Physical mapping of knob-related sequences in some maize determined by FISH (fluorescent *in situ* hybridization) and fiber-FISH

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ABSTRACT

In situ hybridization (ISH) was performed simultaneously to localize and determine the linkage of two labeled probes (TRI and NOR) on the C-metaphase chromosomes of 18 inbred lines and varieties of maize (Zea mays L.). The tandemly repeated DNA sequences TRI (350-bp unit repeats) were labeled with Biotin, while the nucleolar organizer regions (NORs) were labeled with digoxigenin. Analysis of 10 Egyptian lines (Inb2, Inb6, Sd7, Inb8, Sd34, Inb37, Sd58, Inb60, Sd62 and Inb72) and 8 American inbreds and varieties (C103, M017, Parkers flint E742, Parker flint G7390, Tama flint, Wilburs flint, N1018 and DK493). Revealed that the two markers are located on different chromosomes and each of them occupied only one locus in all genotypes. Moreover, extended DNA-fibers (EDFs)-FISH was used to estimate and compare the physical length of the 350 bp repeat locus in the genome of three Egyptian maize inbred lines (Inb2, Sd58, and Sd62) quantitatively. The results revealed that the physical distances of the 350 bp repeat were 939.3, 960.4 and 912.4 Kb, respectively. Furthermore, this technique was employed in accurately estimating the physical sizes of the inter-repeat gaps in each of the studied lines. EDFs-FISH is an effective high resolution tool for quantitative detection of even minute changes in molecular sizes of repeat arrays.

Keywords: *Fluorescence in situ hybridization, NOR, TR-1 (350bp), repetitive DNA sequences, C-metaphase, extended DNA fiber-FISH.*

INTRODUCTION

Fiber-FISH technique has been applied in various ways in genome research in human to analyze overlapping clones (Heiskanen *et al.*, 1994), to detect chromosomal rearrangement (Heiskanen *et al.* 1995b), to determine the physical distance between genes and their 5` to 3` orientation (Heiskanen *et al.* 1995a), to measure the size of the long DNA loci (Shiels *et al.* 1997), and eventually, to expedite positional cloning (Lann *et al.* 1996). Fransz *et al.*(1996)

successfully adopted the fiber FISH technique for plant species.

In general, eukaryotes have genomes much larger than would be expected to code for, and regulate expression of the genes required during development. This has been attributed to the presence of a large amount of repetitive sequences. Cytogenetic and molecular data have so far provided valuable information about the distribution of repetitive sequences along the chromosomes (Fransz *et al.*, 2000).

Among the many economically important plants, maize is one of the most suitable material for development of a physical map by FISH. Maize genome contains 60-80% repetitive DNA sequences interspersed within unrelated repeated DNA or unique sequences (Hake and Walbot, 1980). Studies on the repetitive sequences in the maize genome have mainly focused on interspersed repeated DNA including many transposons and retro-transposons (San Miguel *et al.*, 1998). Cytologically, maize heterochromatin is classified into five types (1) centromeric heterochromatin, (2) NOR heterochromatin, (3) knob heterochromatin, present in the standard (A) chromosome, (4) centromeric knob-like heterochromatin and (5) distal heterochromatin, present in the B chromosome (De Carvalno and Saraiva, 1993). Knobs consist of thousands to millions of tandem 180 and 350 bp repeats (Peacock *et al.*, 1981 and Ananiev *et al.*, 1998b). The heterochromatin at the nucleolar organizar region (NOR) is morphologically similar to knob hetero-chromatin, but it is always located at the secondary constriction on chromosome 6.The nucleolar organizer regions (NORs) contain the major ribosomal genes encoding the 18S and 28S in a tandem fashion (Reeder, 1990). A dramatic progress has been made in mammalian physical map with the development of fluorescence hybridization to extended chromatin fibers or extended DNA fibers (Fransz *et al.*, 1996). The technique involves the release of DNA fibers from lysed nuclei, followed by spreading them across the surface of a microscope slide and hybridization to probes using standard fluorescence protocols.

The present investigation was carried out with three main objectives, (1) to determine the distribution and localization of the 2 heterochromatic loci (TRI) 350 bp and the NOR on the chromosomes of 10 Egyptian and 8 American maize inbred lines and varieties using the FISH technique; (2) To investigate the linkage between these two loci; (3) To apply the fiber-FISH technique to estimate the physical sizes of the TRI (350 bp) knob in three different Egyptian maize lines(Sd62, Sd58 and Inb2) and (4) to estimate the sizes of the gaps on the physical map of *Zea mays*.

MATERIALS AND METHODS

Plant materials

Ten Egyptian maize inbred lines (Inb 2, Inb6, Sd7, Inb8, Sd34, Inb37, Sd58, Inb60, Sd62 and Inb72) and 8 American lines (C103, M 017, Tama flint, Dk493, Parker flint E742, Parker flint G7390, Wilbures flint and N1018), were used for cytological preparations for FISH (Table1). The Egyptian genotypes were provided by the Maize Research Department, Field Crops Research Institute, ARC, Giza, while the American genotypes were obtained from North Central Regional Plant Introduction Station, USA.

Table (1): Name and pedigree of the eighteen Egyptian and American maize varieties and inbred lines.

Inbred line or variety	Pedigree
Inb-2	G4 (American Early)
Inb-6	G102 (Giza Baladi)
Sd-7	American Early Dent / Composite A4
Inb-8	G2210 Early White Composite
Sd-34	American Early Dent / Composite A4
Inb-37	Composite A4
Sd-58	Teplacingo No.5
Inb-60	Rg - 15g - 8 - (syn lapostaxci 64) X Sc14
Sd-62	Teplacingo No. 5
Inb-72	Rg - 29 g s (syn . Laposta X 303) G 216 X M-02 RF
C103	Lancaster sure crop sel (Connecticut)
M017	C1187-2xC103 (Missouri)
Parker flint E742	*
Parker flint G7390	*
Tama flint	*
Wilburs flint	*
N1018	*
DK493	*

* The pedigree of these varieties is not available.

Chromosome preparations for FISH

Seeds of the Egyptian and American lines were germinated to several millimeters in petri dishes in an incubator at 37°C. Roots were cut in 2 mM 8-hydroxy-quinoline for 4 hr. and fixed for several hr. Root tips were treated with Onozoka enzyme and fixed on the slide according to Jiang *et al* (1995) with few modifications as described in details by Adawy (2002).

Preparation of DNA fibers

Extended DNA fibers were obtained by first, isolating of leaf nuclei according to Liu and Whittier (1994). In short, 2 g of fresh leaf tissue were ground to a fine powder in liquid nitrogen, using a precooled mortar and pestle. The powder was then transferred to a 50-ml tube with 20 ml of cold nuclei isolation buffer (NIB) composed of 10 mM Tris-HCl (pH 9.5), 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 4 mM spermidine, plus (1.0 mM spermine, 0.1% (v/v) mercaptoethanol) and gently shaken on ice for 5 min. Subsequently, the solution was filtered sequentially through 180, 120 and 47µm mesh nylon membranes, while on ice. The 22 µm mesh filtration step was omitted in order to ensure a high concentration of nuclei. The filtrate was supplemented with 1 ml of 10% (v/v) Triton X-100 in NIB and then centrifuged at 2000 x g for 10 min at 4°C . The pellet was resuspended in 200 µl NIB, supplemented with an equal volume of 100% glycerol, and stored at -20°C.

Extension of DNA fibers followed the method of Fransz *et al.* (1996) with several modifications. Instead of tilting the slide and allowing the fibers to run down it, the DNA fibers were extended by dragging with a cover slip on Poly L-lysine (Sigma) slides which gave longer and cleaner DNA fibers. For 10 slides, 10 µl of the nuclei suspension was centrifuged at 3600 rpm for 5 min, and the pellet resuspended in 15-30 µl of PBS (10 mM sodium phosphate (pH 7.0) plus 140 mM NaCl). To optimize the quality of the DNA fibers, the number of nuclei that are deposited on the slide can be increase or decreased by adjusting the amount of PBS used to resuspend the pellet. Then, 2 µl of the suspension were deposited on one end of a Poly L-Lysine

microscope slide and dried briefly. After this step, 10 µl of lysis buffer (0.5%(w/v) SDS, 5 mM EDTA, plus 100 mM Tris (pH 7.0) were placed on the nuclei and incubated at room temperature for 4 min; the DNA fibers were then extended using a clean cover slip. The slides were completely air-dried, fixed in 100% ethanol - glacial acetic acid (3:1) for 2 min and finally baked at 60°C for 30 min.

Sources of probes, labeling and hybridization

Two repetitive sequences were used as probes, (TR-I) 350 bp knob and the nuclear organizer regions (NOR). The two probes (350 bp and NOR) were labeled with biotin 11 dUTP, and digoxigenin-11-dUTP, respectively, by nick translation. Preparation of the hybridization mixture was according to Jiang *et al* (1996). The probes and chromosomal DNA were denatured in an oven at 80°C for 5 min, followed by incubation at 37°C in a hybridization chamber overnight. The washes, buffer and protocol used for detection followed Zhong *et al* (1996). The biotin-labeled probe was detected with FITC Avidin followed by incubation in anti-avidin and finally with FITC Avidin (Vector Laboratories). Digoxigenin-labeled probes were detected by mouse anti- Digoxigenin, (Boehringer Mannheim) followed by dig anti-mouse and finally with Rodamine anti-dig (Boehringer Mannheim). The slides were counterstained with 4`,6 diamidino 2 phenylindole (DAPI) in Vectashield mounting medium (Vector Laboratories).

Image capture and analysis

Slides were examined using an Olympus Bx60 fluorescence microscope. Gray scale images were obtained using a Sen Sys CCD camera (photometrics) and then pseudo-colored and merged using IPLab spectrum V. 3.1 software. IPLab spectrum software was also used to make measurements on digitized images. Standard deviations were calculated for all measurement means, except where, 95% confidence intervals (CI) are noted.

RESULTS AND DISCUSSION

Localization of NOR and 350 bp repeat element by FISH

In this investigation, *in situ* hybridization (ISH) was carried out using the two probes 350bp element labeled with biotin and NOR labeled with dig (non-radioactive labeling systems) simultaneously and chromosomes were counterstained with DAPI. These two probes were applied on metaphase chromosomes in ten Egyptian maize lines and eight American maize varieties and inbred lines. In this context, Chen *et al.* (2000) demonstrated and confirmed that simultaneous double color FISH in combination with DAPI counterstaining is a powerful method, not only for physical mapping of DNA sequence but, also for providing chromosomal landmarks. Moreover Maize genome have mainly interspersed repeated DNA including many transposons and reterotransposons (San Miguel *et al.*, 1998). Fransz *et al.* (2000) reported that cytogenetic and molecular data have so far provided valuable information about distribution of repetitive sequences along the chromosome maps consisting of thousands to millions of tandem 180bp and a new 350bp repeats and various retero-transposons. The two labeled probes were simultaneously hybridized on the same slide of metaphase chromosomes of all the tested lines. The results revealed the presence of NOR in only one locus, in only one pair of chromosomes in all tested lines. On the same time, transposable element 350bp (TRI) was located on another chromosome in all varieties and inbred lines. Fig. (1) illustrates the independent assortment of the two probes on the chromosomes, the dig labeling detected

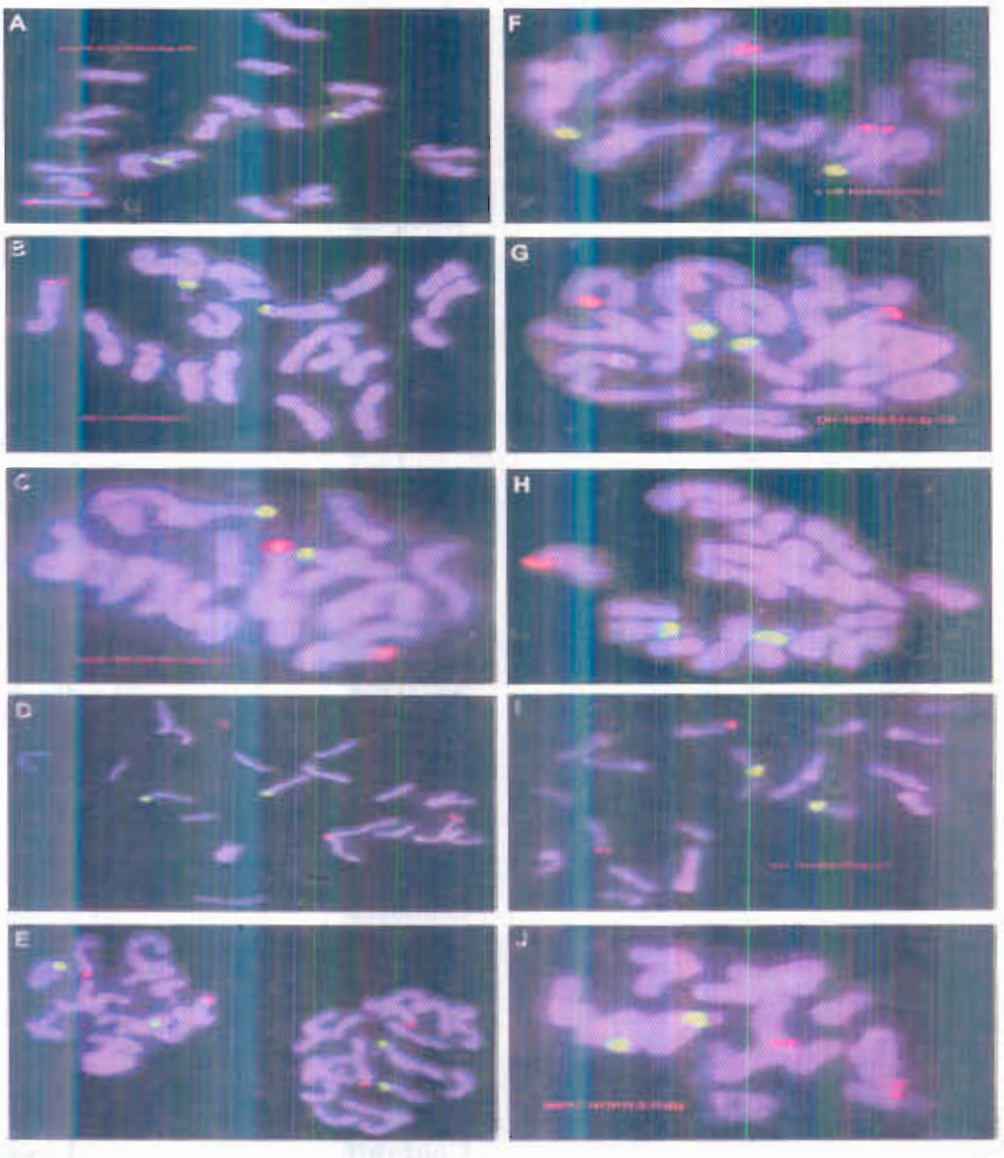

Fig. (1): Two color FISH on metaphase chromosomes of five Egyptian (A-E), and American (F-J) maize inbred lines and varieties using the NOR labeled with Dig 11 dUTP (Red) and 350 bp labeled with Biotin 11 dUTP (Green).

with anti-dioxigenin (red color) and the biotin labeling detected with FITC anti-biotin (green color).

In ten complete metaphase cells examined in detail, we have localized the NOR region on the chromosome pair number 6, and this finding can be used in the future as a detecting tool for the chromosome number 6 of the maize. Similarly, Leitch and Heslop-Harrison (1992), used *in situ* hybridization system in detecting NOR chromosomes number 1,2 and 5 in barley. Based on molecular analysis, Copenhaver and Pikaared (1996b) localized the two NOR loci on chromosomes 2 and 4 in *A. thaliana* and determining their size as 3.5-4 Mb each. Maguire (1983) stated that, arm ratios and NOR location are generally considered a more diagnostic tool for chromosome identification in maize than precise total length, particularly in chromosome number 6 where the total length (μm) in the two homologues was 10.41 and 9.61. In this respect, Reeder (1990) reported that the nucleolar organizer regions (NORs) give rise to the interphase nucleolus and contain the major ribosomal genes encoding the 18S, 28S and 5.8S rRNA molecules. Moreover, Delany *et al.* (1994) added that the importance of the rDNA genes cluster is illustrated by the high degree of conservation of coding sequences, the large number of gene copies, and the localization of these genes at specialized chromosome regions.

Concerning the 350bp element, Ananiev *et al.* (1998b) indicated eight sites of TR-1 elements on the pachytene chromosomes of the Seneca 60 maize inbred line. Adawy (2002) studied the chromosomal distribution of the 180bp and 350bp Knob- associated sequences among the ten Egyptian maize lines using FISH and reported the presence of the TRI repeats in only one locus in all the studied lines. While, Chen *et al.* (2000) found only two clusters of signals from TR-1 repeats in pachytene chromosomes of maize by FISH, one located in the long arm of chromosome 4 at a site approximately 0.89 distal to the centromere and the other co-located with signals from 180bp repeats in the terminal knob (satellite) on the short arm of chromosome 6. The difference between our data and Ananiev s and Chen s data in localizing the 350bp could be due to the use of different genetic material of maize.

Physical mapping of the 350bp element using fiber-FISH.

A major improvement in FISH detection and sensitivity has been achieved with a technique known as FISH to extended DNA-fibers which make use of linearized chromosomal DNA on which FISH signals of different probes directly reflect the physical position of the probe DNA along the DNA molecule. As extended DNA fibers directly display linear positions of DNA sequences in a construct or contig, *in situ* hybridization is particularly informative for evaluating the precise localization and ordering of clones, resolving overlaps and distances, and providing a detailed image of the integrity and coliniarity of probes on chromosomal target DNA.

The data presented in this paper show a detailed molecular cytogenetic analysis of a complete length of the locus 350bp transposable element (TR-1) in three Egyptian maize lines resulting in a physical map of the locus.As shown in Fig(2), fiber-FISH signals of the tandemly repetitive 350bp knob reveal long continuous stretches.

Calibration of the fiber-FISH technique

The 350bp transposable element was hybridized to extended DNA-fibers (EDFs) in order to calibrate and measure the physical length of the 350bp on the three maize inbred lines. The average sizes of signals was

calculated from ten different extended DNA-fibers. As shown in Table (2), the data confirmed that the estimated sizes based on fiber-FISH were very close. One direct application of the fiber-FISH technique is in measuring the physical sizes of DNA loci by converting microscopic sizes (micrometere) into linear DNA sizes (kilobases). According to Watson-Crick DNA model, double stranded DNA molecules can be maximally stretched to 2.97 kb/μm. Previous reports on fiber-FISH resolution ranged from 2.78 to 3.3 kb/μm in both mammalian and plant species (Heiskanen *et al.*, 1995b; Fransz *et al.*, 1996 and Shiels *et al.*, 1997). Jackson *et al* (1998), estimated the average size of signals based on fiber-FISH and found it to be very close to those derived from sequencing analysis.

Table (2) summarizes the average length of locus 350bp and the standard deviation in μm and after converting to kb for each of the 3 maize lines. The average physical length in the genotype Sd62 was 912.4±116 kb, while, in the genotype Sd 58, it was 960±116 kb and in Inb2 was 939±161 kb. McClintock *et al.*, (1981) reported that races of maize have different sets of knobs with a total of 23 locations on the ten chromosomes. The size number and chromosomal distribution of knobs vary among strains but are constant within the same strain.

Table (2): Determination of fiber-FISH length of 350bp-element in three Egyptian maize inbred lines.

Maize lines	Average Length μm	Length Kb	Standard Deviation μm	Standard Deviation Kb.	Number of gaps
SD62	317.9	912.4	32.8	94	2
SD58	334.6	960.4	40.5	116	7
Inb2	327.3	939.3	56.3	161	7

Analysis of gaps in the physical map of the 350bp element.

The physical map contains gaps different from one line to another in the length and number of gaps in each strand.

As demonstrated on the diagram of the 350bp element (Fig. 2), the repeat pattern determined by fiber-FISH is distributed independently in each line, and the number of gaps was 2 in line Sd62 and seven in each of lines Sd58 and Inb 2. Also the length of these gaps was completely different among lines. In line Sd62, the two gaps were 33.6 and 43 kb. The size of gaps in line Sd58 was 9,18, 9,18, 9,22 and 9 kb. While in the line, Inb2, the lengths of the seven gaps were 8.4, 8.4, 8.4, 20, 18, 8.4 and 16.8 kb, based on the genetic distances inside the strand fiber. Thus, extended DNA fibers provide a detailed image of the integrity and colinearity of probes on chromosomal target DNAs. Ananiev *et al.* (1998a), reported that the knobs are considered as stable chromosomal structures characteristic for each line of maize. Jackson *et al.* (1998), Dunham *et al.* (1999) and Hattori *et al.* (2000) used fiber-FISH primarily in estimating gap sizes between assembled contigs and hypothesized that such gaps result from regions of unclonable DNA and are believed to often be associated with low-copy large repeats. Moreover, Fransz *et al.*, (1996), Jackson *et al.*, (2000) and Fransz *et al.*, (2000) confirmed that, fiber-FISH is an effective technique in the physical mapping of repetitive chromosomal regions and these authors demonstrated that large DNA contigs ranging from several hundred kilobase up to 2 Mb can be analyzed by fiber-FISH in a single experiment.

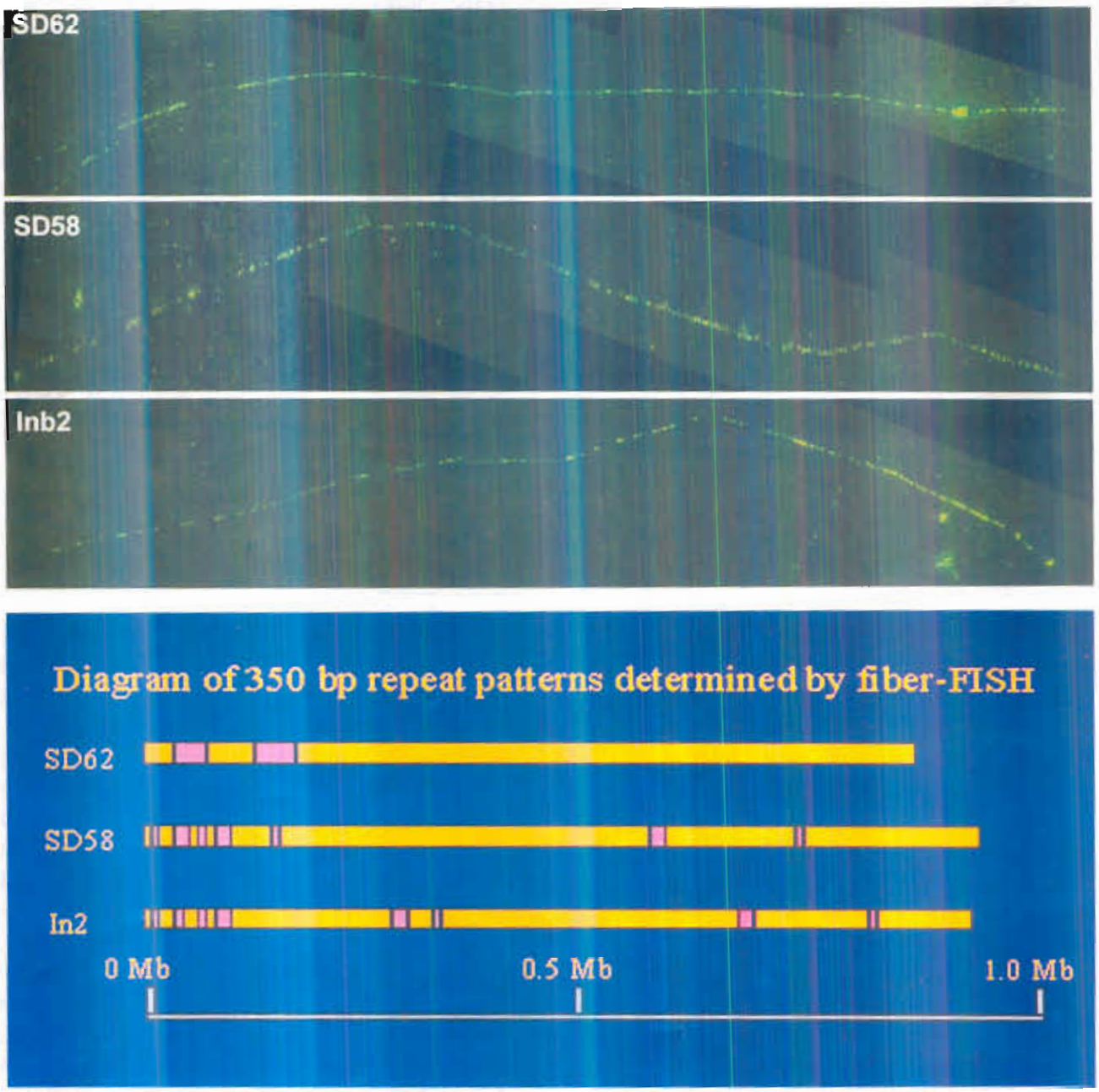

Fig. (2): Fiber -FISH signals from 350bp transposable element (green) after labeling with biotin and detected with FITC Avidin, Anti-avidin and FITC Avidin in 3 Egyptian maize genotypes (Sd 62,Sd 58 and Inb2).

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الملخص العربي

الخريطة الفيزيقية للـ Knob في بعض سلالات الذرة باستخدام تقنية التهجين الفلورسنتي FISH والتهجين الفلورسنتي لخيط واحد من الكروماتين fiber-FISH

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تم استخدام تقنية التهجين الخلوي الفلورسنتي FISH في مرحلة الانقسام الميتوزي لجذور الذرة ونوعين من المسابر ٣٥٠ قاعدة نتروجينية (TRI) ومنطقة منظم النوية (NOR) بعد تعليمهما بالمواد غير المشعة البيوتين (Biotin) والدج (dig)علي الترتيب علي ١٨ صنف وسلالة من الذرة الشامية المصرية والأمريكية لدراسة وجودهما داخل جينوم هذه السلالات . و دراسة عدد المواقع داخل كل سلالة وصنف و أيضا دراسة العلاقة الأرتباطية لهذين المسبرين بعد تهجينهما معا علي نفس الشريحة الزجاجية. كانت السلالات المصرية العشرة هي ٢، ٦، ٨، ٣٧، ٦٠، ٧٢، Sd62, Sd58, Sd34, Sd7 بينما كانت السلالات الأمريكية هى باركرفلنت G7390 و تامافلنت ولبرفلنت و باركرفلنت N1018, M017, E742 C103, DK493 أظهرت نتائج البحث أن هذين المسبرين يقعان علي كروموسومات مستقلة وليست هناك علاقة ارتباطيه بينهما.وكل منهما يحتل موقعا واحدا علي الكروموسومات داخل جينوم جميع السلالات والأصناف المدروسة. أيضا في هذه الدراسة تم استخدام تقنية خيط د ن ا المنفرد (fiber- FISH) لقياس الطول الفيزيقي للموقع (TRI) ٣٥٠ قاعدة نتروجينية داخل جينوم ٣ سلالات مصرية (Sd62, Sd58 والسلالة ٢) . وبينت النتائج أن طول الخيط ٩٦٠,٤ ، ٩١٢.٤ ، ٩٣٩,٣ كيلو قاعدة في الثلاث سلالات على التوالي بالإضافة إلى وجود مسافات بينية (gaps) داخل كل خيط تختلف في عددها وطولها الفيزيقي من سلالة لأخري . ولذا فان تقنية خيط د ن ا المنفرد (fiber- FISH) فعالة ويمكن استخدامها في تحديد أي تغيرات دقيقة جدا داخل التتابعات المتكررة للـ د ن ا.