Characterization of Somatic Chromosome Morphology in Alfalfa, Medicago sativa L.: Comparison of donor plant with regenerated protoclone

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Interest in obtaining karyological information from somatic chromosomes has been stimulated by recent developments in protoplast and tissue culture research. Plants regenerated through cell culture often exhibit characteristics different from the parental material (cf. Larkin and Scowcroft 1981). A certain proportion of this variation has been attributed to changes in chromosome structure and number (Sunderland 1977, Wersuhn and Dathe 1983, Evans et al. 1984).

Presently, research in various laboratories is directed toward regeneration, transformation, and fusion of Medicago species via cell and protoplast culture technologies. Somaclones of diploid and tetraploid Medicago sativa L., alfalfa, may exhibit chromosomal variation from the parental material (Saunders and Bingham 1972, Reisch and Bingham 1981, Johnson et al. 1984). The reported chromosomal variation, other than numerical or structural change, has been unspecified because of the lack of information on somatic chromosome morphology and the difficulty of meiotic studies in tetraploid alfalfa.

The haploid chromosome number, n=16, of tetraploid M. sativa (2n=4x=32) was first reported by Karpechenko (Tischler 1927). This initial count was confirmed by Elders (1926), Fryer (1930) and others (cf. Senn 1938, Lesins and Lesins 1979). Various reports have indicated that four SAT-chromosomes are present in the complemen (Bolton and Greenshields 1950, Bingham 1968, Gillies 1970, Martinez 1976). Recently, alfalfa somatic chromosomes were described by Agarwal and Gupta (1983). However, photographs in their publication did not clearly distinguish chromosome morphology, thus making the resulting karyotype analysis questionable. In order to ascertain specific chromosomes involved in gross structural changes and in losses or additions resulting from tissue culture, a study employing conventional staining and banding techniques was conducted to describe the morphology of the somatic chromosomes of a M. sativa plant. Additionally, a karyotypic comparison was made between the subject plant and a protoclone with an abnormal chromosome number (Johnson et al. 1984) regenerated from the plant.

Materials and methods

The donor plant, designated as RS-K2, is a selection from the Regen-S line, a cultivar of M. sativa with a high proportion of individuals possessing the ability to regenerate from callus.

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Regen-S was developed by E. T. Bingham (Bingham et al. 1975), who has graciously distributed the cultivar to a number of cell culture laboratories. The protoclone, designated as K2-74S, was regenerated according to methodology described in Johnson et al. (1981).

Actively growing root tips from plants grown in clay pots were utilized. Dividing cells in the meristematic region were isolated and analyzed. Specific identification of chromosomes was attempted by conventional acetocarmine staining and various banding methodologies.

**Acetocarmine staining**

The root tips were placed in ice water for 22 to 24 hours and then fixed in a 3:1 mixture of 95% ethanol: propionic acid in which a small amount of FeCl₃ was dissolved to facilitate staining. After 3 to 4 days in fixative, the root tips were hydrolyzed in 1 N HCl at 60°C for 6 minutes, stained in acetocarmine for 3 to 7 days, and squashed in 45% acetic acid.

**Banding methodologies**

The root tips were placed in ice water for 7, 12, or 22 to 24 hours. The shorter immersion periods were used in attempts to produce a high proportion of late prophase cells and/or to reduce contraction in the chromosomes. After pretreatment, the root tips were fixed in a 3:1 mixture of 95% ethanol: glacial acetic acid and refrigerated for 1 to 7 days.

For N-banding, the methodology of Endo and Gill (1984) was followed. Root tips were transferred from the fixative into acetocarmine stain for 1 hour and squashed in 45% acetic acid, with subsequent removal of the cover slip after freezing. The slides were immersed in 45% acetic acid at 60°C for 10 minutes followed by immersion in 95% ethanol for 10 minutes at room temperature (ca. 24°C) and air dried. After drying, the slides were placed in 1.0 M NaH₂PO₄ buffer (pH 4.5-4.8) at 95°C for 2 minutes, rinsed in H₂O, and immersed in Giemsa solution until staining was achieved.

G-banding was attempted according to the methodology of Drewry (1982). After fixation, root tips were placed in 45% acetic acid for 2 to 3 hours and then squashed in the same solution. After removal of the cover slip, the slides were kept at 45-60°C for 12 hours. Slides were then immersed in a trypsin solution (Gibco: trypsin-EDTA 10 × 5.0 ml, Hanks BBS 4.5 ml, H₂O 40.5 ml) at room temperature for 3 to 30 minutes and stained briefly in a filtered Giemsa solution.

C-banding methodology followed descriptions in Rayburn (1984). Root tips were removed from the fixative, placed in distilled H₂O for 2 to 3 minutes and then hydrolyzed in 0.2 N HCl at 37°C for 10 min. Hydrolyzed root tips were then squashed on slides in 45% acetic acid, and the cover slip was removed. After 3 minutes in 5% Ba(OH)₂, at room temperature, slides were rinsed in running distilled H₂O and placed in 2X SSC humidity chambers at 65°C for 45 minutes. After incubation, slides were rinsed successively in 2X SSC and distilled water at room temperature and then stained in 2% Giemsa in a 10⁻² M phosphate buffer (pH 6.8).

For the acetocarmine preparations, the centromeric nomenclature system of Levan et al. (1964) for chromosome designation as modified Schlarbaum and Tsuchiya (1984) was used.

**Results**

Alfalfa root tips immersed in ice for a 22- to 24-hour period were satisfactory for mitotic chromosome studies. This pretreatment period yielded an adequate number of late prophase cells in addition to the commonly found metaphase cells. Shorter periods in ice of 7 and 12 hours were used in attempts to obtain high numbers of cells at mitotic prophase, thus giving banding procedures a relatively large area to exhibit differentiation. However, the shorter time intervals caused chromosomes to become 'sticky' and clump together, making descriptive
Fig. 1. Somatic cell of *Medicago sativa* (RS-K2) showing $2n = 4x = 32$ chromosomes. Secondary constrictions are indicated by sc arrows. Tertiary constrictions are indicated by tc arrows.

Acetocarmine produced acceptable results for observing chromosome morphology (Figs. 1, 4) whereas the various banding methodologies were less satisfactory. N-banding caused one entire arm to stain slightly darker than the other arm in some chromosomes. The majority of chromosomes, however, appeared monochromic. The trypsin digestion used in G-banding was very destructive to the physical chromosome structure in alfalfa. The 20–40 minute interval prescribed by Drewry (1982) for *Pinus* completely eliminated alfalfa chromosomes from the preparation. A shorter immersion period, 3 to 5 minutes, produced centromeric and telomeric bands in a few chromosomes. Even this shortened time period in trypsin appeared to partially digest the chromosomes. C-banding produced the most satisfactory results, with centromeric, telomeric, and intercalary bands observed in several chromosomes. The patterns produced by C-banding and G-banding were similar. However, the C-banding process was less physically destructive to the chromosomes. Although all banding techniques produced some differential staining, the actual differentiation was slight.

Since banding procedures failed to pro-

Fig. 2. Karyotype of *Medicago sativa*: cut from Fig. 1. Chromosomes are arranged according to Table 1. Roman numerals indicate chromosome groups.
duce superior results over conventional staining, a cell from an acetocarmine-stained preparation was selected for karyotype analysis. The cell selected was judged to be the best representative of over 1,000 observed for chromosome morphology.

The complete chromosome complement of M. sativa, 2n = 4x = 32, is shown in Fig. 1. The chromosomes are small, ranging from 1.74–2.46 micrometers in length (Table 1). Fig. 2 is the karyotype cut from Fig. 1 with the chromosomes aligned in groups of four in respect to the autotetraploid nature of M. sativa (Gillies and Bingham 1971; Stanford et al. 1972). Groupings were determined by chromosome morphology, size, and long arm/short arm ratio (Table 1). Sixteen alfalfa chromosomes have centromeres in the median region (m- or M-type) and the remaining chromosomes are submetacentric (msm- or sm-type).

In Fig. 1, arrows indicate four chromosomes possessing secondary constrictions and four chromosomes containing prominent tertiary constrictions. Although chromosomes in these two groups are of approximately the same size, the SAT-chromosomes (Fig. 2, group VIII) are distinguishable from the other chromosomes.
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Chromosomes containing tertiary constrictions (Fig. 2, group IV) by the larger short arm, smaller satellite body, and wider separation of the satellite body from the short arm.

The protoclone, K2-74S, was an aneuploid with $2n=31$ chromosomes (Fig. 3). No tertiary constrictions were observed. A satellite chromosome is missing and three chromosomes have undergone apparent structural changes (Fig. 3). Two chromosomes have abnormally long arms and one chromosome has a somewhat small short arm. The chromosomes are arranged Fig. 4 according to the karyotype analysis procedure applied to the RS-K2 donor plant, with the structurally altered chromosomes being placed in groups from which they likely originated.

Table 1. Measurements in micrometers of the somatic chromosomes of *Medicago sativa* as arranged in Figure 2

<table>
<thead>
<tr>
<th>Chromosome no.</th>
<th>Long arm length (L)</th>
<th>Short arm length (S)</th>
<th>Satellite length</th>
<th>Total length</th>
<th>L/S</th>
<th>Centromere position</th>
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* Tertiary constriction present but not included in length calculation.
** Satellite attached to short arm.
+ Satellite length not included in calculation.

Discussion

In comparison with acetocarmine staining, the various attempts at banding alfalfa chromosomes offered no advantage in specific chromosome identification. Chromosomes subjected to N-banding procedures exhibited less differential staining than those subjected to G- and C-
banding procedures. The occurrence of chromosomes staining with one arm darker than the other arm was previously observed by T. J. McCoy (personal communication), who attempted banding by following a technique of Mok et al. (1974). As observed by McCoy, the differential staining produced by the banding techniques was not superior to aceticarmine staining for specific chromosome recognition.

G-banding also was unsatisfactory for chromosome identification. *Pinus* chromosomes required 20–40 minutes slide immersion in trypsin solution (Drewry 1982), in contrast to the 3 to 5 minute interval found sufficient for alfalfa. Further studies involving less time in trypsin solution or reduction of the trypsin concentration may produce more satisfactory banding.

The C-banding techniques produced the most satisfactory results, with some chromosomes exhibiting telomeric, centromeric and intercalary bands. In comparison with *Triticum* C-banding studies (Gill and Kimber 1974), however, the differential staining was poor. Additionally, not all of the chromosomes exhibited bands with this procedure. Refinement of the procedure may produce satisfactory results, since there have been reports of successful banding of *Phaseolus* (Mok and Mok 1976) and *Solanum* (Mok et al. 1974) chromosomes, which are smaller than those of alfalfa.

The SAT-chromosomes were relatively constant features in the metaphase cells examined. However, the tertiary constrictions were often not visible. This observation prompted the selection of the cell in Fig. 1, because it exhibits the maximum number of distinguishable features of the alfalfa karyotype. The selection of a cell for karyotype analysis not exhibiting tertiary constrictions could produce confusion in future studies relying upon the results of the present study as a basis for comparison.

The chromosomes of groups I and VIII are reasonably distinctive from the other chromosomes in most slide preparations. However, it is difficult to assign some of the other chromosomes to specific groups. For this reason, five classes were designated to aid future investigators in describing which chromosomes or class(es) of chromosomes are involved in structural or numerical changes:

Class 1: This class is comprised of chromosome group I, the very large distinctive submetacentric chromosomes.

Class 2: Chromosome groups II and III comprise this class. Chromosomes in these two groups are approximately the same size and have median centromeres.

Class 3: This class includes the chromosomes of group IV, with prominent tertiary constrictions and the chromosomes of group V. Tertiary constrictions of chromosomes in group IV are not evident in many preparations. In these instances, it is difficult to distinguish the chromosomes of group IV from the nonmetacentric chromosomes of group V.

Class 4: This class is composed of chromosome groups VI and VII. These chromosomes are very small and similar in morphology. Class 4 chromosomes somewhat resemble the chromosomes in group V, except that group V chromosomes are more submetacentric and and somewhat larger in size.

Class 5: The SAT-chromosomes (group VIII) represent this class.

Fig. 5. Idiogram of *Medicago sativa*. Roman numerals correspond with chromosome groups as in Table 1 and Fig. 2. Arabic numbers indicate chromosome classes.
An idiogram reflecting this classification system was developed by averaging the individual chromosome arm lengths in groups I, II and III, IV and V, VI and VII, respectively (Fig. 5).

Applying the classification system to the K2-74S karyotype permits satisfactory segregation of chromosomes into 5 classes, with the exception of group II and the structurally altered chromosomes. Group II chromosomes of K2-74S, in contrast to those of RS-K2, appear to be submetacentric and proportionately larger than group III-chromosomes. This is due to the lesser degree of chromosome contraction of the K2-74S cell in comparison to the RS-K2 cell. Discrepancies between RS-K2 and K2-74S group II chromosomes, however, do not affect the classification system proposed. In both plants, group II chromosomes are more similar in size to group III than to group I chromosomes. Alteration of the classification system is not warranted, since the majority of cells in a slide preparation (using 22- to 24-hour ice pretreatment) are in a highly condensed state, as in Fig. 1.

The size of chromosome 4 precludes classification in any group other than group I (Fig. 4). Apparently, the long chromosome arm has been involved in a translocation and was recipient of a substantial chromosome segment. Inspection of the karyotype reveals no likely candidate for the translocation partner.

According to the classification system, all classes appear to have their full complement of chromosomes, with the exceptions of group VIII, in which a SAT-chromosome is missing, and group III. The remaining two chromosomes exhibiting structural changes could be the end products of a translocation between two chromosomes of group III. Inspection of chromosomes 11 and 12 reveals that if the suspected translocated segment of the long arm of chromosome 11 is added to the shorter arm (less a fraction allowing for an unequal reciprocal translocation) of chromosome 12, then the resulting chromosomes appear to fit well within the size and centromeric ratio characteristic of group III. The possibility that chromosome 12 was a product of a pericentric inversion was also considered. In that case, chromosome 12 could only belong to class 4, which already contains 8 chromosomes. Therefore, this chromosome probably belongs to group III and was involved in a structural change with chromosome 11. Meiotic analysis would provide insight into the relationship between these chromosomes, but meiotic analysis in alfalfa is exceedingly difficult.

The karyotype analysis presented in this study was conducted on a single plant from a selected cultivar. Inspection of photographs from previous cytological studies of different cultivars (Bingham 1968, Mariani 1975) indicate that the chromosomes can be generally segregated into the classes shown in Fig. 3. However, the possibility of intraspecific variation of the karyotype cannot be discounted, because M. sativa is a variable species. The present karyotype analysis and derived chromosome classification system can serve as a basis for describing chromosomal variance in future karyotypic studies of alfalfa, in addition to determining chromosomes involved in gross structural changes.

Summary

Somatic chromosome studies using acetocarmine and various banding procedures were initiated on plants of Medicago sativa (2n=4x=32), alfalfa. Banding methodologies were not superior to a conventional acetocarmine staining procedure in specific chromosome identification. A karyotype analysis was conducted on the acetocarmine-stained chromosomes of a plant (RS-K2) selected from the Regen-S cultivar. Chromosomes were divided into eight groups according to the tetraploid nature of M. sativa. Sixteen chromosomes had metacentric centromeres, with the remaining chromosomes appearing more submetacentric. Four SAT-chromosomes were detected, and chromosomes of one group exhibited tertiary constrictions. Certain chromosome groups were indistinguishable because of similar lengths and arm ratios.
A generalized chromosome classification system was developed to aid in identification of specific changes in chromosome structure and number. Comparison of the karyotypes of the donor plant with a regenerated protocline using the chromosome classification system indicated that translocations and chromosome loss occurred in specific chromosome groups.

Literature cited
