Jpn. J. Genet. (1987) **62**, *pp.* 325-332

Cytological localization of highly repeated DNA sequences, the FokI sequence family and BamHI sequence families, in *Vica faba* chromosomes.

BY Kimitaka YAKURA*, Atsushi KATO** and Shigeyuki TANIFUJI**

* *Department of Biology, Faculty of Education, Kanazawa University, 1-1, Marunouchi, Kanazawa* 920
** *Department of Botany, Faculty of Science, Hokkaido University Sapporo* 060

ABSTRACT

Cytological localization of one of the highly repeated DNA sequences of *Vicia faba*, the FokI repeat sequence family, was determined by in situ hybridization. This sequence family was described in our previous paper (Kato *et al.* 1984) as tandem arrays of 59 bp unit sequences. The distribution pattern of silver grains showed that the FokI family sequences are exclusively localized in certain median regions in the long arms of all five pairs of the subtelocentric (S) chromosomes, but are not found in any portions in the metacentric (M) chromosomes. These median regions in the S long arms correspond closely to the positions of C-band heterochromatin revealed by preferential Giemsa staining. Since M chromosomes also possess C-bands, the absence of the FokI repeat sequences in the M chromosomes indicates that *V. faba* C-band heterochromatin consists of at least two different DNA sequences. *V. faba* chromosomes also contain other repetitive sequences, which have been characterized as the 250, 850, 900, 990, 1150, 1500 and 1750 bp BamHI sequence families (Kato *et al.* 1985). In situ hybridization with nicktranslated 250 and 1500 bp BamHI family sequences indicated that these repeated sequences are generally spread all over the chromosomes.

1. INTRODUCTION

Eukaryotic genomes generally contain large or moderate amounts of highly repeated DNAsequences; they are especially abundant in some plants. Highly repeated sequences have often been detected as satellite DNAs by equilibrium density gradient centrifugation and shown to constitute various types of heterochromatin by means of in situ hybridization using such repeated sequences as a probe. A satellite DNA component of *Scilla sibirica* which consists of multiples of repeating units of 33, 48 and 57 base pairs (bp), was shown to be localized in the heterochromatic regions revealed by cold treatment of plants prior to fixation (Deumling 1981). Peacock *et al.* (1981) showed that *Zea mays* knob heterochromatin contains an array of a 180 bp repeating unit sequence, which is a component of satellite DNAs. Bedbrook *et al.* (1980a, 1980b) have described the physical properties and patterns of sequence divergence of six different cloned repeated sequences of *Secale cereale*, which

were localized predominantly in telomeric heterochromatin. Timmis *et al.* (1975) have shown that cRNA transcribed from a *V. faba* satellite component was spread over all chromosomes. Rowland (1980) also carried out in situ hybridization using cRNA transcribed from *V. faba* whole nuclear DNA under conditions which allow hybridization of reiterated sequence only and showed its widespread distribution in chromosomes and interphase nuclei. A similar result was obtained by Cionini *et al.* (1985) that *V. faba* fast renaturing DNA sequences are interspersed in the genome. Moreover, they showed that a satellite component having a lighter buoyant density than the main band DNA was localized in certain regions which correspond closely to the heterochromatic regions stained with quinacrine mustard. However, the molecular properties of these interspersed repeated sequences, as well as the DNA sequences contained in *V. faba* heterochromatin, have not yet been characterized in detail.

We previously isolated several types of *V. faba* highly repeted sequences. One type, the FokI repeat sequence family, was reiterated as tandem arrays of 59 bp unit sequences, with a copy number of 5×10^6 to 5×10^7 per diploid nucleus; it could be cleaved to the 59 bp monomer and its multimers after digestion with the restriction endonuclease FokI (Kato *et al.* 1084). Another type was a group of interspersed repeated sequences which were shown to be discrete fragments of 250, 850, 900, 990, 1150, 1500 and 1750 bp by digestion with BamHI (Kato *et al.* 1985). Each of these BamHI sequence families comprised about 3% of the total nuclear DNA. In this paper we describe the cytological distribution of these *V. faba* highly repeated sequences.

2. MATERIALS AND METHODS

Preparation of slides

Seeds of *V. faba* (cv. Wase Soramame) were soaked in water for 18 to 24 h and germinated for 2 to 4 days at 25°C in moist vermiculite. Root tips were excised from the seedlings, immersed in 0.1% colchicine for 4 to 5 h, and fixed with an ethanol-acetic acid mixture (3 : 1, v/v) at 4°C overnight. The root tips were next incubated in a solution containing 0.2% pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.) and 0.5% cellulase R-10 (Yakult Honsha Co., Ltd.) at 37°C for 2 h to macerate tissues. Portions containing the meristematic region were dissected from the root tips using forceps under a stereoscopic microscope and squashed in 45% acetic acid on cleaned glass slides (Hutchinson *et al.* 1981). The slides were laid on dry ice and cover-slips were removed. The spread cells were then dehydrated by placing the slides in absolute alcohol for 1 h and air-dried.

Preparation of probes

V. faba nuclear DNA was extracted (Yakura *et al.* 1981) and extensively digested with HaeIII, then subjected to sucrose density gradient centrifugation (Kato *et al.* 1984). A HaeIII relic DNA fraction with a high molecular weight ranging from relic DNA fraction with a high molecular weight ranging from about 7 to 10 kilobase pairs (kb) was collected and subsequently digested with FokI. After electrophoresis on polyacrylamide gel, monomer unit sequences of the 59 bp FokI repeat family were recovered from the gel and purified by a series of phenol extractions. The unit sepuences were ligated up to about 1 kb with T4 DNA ligase (Takara Shuzo Co., Ltd.). The ligated DNA was then nicktranslated with ^{3}H-TTP, ^{3}H-dATP, ^{3}H-dCTP and ^{3}H-dGTP using *E. coli* DNA polymerase I (Macregor and Mizuno 1976; Maniatis *et al.* 1982). The BamHI repeat family sequences of 250 and 1500 bp which have been cloned in pBR 322 (Kato *et al.* 1984) were also labeled by nicktranslation. Specific activity of the ^{3}H-labeled DNA probes was 0.6—1.0×10^7 cpm/μg DNA.

In situ hybridization

The slides carrying dehydrated cells were heated at 70°C for 30 min in 2× SSC (1×SSC=0.15M NaCl, 0.015M Na-citrate), dehydrated through 70% ethanol twice for 10 min and absolute ethanol for 5 min and air-dried. The slides were soaked for 30 sec in 3×SSC/50% formamide preheated to 70°C, followed by dehydration through an ethanol series. To each slide was applied 30 μl of a probe mixture containing $5—8\times10^4$ cpm of the ^{3}H-labeled DNA, 3×SSC, 50% formamide, 1×Denhardt's solution, 10% dextran sulfate and 100 μg/ml sheared salmon testis DNA. In situ hybridization was carried out at 43°C overnight, followed by washing 4 times with 3×SSC/50% formamide at 43°C and washing twice with 2×SSC at room temperature. The slides were dehydrated through an ethanol series, air-dried and dipped in 50% Sakura NR-M2 photoemulsion. After exposure for appropriate times, slides were developed and stained with 7% Giemsa solution for 10 to 15 min. After rinsing witer, photomicrographs were taken.

3. RESULTS

We have previously characterized the FokI repetitive seqeuence of *V. faba* and determined its nucleotide sequence (Fig. 1A) (Kato *et al.* 1984). To discover the cytolological localization of this repeat family, in situ hybridization was carried out. Labeling patterns showed that silver grains are predominantly located in certain median regions of the long arms of all five pairs of S chromosomes (Fig. 2A). The probe, however, did not hybridize to any

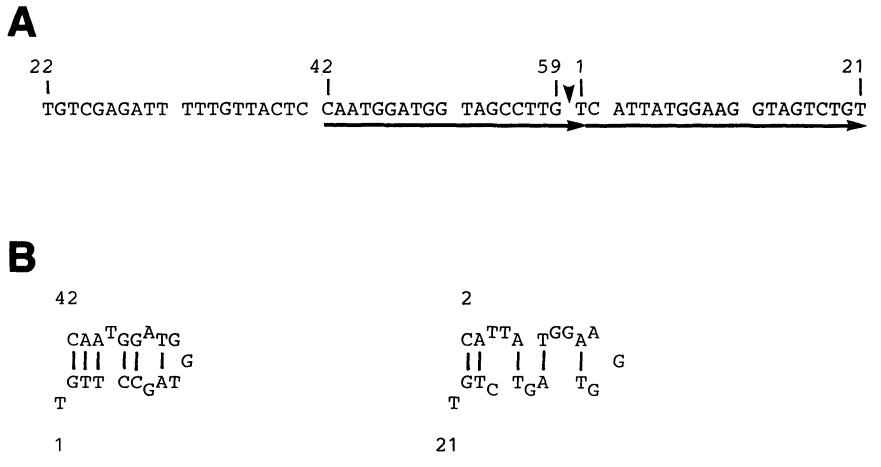

Fig. 1A and B. The nucleotide sequence of the 59 bp unit sequence of the *V. faba* FokI repeated sequence family. Duplicated subrepeats of 19 and 20 bp are indicated by two arrows. The arrow head indicates the cleavage site by FokI. A. The 59 bp repeating unit sequence. B. Dyad symmetrical structures of the duplicated subrepeats.

regions of the M chromosomes. Silver grains were sometimes sparsely seen along the entire length of all the chromosomes. However, these grains were likely due to nonspecific hybridization, because their intensity was at background level and no additional localization in regions other than the median regions of the long arms was found even after autoradiography for a prolonged exposure time. The patterns of interphase nuclei indicated that silver grains are grouped in several clusters (Fig. 2B).

With in situ hybridization of nicktranslated sequences of the 250 and 1500 bp BamHI rapeat families, silver grains were found to be scattered over the interphase and distributed diffusely throughout all the metaphase chromosomes (Figs. 2C and 2D). Careful analysis of many metaphase plates showed that nonhomogeneity of the labeling which might eventually appear in a few plates was accidental, because no chromosomal regions showed preferential labeling in a repeatable way. We previously concluded that seven classes of the BamHI repeat family of 250, 850, 900, 990, 1150 and 1750 bp are interspersed with other unrelated sequences without constituting tandem arrays, on the basis of Southern blot hybridization in which *V. faba* genomic DNA was digested extensively or partialy with BamHI and then hybridized with cloned sequences of these BamHI families (Kato *et al.* 1985). The widespread distribution of ^{3}H-labeled sequences of the 250 and 1500 bp repeated families along the whole length of the chromosomes is consistent with our previous conclusion. Our prior study (Kato *et al.* 1985) also indicated that the distal 250 bp regions of the 850 and 1750 bp BamHI sequence familes were homologous to the 250 bp family sequence. Therefore, it seems probable that the

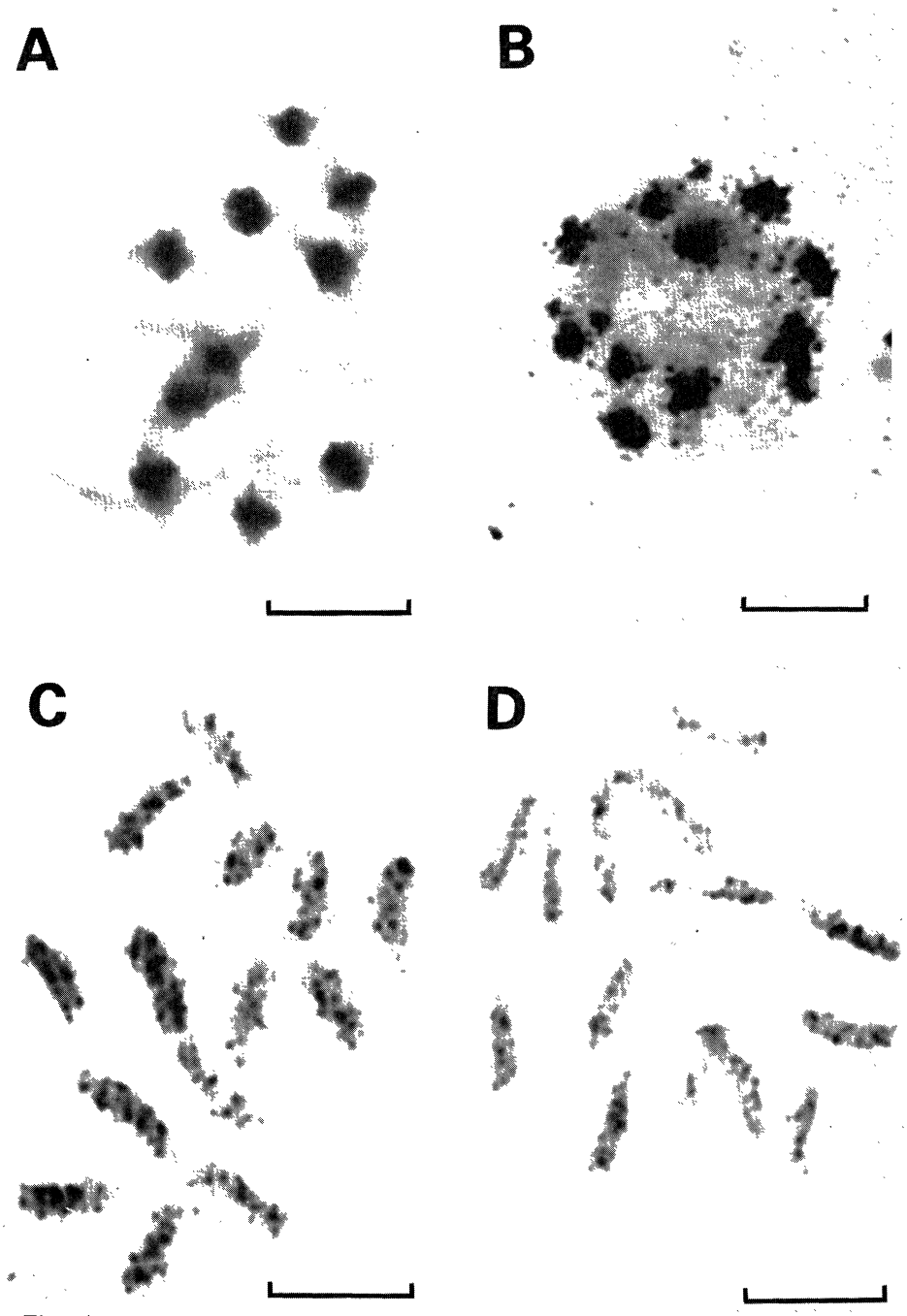

Fig. 2A-D. In situ hybridization of *V. faba* highly repeated DNA sequences. A. Labeling pattern of a metaphase cell hybridized with the nicktranslated 59 bp FokI family sequence. B. Labeling pattern of an interphase nucleus hybridized with the FokI sequence family showing clustered labeling in densely stained chromocenters. C. Labeling pattern of a metaphase cell hybridized with the 250 bp BamHI repeated sequence family, which shows a widespread distribution all over the chromosomes. D. Labeling pattern of a metaphase cells hybridized with the 1500 bp BamHI repeated sequence family. Bars represent 10 μm.

repeated sequences of the 850 and 1750 bp BamHI families are also distributed throughout all the chromosomes.

4. DISCUSSION

V. faba heterochromatin has been revealed by various cytological techniques. Cold treatment of plants brings about the appearance of two unstained bands near the centromere, one in the nucleolar arm and another in the long arm of one metacentric (M) chromosome in a genomic set of six chromosomes (LaCour 1951; Mcleish 1953). The so-called H-segment has been described also for a member of five subtelocentric (S) chromosomes as an irregularly appearing unstained constriction in the median region of the long arm (LaCour 1951). Takehisa (1973), however, reported that median H-segments were observed in approximately half of the S chromosomes.

Heterochromatin blocks can also be revealed by a preferential Giemsa staining technique or its modification. By ordinary denaturation-renaturation Giemsa staining, two regions in the M chromosome, one in the nucleolar arm and another in the long arm, were densely stained, which appeared to correspond to the H segments detectable by cold treatment (Takehisa and Utsumi 1973; Schweizer 1973). In some members of the S chromosomes, proximal regions of their long arms were densely stained (Takehisa and Utsumi 1973; Schweizer 1973). The positions of these proximal Giemsa stained regions, called C-bands, in the M chromosomes and some members of the S chromosomes correspond to those of Q-bands displaying enhanced quinacrine mustard fluorescence (Cionini *et al.* 1985). As mentioned above, a lighter satellite component was characterized as sequences contained in the Q-band regions (Cionini *et al.* 1985). Schweizer (1973), having found that the middle region of the S long arm also became densely stained after application of modified Giemsa staining with additional successive treatments with RNase and 45% acetic acid at 64°C, proposed that there are two or more classes or states of heterochromatin in the genome of *V. faba*. Takehisa (1973) also drew the same conclusion from observing the difference between the M chromosomes and the S chromosomes in the duration of treatments with HCl-acetic acid or cold required to induce the appearance of negatively stained heterochromatin segments. Our present study demonstrated that sequences of the FokI repeat family of *V. faba* highly repeated sequences were localized exclusively in certain median regions of the long arms of all five pairs of the S chromosomes. Since at least half the members of the S chromosomes in a genomic set have no median H-segments and all S chromosomes have Q-bands at the proximal regions in their long arms (with the exception of one S chromosomes having an additional Q-band in its median region), the median regions labeled with silver grains in all the S long arms correspond closely to the median C-bands in the S chromosomes which could be detected only by the modified

Giemsa staining technique.

The FokI repeat sequences did not hybridize to the M chromosomes at all, in spite of the presence of two or probably three C-bands in the M chromosomes, thus indicating that the FokI sequence family is not a component of these heterochromatic regions. Our results confirmed directly that there are at least two types of Giemsa-staining heterochromatin in this species, consisting of different DNA sequences.

Recently Bassi *et al.* (1982) showed that *V. faba* inverted repeated sequences are distributed preferentially to the central regions of the S long arms and both arms of the M chomosome. The labeling pattern of the S chromosomes in their in situ hybridization experiment is very similar to that observed with the FokI repeat family in our present study, but the pattern with the M chromosome was different. Since the 59 bp FokI repeat sequence has a symmetrical structure of two contiguous dyads (Fig. 1B), it is probable that the preparation of inverted repeated sequences obtained by Bassi *et al.* (1982) contained this FokI repeat sequence. However, their preparation contained additional families of symmetrical sequences, since the inverted repeated sequences also hybridized to the central portion of both arms of the M chromosome in which the FokI repeat sequences are not detected (Eig. 2A).

As mentioned earlier, Rowland (1980) has shown by in situ hybridization that *V. faba* highly repeated DNA sequences are spread over the chromosomes. The widespread distribution of fast renaturing sequences along all chromosome arms was also observed by Cionini *et al.* (1985). Their study might be comparable to our own in which the BamHI repeated sequences were hybridized, because each of these sequence families comprises about 3% of the total genomic DNA and the level of reiteration of these sequences is very high. Timmis *et al.* (1975) have shown that sequences of *V. faba* satellite DNA detected after equilibrium density gradient centrifugation in Ag^+/Cs_2SO_4 solution were distributed all over the chromosomes. However, these sequences does not appear to correspond to BamHI repeated sequences, which were also found all over the chromosomes, because our experiments showed that the satellite component separated in an Ag^+/Cs_2SO_4 gradient did not contain the BamHI sequence families. It seems likely that there is another type of interspersed highly repeated sequences in the genome of *V. faba*.

We thank Dr. I. Schubert, Zentralinstitut für Genetik und Kulturpflanzenforschung der Akademie der Wissenschaften der DDR, German Democratic Republic, for his interest and helpful advice. This study was supported by grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES

BASSI, P., CREMONINI, R. and CIONINI, P. G. (1982) Cytological localization of inverted repeated DNA sequences in *Vicia faba*. *Chromosoma (Berl.)* **85**, 453-459.

BEDBROOK, J. R., JONES, J., O'DELL, M., THOMPSON, R. D. and FLAVELL, R. B. (1980a) A molecular description of telomeric heterochromatin in *Secale* species. *Cell* **19**, 545-560.

BEDBROOK, J. R., O'DELL, M. and FLAVELL, R. B. (1980b) Amplification of rearranged repeated DNA sequences in cereal plants. *Nature* **288**, 133-137.

CIONINI, P. G., BASSI, P., CREMONINI, R. and CABALLINI, A. (1985) Cytological localization of fast renaturing and satellite DNA sequences in *Vicia faba*. *Protoplasma* **124**, 106-111.

DEUMLING, B. (1981) Sequence arrangement of a highly methylated satellite DNA of a plant, *Scilla*: A tandemly repeated inverted repeat. *Proc. Nat. Acad. Sci. USA* **78**, 338-342.

HUTCHINSON, J., FLAVELL, R. B. and JONES, J. (1981) Physical mapping of plant chromosomes by *in situ* hybridization. In: *Genetic Engineering* (eds. Setlow, J. K. and Hollander, A.) Vol. 3, pp. 207-222, Plenum Press.

KATO, A., YAKURA, K. and TANIFUJI, S. (1984) Sequence analysis of *Vicia faba* repeated DNA, the FokI repeat element. *Nucl. Acids. Res.* **12**, 37-48.

KATO, A., Y., YAKURA, K. and TANIFUJI, S. (1985) Sequence analysis of *Vicia faba* highly repeated DNA: the BamHI repeated sequence families. *Plant Mol. Biol.* **5**, 41-53.

LACOUR, L. F. (1951) Heterochoromatin and the organization of nucleoli in plants. *Heredity* **5**, 37-50.

MACREGOR, H. C. and MIZUNO, S. (1976) *In situ* hybridization of "nick-translated" ^{3}H-ribosomal DNA to chromosomes from salamanders. *Chromosoma (Berl.)* **54**, 15-25.

MANIATIS, T., FRITSCH, E. F. and SAMBROOK, J. (1982) Enzyme used in molecular cloning. In: *Molecular Cloning*, pp. 97-148, Cold Spring Harbor Laboratory.

MCLEISH, B. A. (1953) The action of maleic hydrazide in *Vicia*. *Heredity* **6**, 125-147.

PEACOCK, W. J., DENNIS, E. S., RHOADES, M. M. and PRYMOR, A. J. (1981) Highly repeated DNA sequence limited to knob heterochromatin in maize. *Proc. Nat. Acad. Sci. USA* **78**, 4490-4494.

ROWLAND, R. E. (1980) Distribution of repetitive DNA sequences in *Vicia faba* chromosomes. *Exp. Cell Res.* **126**, 444-448.

SCHWEIZER, D. (1973) Differential staining of plant chromosomes with Giemsa. *Chromosoma (Berl.)* **40**, 307-320.

TAKEHISA, S. and UTSUMI, S. (1973) Visualization of metaphase heterochromatin in *Vicia faba* by the denaturation-renaturation Giemsa staining method. *Experimentia* **29**, 120-121.

TAKEHISA, S. (1973) Two types of negative heterochromatin in *Vicia faba* as related to nonrandom distribution of chemically induced chromosome aberrations. *Mutation Res.* **17**, 267-269.

TIMMIS, J. N., DEUMLING, B. and INGLE, J. (1975) Localisation of satellite DNA sequences in nuclei and chromosomes of two plants. *Nature* **257**, 152-155.

YAKURA, K., FUKUEI, K. and TANIFUJI, S. (1978) Chromatin subunit structure in different tissues of higher plants. *Plant and Cell Physiol.* **19**, 1381-1390.