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***In situ* Hybridization of β -Tubulin to Alfalfa Chromosomes**

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In situ hybridization techniques have been employed to identify two chromosomes of alfalfa. A heterologous sequence of β -tubulin was labeled with biotin using nick translation to produce a nonradioactive probe which was hybridized *in situ* to chromosome squashes of diploid ($2n = 2x = 16$) cultivated alfalfa. The streptavidin-horseradish peroxidase complex was used to localize the probe on the chromosomes. Specific hybridization was observed on two pairs of chromosomes, one small metacentric chromosome and one submetacentric chromosome. Video enhancement technology was used to improve the visualization of the hybridization sites. Radioactively labeled β -tubulin probe also hybridized to specific bands on a Southern blot of alfalfa DNA. This procedure is especially use-

ful for plant species which have been difficult to karyotype using conventional procedures due to small chromosome size and/or high basic chromosome number. Refinement of this technique is underway to identify all 8 pairs of chromosomes for the construction of a standardized alfalfa karyotype.

Alfalfa (*Medicago sativa* L.) is the number 1 forage crop in North America, and it is fourth in total crop acreage (Barnes et al. 1988). The potential for the improvement of alfalfa is enhanced by its capacity to regenerate *in vitro* from protoplasts (Kao and Michayluk 1980) and to be transformed by *Agrobacterium* (Shahin et al. 1986). However, the benefits from using these technologies are diminished due to the lack of a chromosomal linkage map and basic genetic knowledge of alfalfa.

Potential utilization of the biotechnology available for the identification of important agronomic traits and thus the improvement of alfalfa will require the construction of a chromosomal gene map. Once developed, the chromosomal gene map can be used to understand the alfalfa genome. Alfalfa is an autotetraploid ($2n = 4x = 32$) and expresses tetrasomic inheritance. A genetic linkage map using classical methods has not been developed for this species. However, a few known chromosome linkages have been derived through trisomic studies (Kasha and McLennan 1967).

Cultivated alfalfa can be haploidized to the diploid level ($2n = 2x = 16$) by crossing $4x$ plants with pollen from $2x$ plants (Bingham and Saunders 1974). A karyotype of pachytene chromosomes has been established for diploid alfalfa (Kasha et al. 1970); however, pachytene analysis is very laborious in alfalfa. A karyotype of somatic chromosomes of diploid or tetraploid alfalfa has not been developed due to the small size of the chromosomes (2 to 5 microns in length) and the difficulty in identifying all 8 pairs of homologous chromosomes [4 pairs of chromosomes are metacentric and relatively the same size (Kasha et al. 1970)].

The classical methods of identifying chromosomes are by morphology (size, location of the centromere, and arm lengths) and banding techniques such as G- and C-banding. These techniques have not been successful in identifying individual mitotic chromosomes of alfalfa (McCoy and Bingham 1988). *In situ* hybridization of labeled DNA probes to mitotic chromosomes has been used to identify specific chromosomes in wheat (Rayburn

and Gill 1986). This technique has been shown to be sensitive enough to identify single copy DNA in *Crepis* (Ambros et al. 1986).

The objective of this paper is to determine if *in situ* hybridization techniques could be used to identify the location of β -tubulin on alfalfa chromosomes.

Materials and Methods

Southern Blots

Genomic DNA was extracted from alfalfa plants identified as having resistance (R) or susceptibility (S) to races 1 and 2 of anthracnose caused by the organism *Colletotrichum trifolii*. Stems and leaves were collected from greenhouse grown plants at the prebloom stage. The plant material was frozen in liquid nitrogen and freeze dried immediately after harvest. The DNA was extracted by the C-TAB method (Keim et al. 1988). Digests of 2 μ g DNA samples by restriction endonucleases Eco RI and Hind III (Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin 53711 and Boehringer Mannheim Biochemicals, 9115 Hague Road, PO Box 50816, Indianapolis, Indiana 46250, respectively) were electrophoresed in 0.8% agarose gels. Following acid depurination and denaturation, the DNA was transferred to Biotrans[®] nylon membranes (ICN Biochemical, Inc., 3300 Hyland Avenue, Costa Mesa, California 92626) by the method of Southern (1975). A 10 Kb DNA sequence of β -tubulin from *Chlamydomonas reinhardtii* (Youngblom et al. 1984) was used as the probe for hybridization. Plasmid DNA was prepared by the alkaline lysis method (Sambrook et al. 1989) and was labeled with [α^{32} P] dCTP by the method of random primed DNA labeling (Feinberg and Vogelstein 1984) using a kit from Boehringer Mannheim Biochemicals, according to manufacturer's instructions. The blot was prehybridized for 6 h at 37°C in 50 ml of prehybridization solution (5 \times Denhardt's solution, 5 \times SSC, 50 mM sodium phosphate at pH 6.5, 0.1% SDS, 30% formamide, 5% dextran sulfate, 250 μ g/ml denatured sheared salmon sperm DNA) with gentle agitation. The denatured probe was added to 40 ml of the prehybridization solution, and the hybridization proceeded overnight. The blot was washed twice for 5 min in 2 \times SSC, 0.1% SDS at room temperature, then in 0.2 \times SSC, 0.1% SDS twice for 5 min at room temperature, twice for 10 min at 50°C, and finally 5 min at 60°C. The blot was exposed to X-OMAT AR film with one intensifying screen for 6 h at -70°C.

In situ Hybridizations

Chromosome squashes of cultivated diploid ($2n = 2x = 16$) alfalfa (CADL), obtained from E. T. Bingham, University of Wisconsin, Madison, Wisconsin were made for *in situ* hybridization experiments to identify chromosomes. A single root tip was harvested per germinating CADL seed. The root tips were pretreated in ice water for 24 h and fixed in 3:1 (alcohol:glacial acetic acid) for a minimum of 24 h. A single root tip was placed on a microscope slide for chromosome squash preparation using 1% acetocarmine and the slides were stored at -70°C.

The 10 Kb DNA sequence of β -tubulin was used as the probe for hybridization to chromosomes. The plasmid DNA was nick translated and labeled with biotinylated dUTP, using a nick translation kit from BRL, following manufacturer's instructions. Detection of the biotinylated probe was performed by using a Detek 1-hrp kit from Enzo Biochemical, Inc. (325 Hudson Street, New York, New York 10013).

In situ hybridization of the β -tubulin probe to alfalfa chromosome squashes was accomplished by applying 20 μ g/ml of labeled β -tubulin DNA in a mixture of 50% formamide, 10% dextran sulfate, and 10 μ g/ml of sheared salmon sperm DNA in 2 \times SSC. The probe DNA was denatured at 100°C for 10 min. Slides, previously stored at -70°C, were air dried, placed in 70% formamide in 2 \times SSC at 70°C for 3.5 min, and then rapidly dehydrated in an alcohol series (70%, 95%, and 100%) at -20°C. Twenty μ l of the probe mixture was applied to each slide and a coverslip was placed over the mixture. The slides were placed on small plastic weighing boats which were resting on filter paper saturated with 2 \times SSC in glass petri dishes (humidity chambers) and incubated for a minimum of 12 h at 37°C. After incubation the coverslips were removed and the slides were rinsed in 2 \times SSC at room temperature for 5 min, 2 \times SSC at 37°C for 10 min, 2 \times SSC at room temperature for 5 min, 0.1% triton X-100 in PBS for 2 min, and PBS at room temperature for 5 min. Slides were then incubated at 60°C in 3% bovine serum albumin, fraction 5 (BSA) (Sigma Chemical Company, PO Box 14508, St. Louis, Missouri 63178) in PBS for 1 h, after which the slides were briefly rinsed in PBS. Slides were allowed to drain but not dry.

The site of *in situ* hybridization was detected by binding of a streptavidin-horse-radish peroxidase complex (Enzo) to the biotinylated probe. A 100 μ l aliquot of the streptavidin-horse-radish peroxidase com-

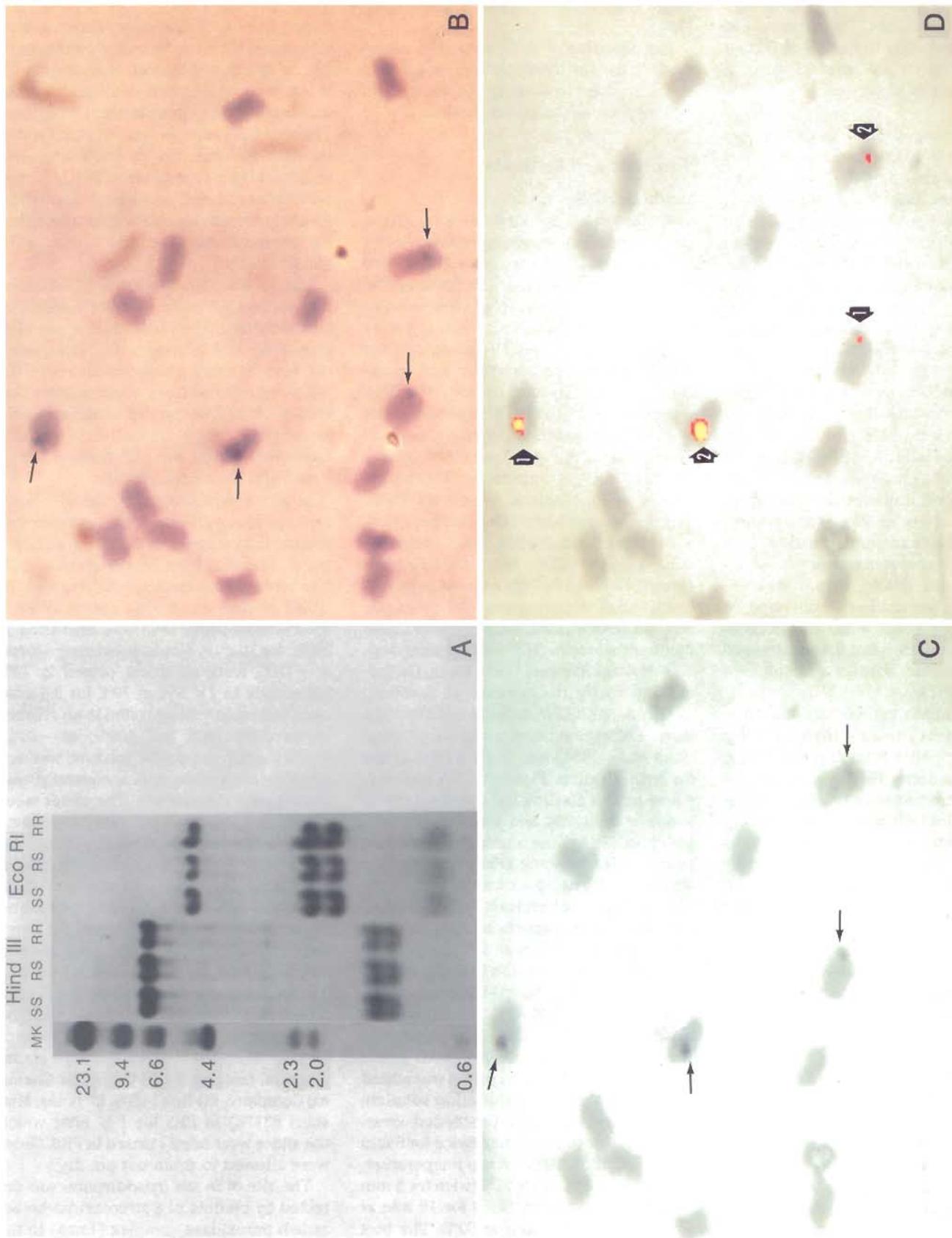


Figure 1. (A) Southern blot of DNA from three different alfalfa plants resistant and susceptible to races 1 and 2 of anthracnose (designated as SS, RS, and RR), digested with Hind III and Eco RI, probed with β -tubulin. The left lane is a marker (MK) λ DNA digested with Hind III and end labeled with [32 P]dCTP. The size of the fragment is indicated to the left in Kb. (B-D) *In situ* hybridization of biotin labeled β -tubulin to the same diploid ($2n = 2x = 16$) CADL alfalfa chromosome spread. (B) Chromosomes as viewed through a microscope; arrows indicate hybridization sites. (C) Enhanced image utilizing video enhancement techniques. (D) Pseudocolor enhanced image to show location of β -tubulin, (arrow 1) identifies a pair of small metacentric chromosomes and (arrow 2) identifies a pair of submetacentric chromosomes.

plex was applied to each slide. Coverslips were replaced and the slides were incubated in a humidity chamber at 37°C for 30 min. Coverslips were removed and the slides rinsed in 2 × SSC for 5 min at room temperature. After a brief rinse in PBS, 410 μl of a solution of 0.05% diaminobenzidine tetrahydrochloride (DAB) (Sigma) and 4% hydrogen peroxide was placed on the slides for 5 min. Slides were rinsed in PBS and immediately stained with 2% Giemsa (Fisher Scientific Company, 711 Forbes Avenue, Pittsburgh, Pennsylvania 15219) for 1 min. Slides were then air dried and mounted in permount overnight. The hybridization sites appeared as brown stained spots on the chromosomes. A minimum of 20 cells/root tip were observed and a total of 10 plants were analyzed.

Image Analysis

Image analysis of the probed chromosome squashes was conducted utilizing a RAS 1000 computerized video enhancement system (Amersham Corporation, 2636 South Clearbrook Drive, Arlington Heights, Illinois 60005) and software developed in association with Loats Associates, Inc. (PO Box 528, Westminster, Maryland 21157-0528). Image analysis consisted of projection of the chromosome image through a Zeiss Universal microscope to a newvacon black and white video camera. The video image was digitized (512 × 480 pixel × 256 gray shades) and the information stored in the computer. Stored images were colorized (pseudocolor) on an analog monitor according to their densitometric measurements. Photomicrographs were produced from a 35mm camera mounted on the microscope and from the video enhanced images with a Polaroid Freeze-frame Video Image Recorder (Polaroid Corporation, Cambridge, Massachusetts 02139).

Results and Discussion

Specific bands were detected on the Southern blot demonstrating that β -tubulin identified specific sequences of alfalfa DNA (Figure 1A). Multiple bands were observed in the Southern blot. This is consistent with other eukaryotes, in that tubulin genes exist in multigene families (Alexandraki and Ruderman 1981; Cleveland et al. 1980; Lee et al. 1983; Lopata et al. 1983; Seebeck et al. 1983).

The locations of homology of the probe to the chromosomes were observed as brown spots caused by the staining of the streptavidin horseradish peroxidase complex to the biotinylated DNA on the blue

counterstained alfalfa chromosomes (Figure 1B). Four hybridization locations were identified on 2 different pairs of chromosomes (Figure 1B, arrows). The β -tubulin probe hybridized to the terminal end of one pair and subterminal region on the other pair of chromosomes. Due to the small size of alfalfa chromosomes, video enhancement techniques were used to improve localization of the probes (Figure 1, C and D). Video enhancement and image analysis of the biotin labeled alfalfa chromosomes allowed the chromosomes to be enlarged so that the hybridization sites could be identified and color enhanced. The chromosome pair with the hybridization site on the terminal end is one of the small metacentric chromosomes which has been difficult to distinguish from the other small metacentric chromosomes (Figure 1D, arrow 1). The chromosome pair with the subterminal hybridization site is a submetacentric chromosome (Figure 1D, arrow 2). Additional DNA probes need to be developed to identify each of the 8 pairs of chromosomes in order to positively identify the specific locations of the β -tubulin gene in alfalfa.

The use of nonradioactive biotin to label DNA probes is a rapid and reliable method for detecting gene sequences on alfalfa chromosomes. The procedure is rapid and is performed on a single microscope slide. Video enhancement techniques are becoming widely used in the biomedical field (Loats et al. 1988), and thus have been shown to have applications in plant cytogenetics. The β -tubulin probe can be used as a marker to identify individual alfalfa chromosomes, due to differences in its location on the chromosome pairs. This procedure is especially useful for plant species such as alfalfa which have been difficult to karyotype utilizing conventional procedures due to small chromosome size and polyploid nature. Researchers are pursuing development of additional probes to identify all of the alfalfa chromosomes. Correlation of this information to the pachytene karyotype will make it possible to develop a standardized karyotype of alfalfa.

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