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# Molecular divergence of alfalfa somaclones

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**Summary.** Plantlets were regenerated from alfalfa callus following passage through a tissue culture medium which contained gibberellic acid. A proportion of these plantlets showed obvious morphological variations. Leaflet, stem and petiole tissue of these plants were extracted to yield a soluble protein homogenate which was characterized by polyacrylamide gel electrophoresis. Over 18 individual protein bands were resolved and visualized by staining with coomassie blue G250. Electrophoretic gels from regenerated plantlets and from the parent plant were scanned spectrophotometrically and analyzed. The relative quantity of each of the proteins resolved from plants was correlated with proteins of other plants via the Pearson's product-moment correlation. Cluster analysis was then performed using these correlation coefficients to judge relatedness among somaclones and the parent plant. Two of 22 somaclones (9.1%) differed significantly from the parent and from the other somaclones judged by quantitative protein pattern variations. Three distinctive lineages through tissue culture produced plantlets. Using a discriminant analysis strategy somaclones could be grouped according to lineage with 80.8% accuracy based upon distinctions between protein electrophoretic patterns. Two of the somaclone lineage groupings showed no overlap with the parental grouping which indicated significant molecular divergence of these plantlets as judged by quantitative protein differences.

**Key words:** Somaclones – Electrophoresis – Tissue culture – *Medicago sativa* L. – Cluster analysis – Discriminant analysis

## Introduction

The use of cell and tissue culture for propagation of plants and introduction of new variability recently has attracted much attention. It is well recognized that cells in tissue culture are often genetically and karyotypically altered. Time in culture is thought to play an important role in the amount of variation observed in plants regenerated from tissue culture (McCoy et al. 1982). Hartman et al. (1984) report elevated ploidy level in plants regenerated from long term cultures of alfalfa while they observed the normal tetraploid number of chromosomes in plants regenerated from shorter term cultures.

There may also be changes in chromosome number which do not involve the entire genome. Aneuploidy has been observed repeatedly in plants regenerated from in vitro cell cultures. Examples include ryegrass (Ahoowalia 1983). *Haworthia* (Ogihara 1981), celery (Orton 1983; Murata and Orton 1983) and alfalfa (Johnson et al. 1984; Groose and Bingham 1984).

In addition to these observable karyotypic changes, single gene mutations may also be induced by in vitro conditions. The study of Buiatti et al. (1985) indicates that spontaneous mutations may occur in tissue cultures at frequencies several times greater than those observed in conventional mutagenesis experiments. Evans and Sharp (1983) identified 13 putative nuclear gene mutations among 230 regenerated tomato plants. Selfing of these regenerates produced R1 generations for which 12 of the 13 traits segregated in 3:1 ratios which suggested simple dominant or recessive mutations. Analogous gene action was reported in lettuce by Engler and Grogan (1984) where 10 selfed regenerates produced progeny which segregated in a 3:1 ratio.

Larkin et al. (1984) described somaclonal variation in wheat which they judged from segregation data to be due to both dominant and recessive mutations. Though normally found in the heterozygous state, some true breeding and presumably homozygous, mutants could also be observed. The variation in plant tissues after in vitro subculture observed by

Larkin and coworkers was not explainable by loss or addition of chromosomes as they could not detect gross karyotypic changes.

One of the areas in which the variation which results from tissue culture seems to promise the greatest utility is in plant responses to disease, and to environmental and chemical stresses. The somaclonal variation discovered in tissue culture has proven to be a valuable tool in the selection for plant tolerances and resistances to herbicides and toxins. Chaleff and Keil (1982) selected for resistance to the herbicide pichloram in cultured tobacco cells and obtained regenerated plants not only resistant to pichloram but to hydroxyurea. Somaclonal sugarcane plants derived from callus cultures showed a very high frequency of tolerance to eyespot toxin (Larkin and Scowcroft 1983). This expression of tolerance could be increased through continued selection in culture and could be built upon by successive cycles in culture. Smith and McComb (1983) also have used in vitro techniques in the study of the variation in relative salt tolerance in alfalfa following repeated subculturing. In general, passage of plant tissues through in vitro culture conditions appears to accelerate the expression of new traits, presumably by an increased propensity for genetic alterations.

Electrophoretic analyses of proteins and enzymes have been used in many studies as means of identifing genetic changes and manifestations of gene action at the protein level. Especially pertinent in demonstrating the power of PAGE (polyacrylamide gel electrophoresis) techniques are the studies of McDaniel and Ramage (1970) and of Suh et al. (1977) in which protein PAGE was used to differentiate aneuploids of barley and sorghum from each other and from the disomic lines. Aneuploids which contained different extra chromosomes exhibited distinctive soluble protein patterns. PAGE of proteins has proven to be a powerful tool in biochemical genetic analyses in these and other plant systems.

Although a number of studies have focused upon the genetic basis of somaclonal variation, there is a paucity of information with regard to the biochemical basis for this phenomenon. In this study we have analyzed electrophoretically the changes in cellular proteins which take place in alfalfa somaclones as they arise, and we have quantitated the alternative paths which this variation may follow by discriminant function analysis.

## Materials and methods

*Regeneration*

Petiole and stem tissue from alfalfa (*Medicago sativa* L.), variety 'Regen S', was surface sterilized in 10% bleach (0.525% sodium hypochlorite) for 10 min then cut into 3 to 5 mm pieces before further sterilization in 1% bleach for 1 min. Tissue was then rinsed twice in sterile distilled water and placed on B-II solid medium (Bingham et al. 1975). After one month of initiation on this medium containing a high level of 2,4-Dichlorophenoxyacetic acid (2,4-D) (Walker et al. 1979) the tissue was transferred to B5-GA3. This regeneration medium consists of Gamborg's basal B-5 medium (Gamborg 1970) with 1 mg gibberellic acid (GA3)/liter medium as the only plant growth hormone present. Regen S seed was provided by E. T. Bingham of the University of Wisconsin.

*Electrophoresis*

Proteins extracted from parent plants and regenerated plantlets were analyzed by PAGE. All plants were maintained in growth chambers under low light conditions prior to analysis. Soluble proteins were extracted from leaflet and stem tissues using a buffer consisting of 0.1 M tris, 0.5 M sucrose, 0.1% ascorbic acid, and 0.1% cysteine. The ratio of leaf tissue to buffer was 0.25 g to 2.0 ml. The tissue was macerated in this buffer, then frozen to disrupt cell structure. Cell debris was pelleted by centrifugation in a Sorvall SS-34 rotor at 4,000 × g for 20 min.

Electrophoresis was carried out on a standard pH 8.9, 7% discontinuous, polyacrylamdie slab gel (Davis 1964). The 0.75 mm thick gels were run in pairs using constant current at 3 to 5 amps (ISCO electrophoresis power supply) for 4 to 5 h. The temperature was maintained at approximately 4C by an Endocal Neslab water cooling system. Gels were then fixed and stained in a solution of 12% trichloroacetic acid (TCA) and coomassie blue G for about 24 h. Destaining of gels was achieved by several washes in 7% acetic acid.

Gels were cut into strips and scanned using a Beckman model 3600 spectrophotometer and a Beckman gel scanner accessory. Quantitative estimations of individual proteins were obtained by measuring the relative peak height for the 18 protein peaks which were consistantly identifiable between densitometer scans of all electrophoretic runs.

*Cluster analysis*

A program for a Sage II computer was written (Baertlein 1984) which calculated Pearson's product-moment correlations for the electrophoretic data and derived a relatedness coefficient for each somaclone in comparison to every other somaclone and to the parent plant from which it was derived. A complete description of this program which may be adapted to run on other computer systems is available from either author. Electrophoretic data from the parent plant discussed here were replicated four times. Replicate electrophoretic runs for individual somaclones were not possible as not enough tissue was available on any single somaclone. These correlation coefficients were then used in a cluster analysis in order to construct a phenogram based upon protein variation. The means of the correlation coefficients of plants within clusters were used to describe the degree of relatedness between clusters. The phenogram provides a graphic representation of relatedness of somaclones to each other and to the parent plant based on electrophoretic protein data.

A series of four parents representing two alfalfa varieties and a total of 109 somaclones were analyzed (Baertlein 1984). Here we present only selected representative parent-somaclone lineages from the larger study for brevity.

*Discriminant analysis*

A Mahalanobis based discriminant analysis program available through SPSS (Statistical Package for the Social Sciences) (Nie et al. 1975) was used to determine whether clones of known descent through subculturing from the original parent plant could be classified on the basis of a subset of the protein bands for which quantitative data were taken. Three of the 18 proteins, transformed as

$$\log_e (x+1)$$

in order to eliminate zero values, were chosen according to the statistical design of the computer program based upon their relative effectiveness in distinguishing between the plant

groups. The three somaclone groups consisted of plantlets derived from three different lines of descent through tissue culture, while the fourth group consisted of replicate runs of the parent plant. Using these three proteins from each clone as parameters, three arithmetic functions were determined. A numerical value was computed for each plant using each of the three functions (the X, Y, and Z coordinants for that plant). Statistically, 95% of the plants belonging to a group would be expected to fall in the area described by an ellipsoid having three axes equal to the standard deviation for the X, Y, and Z coordinant with a centroid at a point described by the mean of the X, Y, and Z coordinant values. For clarity, we plotted these data in 2 dimensions.

The discriminant analysis program next uses the three functions to classify the plants into the four groups defined by the functions without relying on prior knowledge of plant lineage. The frequency with which the plants are correctly classified according to group serves as a statistical measure of how precisely defined these groups actually are based upon protein information.

## Results

Of nine plants, all were regenerable by our present method (see "Materials and methods"). This was a higher regenerability than we previously achieved in our laboratory with any of three other published regeneration procedures for alfalfa using the same nine plants as starting materials. (Method of Bingham et al. 1975–33% regeneration; method of Johnson et al. 1981 – 33% regeneration; method of Stavarek et al. 1980 – 0% regeneration). A variation in our regeneration protocol in comparison with other alfalfa regeneration protocols was the inclusion of 1 mg/l $GA_3$ in the regeneration medium, which appeared to potentiate regeneration. $GA_3$ has also been shown to be effective in the regeneration of potato from tissue culture (Firman 1984).

Morphological variation in somaclones often was observed in the form of reduced leaf size and variation in leaflet number. The most striking variant phenotype we observed is shown in Fig. 1. The plant on the left has the typical appearance of a regenerate. The compressed planar habit of the plant on the right has resulted in higher leaf to stem ratio which could be an agronomically useful trait for forage production, if maintained following transfer to the field. This trait has tended to become less pronounced with continued growth in several planar variants observed to date.

Morphological observations are difficult to repeatedly quantitate. Such morphological variations are often the result of complex biochemical pathways involving many gene products, any number of which may be altered compared to wild type. In the present study, we chose to analyze total green tissue protein electrophoretic banding patterns in the form of spectrophotometric gel scans in order to look directly at gene products and thereby obtain an estimate of genetic

**Fig. 1.** Somaclonal variant with a compressed planar morphology (*right*) and a somaclone showing a normal phenotype (*left*)

variation judged at the protein level. A representative electrophoretic gel is shown in Fig. 2. Four dark staining protein bands which can be used as benchmarks when locating other bands can be readily noted. Differences in total protein content were observed as extracts of some clones show darker overall banding patterns. Since insufficient plant tissue was available to accurately determine protein content before electrophoresis, like amounts of protein loaded in each well were estimated based on the fresh weight of the plant and varied somewhat. For this reason, the data were normalized as part of the statistical process. Normalization was designed to eliminate any bias due to small differences in the quantity of protein loaded on gels to enable a conservative estimate of actual quantitative protein variation.

The results of the cluster analysis and plotting of the phenogram (Fig. 3) show a directional (vectorial) change in protein constitution of somaclone tissues as compared to the parent. All of the clones except one fall to the left of the four replicate electrophoretic runs of the parent plant. The level of variation in the parent runs indicates the level of experimental error (all four runs cluster above a correlation coefficient of +0.95). The 99% confidence level at which clones are no longer considered to originate from the same population falls at a correlation coefficient of +0.57. Therefore two of the 22 somaclones, or 9.1%, based upon mean correlation coefficients of clusters, are statistically signifi-

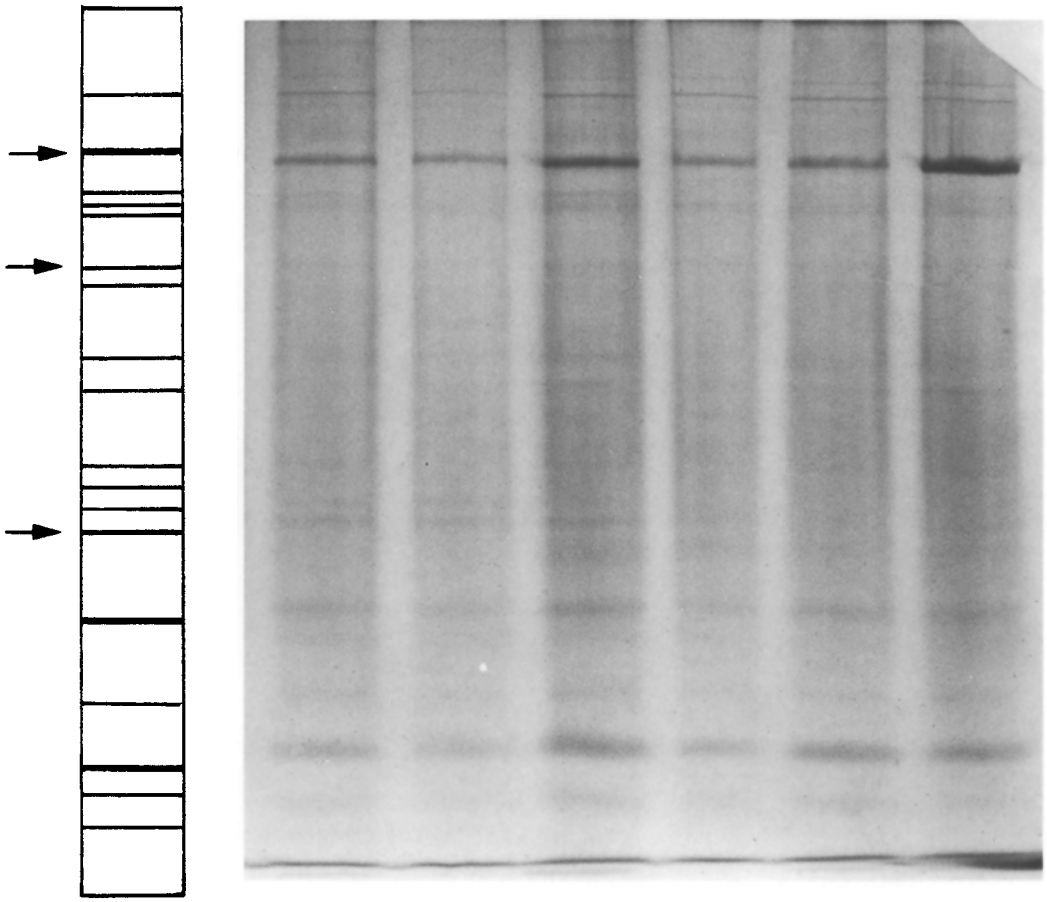

**Fig. 2.** PAGE slab gel from somaclonal tissue extracts stained for total protein using coomassie blue. G250 (see "Materials and methods"). Protein bands used for cluster analysis are diagrammed on the *left* and those used in descriminant analysis are marked with *arrows*

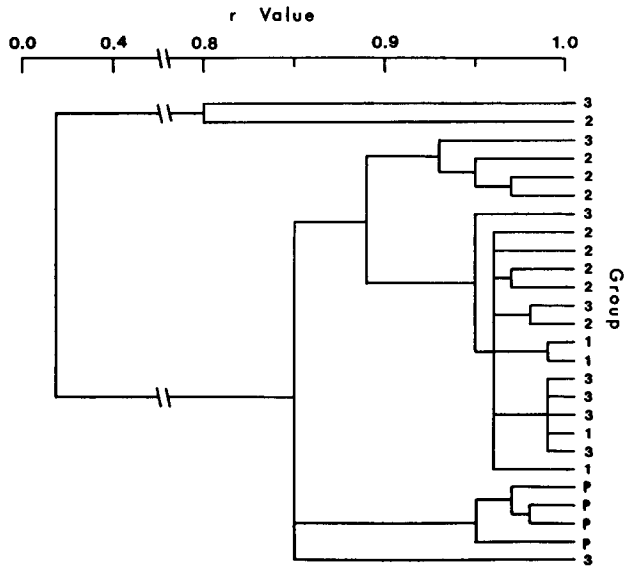


**Fig. 3.** A phenogram based upon cluster analysis of the 22 somaclones and 4 electrophoretic runs of the parent plant. The 99% level of significance falls at an r value of +0.57

cantly different from the parent and from the other somaclones as judged by protein phenotypes.

Identical experiments as described here for somaclones derived from a single parent plant, were conducted for somaclones from four other parent plants. Analysis of these plants yielded a mean frequency of variants of 6.9% (6 of 87). The overall frequency of protein variants for all five parent plant derivative clones was 7.3% (8 of 109) (Baertlein 1984).

Through discriminant analysis we created three mathematical functions which were used in the classification of sequential tissue samples of the parent plant and of the three groups of somaclones which differed in lines of descent through passages of tissue culture. These functions were based upon the three most useful proteins for discrimination between the three groups. These specific proteins are identified with arrows in Fig. 2. The uppermost of these proteins exhibits a migration rate identical to the chloroplast protein, fraction 1, and could be subject to quantitative alteration as a result of variations in light intensity. We ran a

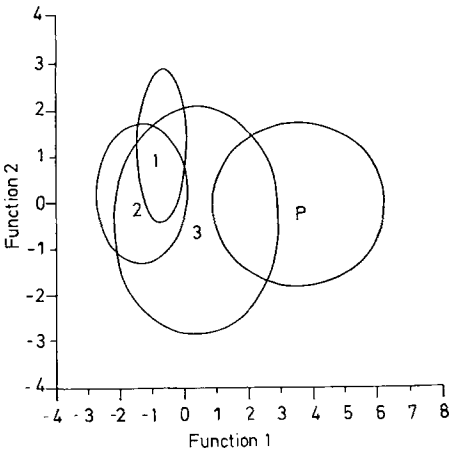


**Fig. 4.** Two-dimensional plot illustrating the dispersion of somaclones and parent protein patterns based upon two functions derived through discriminant analysis. Centroids of each grouping are marked with the group number or P for parent grouping. The axes of the ellipses are equivalent to the standard deviation for the distribution of data points in each dimension

**Table 1.** Frequency of correct classification of somaclones (S) and parent electrophoresis runs according to group by discriminate analysis. Overall frequency of correct classification is 80.8%

| Actual group | Total no. of plant (cases) | Predicted group membership (cases) | | | |
|---|---|---|---|---|---|
| | | Parent | S-1 | S-2 | S-3 |
| Parent | 4 | 4 | 0 | 0 | 0 |
| S-1 | 4 | 0 | 3 | 0 | 1 |
| S-2 | 9 | 0 | 1 | 8 | 0 |
| S-3 | 9 | 1 | 1 | 1 | 6 |

discriminant analysis in which this protein was excluded with results similar to those reported here, which indicated that small light intensity differences which may have occurred in environmental chambers during culture and regeneration were not a factor in these experiments.

Each plant may be assigned a position in three space based upon the values computed for it using the three functions developed in our analysis. From the means and standard deviations of the values for each coordinate derived in this manner, groupings may be constructed. Figure 4 graphically represents these groupings in two dimensions based upon the two most descriptive functions. This is done for convenience of interpretation and as plotting the third dimension was found to add little information in this instance. One can see that the replicates of protein data of the parent fall within a reasonably distinct grouping overlapped by only the most disperse somaclone group. As in the cluster analysis, there seems to be a directional divergence of somaclones away from the parent. The centroids of all somaclonal groupings fall to the left of that of the parent grouping in this graphic representation, and two of these somaclone groupings are significantly different from that of the parent.

Although judging from Fig. 4 there appears to be considerable overlap between groupings, the classification of plants based solely upon values obtained for each plant using the three functions, was 80.8% correct (Table 1). The parent data were classified 100% accurately and in only a single case was a somaclone classified incorrectly as a parent. This somaclone belongs to the most variable group (group 3). Somaclone group 3 showed the highest rate of misclassification, 33.3%; and contained a clone incorrectly identified as belonging to each of the other three groups. This is to be expected, as individuals in group 3 overlap all other groups to a great extent.

The two somaclones shown statistically to be variants using cluster analysis belong to groups two and three. Although the patterns of the 18 proteins of these somaclones vary significantly from those of all other somaclones and the parent plant, they do not vary discordantly with their respective group pattern based upon the three discriminating proteins. This is substantiated by the correct classification of both these variants according to their respective group.

## Discussion and conclusions

From protein electrophoretic data we were able to identify two alfalfa somaclones which exhibit protein banding patterns which are significantly different from the parent plant and from the other clones. Protein variants this extreme occurred at a frequency of 9.1% in the population. These data suggest that subtle genetic alterations have occurred within the population of somaclones which we obtained after consecutive subculturing. Such changes could have resulted from alterations in the regulation of protein synthesis analogous to those described by McDaniel and Ramage (1970) and Suh et al. (1977) for electrophoretic protein alterations in aneuploids. A full understanding of genetic control mechanisms operative in alfalfa somaclonal variants will require the analysis of progeny to substantiate the heritable nature of the observed variant proteins. No breeding studies could be performed here as we were obliged to sacrifice the entire plantlet to obtain sufficient proteins for electrophoretic analysis.

Discriminant analysis could distinguish three decendant lineages through tissue culture derived from three distinct calli derived from a single parent plant.

These somaclonal groupings could be distinguished from each other and from the parent plant from which they were derived. Our observations of protein variation suggest a tendency for vectorial genetic changes within these separate cultures. This theory is also supported by the tendency of the clones to deviate directionally from the parent as judged by both the cluster analysis (based upon 18 proteins) and by the discriminant analysis (based upon three proteins chosen for their ability to distinguish the groups). Such a directional nature is suggestive of an accelerated evolutionary change, perhaps potentiated by the environment of tissues in culture.

The fact that the three different classes of somaclones could be distinguished from each other through discriminant analysis would support the possibility of altered gene expression or gene regulation. All subcultures were treated identically yet it is possible to separate those plants derived from different initial cultures from the same parent on the basis of protein constitution. Discriminant analysis has enabled us to separate effectively somaclones in alfalfa based upon protein phenotypes. The statistical techniques which we have developed should be readily applicable to evaluation of somaclones in other plant systems and in the characterization of other variant traits. The application of statistical tools to the previously largely subjective evaluations of plant tissue cultures is a significant step in the growth of this emerging area of biotechnology.

To verify the genetic nature of the variation described here, breeding experiments will be necessary to ascertain the mode of inheritance of the differences observed and their stability of expression. Experiments are now in progress to regenerate sets of clones from different parent plants and to produce asexual propagules as sources of tissue and for breeding.

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