent progress in *Brassica*
6 genome sequencing projects have provided useful sequence information for cytogenetic studies (review:
7 Heslop-Harrison and Schwarzacher 2011) and *Brassica* bacterial artificial chromosome (BAC) clones
8 have been used for hybridization as FISH probes to physically localize specific sequences on *Brassica*
9 chromosomes (Howell et al. 2005, 2008; Nicolas et al. 2007, 2008; Feng et al. 2009; Kim et al. 2009;
10 Szadkowski et al. 2010, 2011; Xiong and Pires 2010; Xiong et al. 2011). As in most plant species,
11 transposable elements are abundant in the *Brassica* genome, and some of these are genome specific
12 (Alix et al. 2005, 2008; Lim et al. 2007); there are also tandemly repeated DNA motifs which show
13 specificity to some chromosomes and genomes (Harrison and Heslop-Harrison 1995). Along with other
14 genome-specific sequences, this means that total genomic DNA can be used to distinguish genomes
15 (Snowdon et al. 1997), but because of the relatively small chromosomes and limited evolutionary
16 divergence of the A, B and C genomes, it is difficult to confirm the origin of all chromosomes
17 throughout their length. However with the greater evolutionary distance between genera, *Brassica* and
18 *Raphanus*, the separation is robust. Total genomic DNA of radish has been used as a probe to
19 distinguish the radish R genome from A and C genomes of *Brassicac*s (Snowdon et al. 1997;
20 Benabdelmouna et al. 2003; Chen and Wu 2008; Akaba et al. 2009). Budahn et al. (2008) also used a
21 radish specific probe, pURsN, *in situ* hybridization, to identify radish chromosome additions in oilseed
22 rape.

1 The aim of the current study was to localize physically the radish *Rfk1* gene, the chromosome
2 constitution, and the putative flanking region of radish chromatin in the turnip rape genomic
3 background using GISH and BAC-FISH. Localization of the restorer gene aimed to understand the
4 nature of its instability in the A genome and to suggest tools for selection and breeding towards the
5 functional hybrid system for turnip rape.

6

7

8 **Material and methods**

9

10 **Plant material**

11

12 The breeding line, 4021-2 Rfk, of spring turnip rape (*Brassica rapa*) was selected for the chromosome
13 preparations. Details of the breeding work of this restorer line 4021-2 Rfk has been described
14 previously (Niemelä et al. 2010). The open pollinated turnip rape (*Brassica rapa*) line of Finnish origin,
15 4021B (AA, 2n=20), was used as a negative control for chromosome preparations. Both breeding lines
16 had the same genetic background except the 4021-2 Rfk was carrying the Kosena *Rfk1* restorer gene.
17 The 4021-2 Rfk was produced through traditional backcrosses, where the spring oilseed rape (*Brassica*
18 *napus*) breeding line RfA4 (Plantech Research Institute Japan) having the Kosena *Rfk1* gene was used
19 as a donor parent. The homozygous (Rfk1, Rfk1) plants were selected from BC6F4 progeny before
20 flowering stage using TaqMan qPCR (Niemelä et al. 2010) and they were cross pollinated to form the
21 fertility restoring 4021-2 Rfk line for the present study.

22

1 Chromosome preparation

2

3 Chromosome preparations were made from the root tips of turnip rape lines 4021B and 4021-2 Rfk
4 using standard techniques (Schwarzacher and Heslop-Harrison 2000). In brief, seedling root tips were
5 incubated in 2 mM 8-hydroxyquinoline for 3 h before fixation in fresh 3:1; ethanol : acetic acid. After
6 storage and rinses, roots were digested in an enzyme solution [0.1% (w/v) cytohelicase (Sigma-Aldrich,
7 Steinheim, Germany), 0.1% (w/v) cellulase Onozuka RS (Sigma-Aldrich, Steinheim, Germany), 0.1%
8 Pectolyase Y23 (Sigma-Aldrich, Steinheim, Germany) in 10 mM citrate buffer, pH 4.8 for 90 min at
9 room temperature, and then squashed in 60% (v/v) acetic acid. After freezing, coverslip removal and
10 dehydration through an alcohol series, slides were selected under phase-contrast or after staining with
11 DAPI (4,6-diamidino-2-phenylindole, Sigma), dehydrated, dried and stored at -20°C until hybridization.

12

13 Probe labelling

14

15 For the genomic probe, plant DNA was extracted from greenhouse-grown young leaves of radish
16 ‘Daikon’ using DNeasy Plant Maxi Kits (Qiagen). DNA was sonicated to fragments of about 500 bp
17 and labelled by random priming with biotin-11-dUTP (Roche) and digoxigenin-11-dUTP (Roche). For
18 the BAC clone probe, BAC64 (Desloire et al. 2003) from Genoplante-Valor, was kindly provided by
19 INRA-CNRGV (Centre National de Ressources Genomiques Vegetales, Castanet-Tolosan, France); the
20 fertility restoration locus *Rfo* (homologue to *Rfk1*) is in *R. sativus* BAC64 (contig 127 kb, accession
21 number AJ550021). BAC DNA was isolated with a NucleoBond Xtra Midi kits (Macherey-Nagel) and
22 PCR using the primer pair (forward primer, 5'-TCATCCCCCAAATGATAGAT-3'; reverse primer, 5'-
23 GAAGCTGCAAAGTGGGTTTC-3') designed for the *Rfk1* gene was carried out to verify the BAC64

1 identity. BAC DNA was sonicated to fragments <1kb, and labelled with biotin-11-dUTP or
2 digoxigenin-11-dUTP using the Invitrogen BioPrime CGH labelling kit. Two ribosomal DNA probes,
3 5S and 45S, were labelled with Alexa-647-dUTP (Invitrogen).

4

5 *In situ* hybridization and signal detection

6

7 *In situ* hybridization was performed according to Schwarzacher and Heslop-Harrison (2000) and
8 Schwarzacher (2008) with slight modifications. Up to three different probes were used in each
9 hybridization, labelled with biotin-11-dUTP, digoxigenin-11-dUTP or Alexa-647-dUTP. Slides with
10 chromosome spreads were re-fixed in ethanol:acetic acid 3:1, treated with RNase (100 µg/ml) solution
11 and then with pepsin (5 µg/ml in 0.01 M HCl) to remove cytoplasm observed surrounding the mitotic
12 chromosome spreads. Preparations were fixed with paraformaldehyde, dehydrated in an ethanol series
13 and air dried. The hybridization mixture consisted of 40% formamide, 2xSSC, 10% dextran sulphate,
14 1µg of salmon sperm DNA, 0.125 mM EDTA, 0.125% SDS and 1-4 µl (25 to 60 ng) of each labelled
15 probe with the final volume of 40-42 µl of mixture for each slide. The hybridization mixture was
16 denatured at 85°C for 10 min, cooled on ice for 10 min and then applied to the slides. The slides with
17 the chromosomes and probes were then denatured at 75°C for 7 min and hybridized at 37°C for 16 h
18 using a modified thermal cycler. The post-hybridization washes were carried out following with a low-
19 stringency wash using 0.1xSSC without formamide at 42°C. The hybridization sites were detected
20 using a mix of anti-digoxigenin conjugated to FITC (Roche) and streptavidin conjugated to Alexa 594
21 (Invitrogen). The preparations were counterstained with DAPI and mounted in AF1 medium (Citifluor,
22 London, UK). Slides were examined with an epifluorescence Zeiss Axiophot microscope and images
23 were captured with a ProgRes C12 cooled CCD camera. Images were processed using Adobe

1 Photoshop CS4 using only functions including contrast and brightness adjustment that affect the whole
2 area of the image equally. For the *in situ* hybridization 4 out of 20 restorer line 4021-2 Rfk preparations
3 and 2 out of 8 control line 4021B preparations were selected. In each *in situ* hybridization, 3 to 10
4 metaphase chromosome sets were studied.

5

6

7 **Results**

8

9 Both genomic and BAC clone probes used in this study were hybridized with the chromosome
10 preparations of the restorer line 4021-2 Rfk. Genomic *in situ* hybridization with *Raphanus* DNA
11 revealed that the fertility-restoring turnip rape line 4021-2 Rfk plants were either monosomic
12 ($2n=2x=20+1R$) or disomic ($2n=2x=20+2R$) addition lines with one copy or two copies of an alien
13 chromosome originating from *Raphanus* (Japanese radish). In the control turnip rape line 4021B,
14 without the restorer trait, no strong signal was detected in any of the mitotic chromosome spreads
15 studied.

16 *In situ* hybridization with the BAC64 clone carrying the fertility restoration locus *Rfo* resulted
17 in strong double dots of hybridization signal, one on each sister chromatid (Figs. 1a, 1c, 1f), on the
18 radish chromosome identified by GISH (Figs. 1b, 1d, 1e). Thus the fertility restoring *Rfk1* gene,
19 homologue to *Rfo*, was located on this chromosome. The two signals of BAC64 were more specifically
20 located in the sub-terminal parts (Figs. 1a, 1b) of the radish chromosome arm. With the image of
21 control line, 4021B, no strong signal was seen.

22 In addition to the signals detected in radish chromosomes as a result of hybridization with
23 BAC64 probe, two pairs of weaker signals (Figs. 1a-1d, 1g) were also sometimes visible on two

1 chromosomes of the turnip rape A genome, indicating the location of a homoeologous region to *Rfk1*
2 on the A genome. A BLAST analysis comparing the full length sequence of *R. sativus* BAC64 clone
3 (AJ550021.2) with the whole genome of *B.rapa* subsp. *pekinensis* (The *Brassica rapa* Genome
4 Sequencing Project Consortium 2011) identified a *B. rapa* subsp. *pekinensis* BAC clone KBrB025K04
5 (AC189288.2) with high homology. The whole sequence coverage between BAC64 clone and BAC
6 KBrB025K04 clone was 45%. The BAC KBrB025K04 is situated on the largest chromosome, linkage
7 group A09 (6.10.2011) and it carries the fertility restorer gene (*Rf*) (Pentatricopeptide repeat-containing
8 protein, fertility restorer B) (KBrB025K04CG0180), which has 90% homology with *R. sativus ppr-B*
9 gene (*Rfo/Rfk1* gene) situated in BAC64 (88044-88063, 88191-90235). This *B.rapa* fertility restorer
10 gene *B* (KBrB025K04CG0180) is homologue to *R.sativus ppr-B* gene (Mora et al. 2010), but is unable
11 to restore fertility. The radish *Rfo* locus consists of three close related genes in tandem, named *ppr-A*,
12 *ppr-B* and *ppr-C*, which the *ppr-B* has the fertility restoration activity (Brown et al. 2003; Desloire et al.
13 2003; Koizuka et al. 2003). The homology between *R.sativus* BAC64 clone and *B.rapa* linkage group
14 A09 was visualized in a dot-plot matrix (Fig. 2), around 7.1Mb from the end of the 37.12Mb assembly,
15 to show the homology of all three ppr genes in BAC64 (*ppr-A* gene situation: 80291-80313, 80454-
16 82484; *ppr-B* gene situation - see above; *ppr-C* gene situation: 101320-102240, 102502-102609) with
17 *B.rapa* linkage group A09 and the dot-plot analysis demonstrates that the region covering all the ppr
18 genes has two copies in the *B.rapa* background.

19

20

1 Discussion

2

3 The results define the physical position of a chromosome region containing a fertility restorer gene
4 *Rfk1* located on an additional radish chromosome in the A genome of spring turnip rape (*Brassica*
5 *rapa*). *Rfk1* is a valuable gene for breeding because it allows development of hybrid varieties for turnip
6 rape. The genomic radish probe clearly hybridized to the chromosome/chromosomes of radish in the
7 fertility restoring turnip rape line 4021-2 Rfk (Figs. 1b, 1d, 1e, 1g). The signals were strong and the
8 whole chromosome was evenly labelled allowing characterization of the radish addition in the turnip
9 rape genome.

10 The BAC carrying the *Rfk1* locus, BAC64, was used a robust probe for identifying the locus in
11 *Brassica* and *Raphanus*, with little cross hybridization elsewhere in the genome. BAC clones
12 frequently contain many repetitive sequences which are homologous in the target genome, requiring
13 high stringencies and blocking in FISH (Kim et al. 2002; Schwarzacher 2008) to identify loci of the
14 specific traits linked to that BAC clone. Similar clear BAC-FISH signal results were also found by
15 Feng et al. (2009) when localizing the *Rfo* gene in oilseed rape genome using two different *R.sativus*
16 BAC probes (G62 and B420) linked to the *Rfo* locus.

17 In the backcross progeny from *B.napus* to *B.rapa* (Niemelä et al. 2010), the segregation ratio
18 followed 30:70 instead of the expected 50:50 ratio, perhaps due to location of the *Rfk1* gene as a
19 segment of an extra region of radish chromosome in the turnip rape A genome. The GISH results
20 showing both monosomic and disomic additions in the offspring, previously selected for homozygosity,
21 demonstrate the instability of the addition chromosome (Niemelä et al. 2010). During two generations
22 of selecting and intercrossing 100% homozygous plants (analysed by TaqMan qPCR) the offspring
23 segregated to 90% homozygous and 10% hemizygous plants (data not shown). Budahn et al. (2008)

1 reported that plants having disomic additions of radish chromosomes in rape-radish lines were expected
2 to have high stability, and in some cases, disomic additions can be stable (Chevre et al. 1991). However,
3 here the disomic addition of radish chromosome in the A genome of turnip rape was not stable, making
4 them unsuitable for hybrid seed production. In several studies, instability of alien chromosomes has
5 been found in hybrids obtained from intercrosses between different *Brassica* species (Chevre et al.
6 1991; Peterka et al. 2004; Wei et al. 2010). Ge and Li (2007) and Ge et al. (2009) have demonstrated
7 that the epigenetic phenomenon known as nucleolar dominance plays a role in alien chromosome
8 stability in *Brassica* species. The similarity or difference in between these components in parental
9 material could affect to the chromosome structure and function during mitotic and meiotic divisions in
10 hybrids. To achieve complete stability of the *Rfk1* gene, also exploited in practical breeding, the
11 integration of the gene to the A genome chromosomes of turnip rape may be required. The *Rfo* gene,
12 which is stably integrated in the oilseed rape C genome (Hu et al. 2008; Feng et al. 2009), is now used
13 for hybrid seed production (Budar et al. 2004).

14 In *in situ* hybridization with BAC64 clone the 90% homology between the studied *R.sativus*
15 *ppr-B* gene and *B.rapa* spp. *pekinensis* KBrB025K4CG0180 gene shows that these genomic regions
16 are homologous between A and R genomes, which could increase the opportunity of transferring the
17 fertility restoring trait from additional radish chromosome to turnip rape chromosome via
18 homoeologous recombination. Tang et al. (2008) found also multicolour BAC-FISH very useful in
19 identifying chromosomal regions between tomato (*Solanum lycopersicum*) and potato (*Solanum*
20 *tuberosum*), which gives the information utilized in breeding techniques in introgressing genes from
21 wild *Solanum* species into cultivated crops. The clear BAC-FISH signal of the radish chromosome as
22 well as the weak BAC-FISH signal at the turnip rape chromosome (A09) was found from the sub-
23 terminal region. According to Kim et al. (2002) and Wang et al. (2007) BAC hybridization shows that

1 intergenomic introgressions often occur at distal parts of chromosomes.. In our case it would be
2 desirable to have the homologous fertility restoring region in chromosome area with higher levels of
3 recombination activity. Based on recent study of Shirasawa et al. (2011) *R.sativus* and *B.rapa* share
4 large homologous genomic regions but the order or composition of these genomic segments do not
5 correspond. This high genetic homology in between *R. sativus* and *B.rapa* would facilitate transferring
6 the fertility restoring trait from radish to turnip rape, but the genetic information that regulates the
7 homologous pairing during meiosis should be interrupted to favour recombination between
8 nonhomologous A and R genome. The increasing knowledge of the mechanisms and genes, that control
9 crossovers provide useful tools for plant breeders to promote homeologous recombination in case of
10 exploiting useful traits through interspecific crosses (Snowdon 2007; Nicolas et al. 2008; Wijnker and
11 de Jong 2008). According recent studies in *B.napus* one possible way to increase homeologous
12 recombination is manipulating the plant karyotype (Nicolas et al. 2009; Leflon et al. 2010). They
13 found that special *PrBN* (*Pairing Regulator in B.napus*) gene regulates homeologous pairing in
14 *B.napus* haploids and that depends on a plant's chromosomal composition. According these results they
15 suppose that the increase in recombination could be due to change in ploidy level and it might work in
16 more general trend also. Thus using for example a triploid hybrid of oilseed rape breeding line RfA4
17 (having Rfk1 gene) and turnip rape breeding line 4021-2 Rfk in a crossing program, could facilitate
18 overall homeologous recombination between A, C and R genome chromosomes. However, Mason et al
19 (2010) found that more complex interactions between genomic structure and alleles are involved
20 controlling homeologous pairing in *Brassicac*s. The irradiation technology has also exploited
21 successfully when developed R2000 *B.napus* Ogu-INRA restorer line (Primard-Brisset et al. 2005).
22 The *Rfo* gene was integrated into C genome of *B.napus* by forcing recombination between radish and
23 rapeseed using ionising irradiation.

1 To have a functional Ogu-INRA CMS/Rf hybrid system for turnip rape, it would be ideal to
2 substitute the putative homeologous representative regions in the A genome with radish chromosome
3 area having the restorer gene. Additional breeding techniques, like changing the ploidy level or using
4 irradiation, to increase the recombination level between R and A genomes may be required. Now the
5 BAC64 as a BAC-FISH probe is reliable for the selection of the turnip rape plants carrying *Rfk1* gene.

6

7

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9

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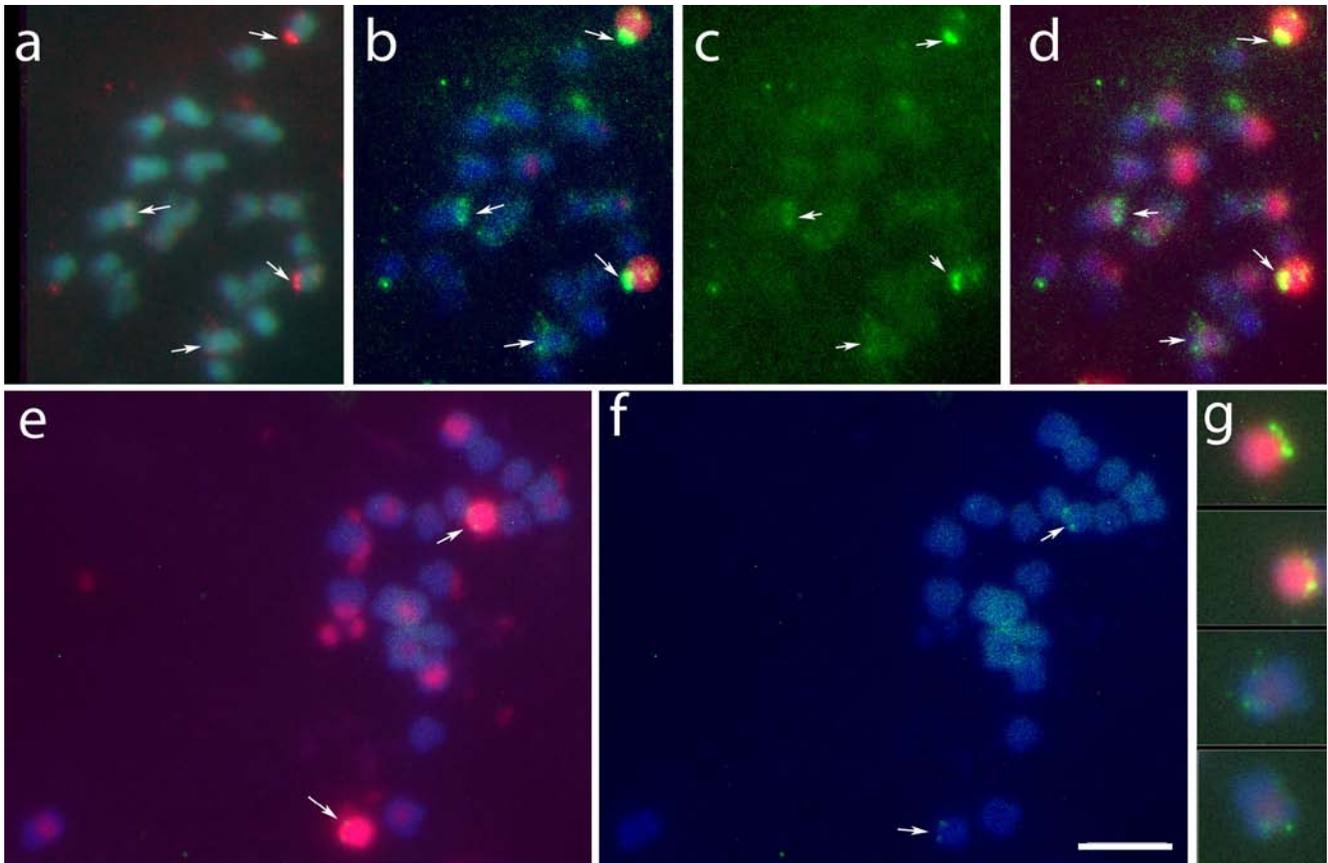
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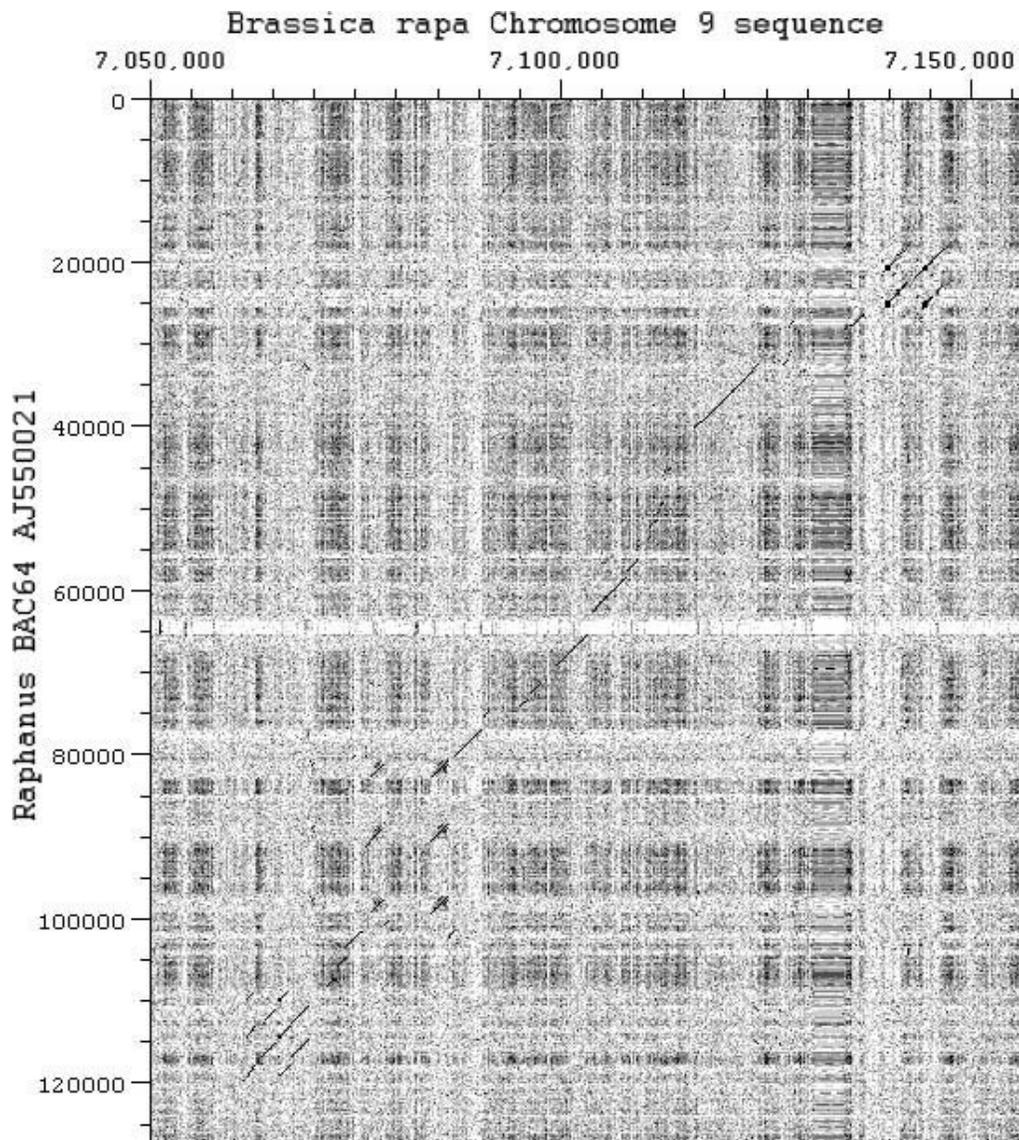


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3 Figure 1. Fluorescence *in situ* hybridization with genomic radish DNA and radish BAC64 clone probe
 4 to locate the radish chromosome region carrying fertility restorer *Rfk1* gene in *Brassica rapa* spring
 5 turnip rape genome. All the images are from a disomic ($2n=2x=20+2R$) addition line; chromosomes
 6 counterstained blue with DAPI. (a) Double dot signals of BAC64 clone (red) in sub-terminal area on
 7 two pairs of sister chromatids; strong signals on additional radish chromosome pair and weaker signals
 8 on *B.rapa* chromosome pair (b) Same metaphase as in image (a). BAC64 clone double dot signals
 9 green and additional radish chromosome pair labeled red with genomic radish DNA. (c) BAC signal
 10 from (b) in green. (d) Image (b) with far red signals shown in red of the 45S rDNA probe. (e) A second
 11 metaphase showing the labeled radish chromosome pair red, BAC-FISH signals (green) on the radish-
 12 origin chromosomes and far red signals of 45S ribosomal DNA. (f) As (e) showing sub-terminal BAC
 13 signal on radish-origin chromosome pair. (g) Isolated chromosomes from a single metaphase showing
 14 the BAC64 clone double dot signals (green) in two additional radish-origin chromosomes (red signal)
 15 and weaker BAC64 double dots signals (green) on two turnip rape chromosomes. Bar 5 μ m.

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1 Figure 2. Dot-plot matrix of *Raphanus sativus* BAC64 clone sequence (AJ550021) and *Brassica rapa*
 2 chromosome A09 sequence. The sequence from the 125kb long BAC is homologous to a region 7Mb
 3 from the end of the 37 Mb long chromosome sequences, consistent with the sub-terminal location
 4 identified by *in situ* hybridization.