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PROTEIN AND ENZYME VARIATION IN INDUCED MUTANTS OF TOBACCO*

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Introduction.-- The induction of mutations in higher plants by radiation and other mutagens has been under investigation for many years. In species of economic importance a number of mutant lines of high productivity, resistance to disease, ornamental value, and phylogenetic significance have been isolated.¹ However, there is a paucity of studies on the biochemical bases of the observed physiological and morphological changes produced. Proteins are direct products of the genetic information transcribed from the DNA to the RNA, and determine both morphology and function. A detailed study of the enzymes and other proteins of phenotypically altered mutant types should not only provide evidence for the biochemical bases of the observed changes but should also lead to meaningful inferences regarding the alterations which have occurred in the genetic material itself. With these objectives the present study of protein and enzyme variation in radiation-induced mutant lines of Nicotiana tabacum was undertaken.

The mutant lines investigated were mainly derived from seeds irradiated with thermal neutrons and subsequently continued for eight generations by self-pollinating single plant selections for tallest and shortest individuals. Polyacrylamide gel electrophoresis was used to investigate the protein variation. This is a relatively uncomplicated technique which allows the resolution of individual proteins or groups of proteins from rather crude tissue extracts containing a complex mixture. It offers the possibility of detection of mutationally altered proteins and is therefore suitable for screening a large number of variant individuals or populations.

In the leaf extracts of the mutant and parent types, the electrophoretic patterns of esterases, peroxidases, leucine aminopeptidase (LAP), and of the proteins stainable with Amido Black were investigated. Peroxidases were investigated because of their role in the oxidation of the plant growth regulator, indole-3-acetic acid (IAA). The choice of the other enzymes was arbitrary, depending upon the availability of suitable enzyme assay methods for localizing the enzymes on the gels.

Materials and Methods.-- The mutant lines of Nicotiana tabacum have a complex history of irradiations and selections which will be published in detail elsewhere, but may be summarized as follows. The seeds are of a variety known as Texas Cuban High Nicotine and is highly homozygous. Between 1950 and 1956 seeds were irradiated every other generation with 5000 r of β -irradiation and were selected in each generation for both tall and short types. No phenotypic response to the selection was detected and in 1957 seeds of the β -irradiated lots, as well as a control lot, were subjected to thermal neutron irradiation for 20 hrs at a flux of 7.15×10^8 neutrons/cm²/sec. Selection was again practiced every generation for tallest and shortest plants, and lines were developed by self-pollinating selected individuals through eight generations. Selected lines S-1 and T-5 were derived from neutron-irradiated stocks only while selected lines S-2, S-3 and T-3 had a history of both β and neutron irradiation. A second thermal neutron irradiation was applied in 1960 to the " β + 1 neutron exposure" stocks and, selections carried out through eight generations of inbreeding, gave rise to selected line T-4. These

six lines, as well as two unirradiated control lots that were subjected to similar selection pressures, are listed in Table 1.

All the lines were sown at the same time and raised under the same growing conditions. Young leaves collected between 9 a.m. and 12 noon from field-grown plants were immediately washed, frozen and stored at -70° C. The plants were of the same physical age when the leaves were collected. Subsequent procedures, until the completion of electrophoresis, were carried out in a cold room at 5° C. Protein extracts from the leaves were obtained using a method of Morton² (Procedure F) with modifications. After removing the midribs, 40 grams of leaf tissue was finely chopped and then macerated (using a mortar, pestle and silica) in 40 ml of 0.01 M sodium pyrophosphate buffer containing 1% 2-mercaptoethanol. The slurry was further disrupted in a Waring Blender for two minutes, cooling after each 30 seconds of blending. It was added slowly to 10 volumes of acetone at -15° C with rapid stirring, held at this temperature for 15 minutes, and the sediment then collected by vacuum filtration. The sediment was washed twice by resuspending in 450 ml of acetone at -15° C, then dried for an hour. The dried material was ground to a fine powder in a mortar before suspending in 120 ml of n-butanol at -15° C for 15 minutes. Again the sediment was collected by vacuum filtration, washed twice with acetone at -15° C, and dried for one hour. The dried material was powdered in a mortar and again extracted with sodium pyrophosphate buffer for 1-2 hours. The suspension was centrifuged for 10 minutes at 5000 x g, the sediment discarded,

and the supernatant again centrifuged at 35,000 x g for 30 minutes. The supernatant was collected and dialyzed against 100 x volumes of 0.01 M phosphate buffer, pH 7.7, containing 0.2% mercaptoethanol, for 72 hours with the buffer changed every 24 hours. After dialysis the extract was again centrifuged at 35,000 x g for 30 minutes. Protein estimations made on some extracts by the method of Lowry et al.³ established that the protein content of the extracts was in the range of 0.3 to 0.4 mg/ml.

Electrophoresis was performed using the procedures outlined by Ornstein⁴ and Davis.⁵ Standard 7.5% gels were used in pyrex glass columns of 10 cm length and 0.6 cm inner diameter. The extracts were made to 0.1 M sucrose to facilitate layering of the sample over the large-pore gel. The quantity of extract layered on each gel was varied in the range of 50 to 300 microliters. The front was allowed to migrate 5.5 cm from the beginning of the separation gel.

The procedures described by Scandalios⁶ for starch gels were used to demonstrate peroxidases, esterases, and LAP on the acrylamide gels. Esterases using α - and β -naphthyl acetate as substrates, and LAP were demonstrated after about 15 minutes of incubation of the gel in the reaction mixture. Peroxidase activity was shown in about 1 minute. The intense color of benzidine blue, an intermediate product of the oxidation of benzidine, provides high resolution but is unstable. Therefore, the gels were photographed within 10 minutes with Polaroid color film Type 58. One per cent Amido Black in 7% acetic acid was used as a general

protein stain. Gels were incubated in the mixture for 1 hr, and then destained electrophoretically.

Slight variations occur in the distance from origin to front. Furthermore, the gels expand when stored. In order to make definitive comparisons among the patterns obtained from the different lines, an R_f value was calculated for each band, based upon the migration of the band relative to the front. The mean of observations on gels obtained from four or five independent electrophoretic runs from two separate extractions was used to compute the R_f value for each band.

Results.-- Proteins: The electrophoretic patterns of the proteins stainable by Amido Black are shown in Figure 1. The tall irradiated lines, T-3 and T-4, and the short irradiated lines, S-1 and S-2, display patterns identical to the parental types, P-1 and P-2. However, the patterns of T-5 and S-3 differ from the parentals. Both types are missing a band at 5.3, and T-5 lacks a band at 1.9.

Esterases: The esterase patterns (Fig. 2) obtained using α -naphthyl acetate as the substrate are identical for the parental types, the tall mutants, and S-1. A major band at 4.2 is missing in S-2. The three minor bands seen in the region of 4.8 to 5.5 in the other types are replaced in S-3 by a semi-major and a minor band.

Similar patterns are observed when β -naphthyl acetate is used as the substrate.

Leucine aminopeptidase: The LAP patterns are shown in Figure 3. The parental and mutant types, with the exception of S-2, show five bands. S-2 has an additional band at 6.7.

Peroxidases: The patterns of the peroxidases (Fig. 4) present the greatest diversity. Each of the short mutants differs from the parental types and the tall mutants. One of the tall lines, T-5, differs from the parents and other tall lines. The differences are both qualitative and in the intensity of staining. S-2 has only 5 bands, as opposed to 7 in the other types. T-5 differs from the parental types in having a smaller band at 5.75. S-1 and S-3 possess four bands in the region of 5.0 and 6.1 with the same R_f values as those of the parents. However, the intensity of staining in the region is less. Only one band has an equivalent to the parental quantity in S-1 and all have less in S-3.

Discussion.-- The unirradiated tall and short control selections do not differ significantly in their mean height. As well, differences between two of the mutant lines, T-3 and T-5, and the selected control are not significant. However, all of the short mutant lines differ significantly from the mean of the short control.

The experimental results show that the unirradiated tall and short control selections do not show variations in their electrophoretic patterns for the proteins and enzymes studied. This is also true for the two irradiated tall lines, T-3 and T-4; all other mutant lines differ from the parental types and from each other.

The fact that T-4 differs in mean height but not electrophoretically from the controls suggests that either the induced genetic changes do not involve the proteins and enzymes sampled or that the technique is not sensitive enough to detect the alterations. Conversely, the fact that

T-5 differs electrophoretically but not in mean height suggests that the changes observed are in proteins not directly involved in processes affecting growth in height, but rather represent heritable changes not yet assessed in the phenotypes. Since all the short lines differ significantly in mean height from the controls, it is likely that the changes in protein observed may be related to growth. With the techniques employed in this experiment, only a small fraction of the total proteins which must be present in a plant of N. tabacum during its life cycle are present in the extract which is layered on the gel. In addition, only a portion of the proteins in the extract are demonstrated with the particular enzyme assay techniques employed. Furthermore, even proteins identified on the gels at the same site for all lines may contain differences which cannot be resolved by the technique used. A change in migration can be brought about only if a significant change has occurred in the charge, size, or shape of the molecule. Shaw⁷ has pointed out that a single amino acid change will produce a change in the net charge of the molecule in only 27.56 per cent of the cases. Hence, the observed changes probably represent only a small fraction of the total alteration in the proteins and enzymes which have been brought about by changes in the genetic material induced by radiation.

The significance of the observed electrophoretic differences cannot be interpreted with difinity. Theoretically, each band on the gel represents either a single protein or a group of proteins with similar charge and molecular size. Those stained with Amido Black represent pro-

teins which may or may not have enzymatic properties. The bands demonstrated by the enzyme assay techniques for esterases and peroxidases represent closely related but distinct enzymes, some of which may be isozymes. In the case of leucine aminopeptidase, the bands probably represent isozymes of a single enzyme.

Absence of a particular band in the mutant lines signifies that the genetic information for the particular protein or enzyme is lacking in the genotype, or that it has been suppressed, or that the rate of formation has been reduced so that the quantity present is insufficient to detect on the gels.

The appearance of a new band in the electropherogram for LAP in one of the mutant lines possibly represents a new isozyme.

The changes in the peroxidases, where some of the bands have the same mobility but show marked differences in stainability, represent quantitative differences. The absence of two peroxidase bands in S-2 and variation in the intensity of staining for three other bands in short selections S-1 and S-3 suggest a possible causative involvement of these enzymes with short phenotypes. Peroxidases are known to oxidize the growth regulator, IAA, and could thus limit plant growth by reducing the amount of this growth substance. With the present evidence it cannot yet be concluded whether the observed changes in the peroxidase patterns are causal to, result from or are unrelated to the short stature of the phenotypes. Peroxidase patterns studied in developmental stages of these short mutants may help in elucidating the relationship.

Summary.-- Altered proteins and enzymes have been demonstrated to occur in radiation-induced mutant lines of a higher plant. Acrylamide gel electrophoresis was used to detect electrophoretic variants among selections for plant height in the eighth inbred generation derived from irradiated seeds and unirradiated controls of the self-pollinating species Nicotiana tabacum. Gels prepared with extracts of comparable leaf material of 3 tall and 3 short selections from irradiated stock, as well as a tall and a short control selection, were stained for proteins with Amido Black and for esterases, peroxidases and leucine amino-peptidase. No variation in protein and enzyme patterns was observed in the tall and short control lines, or in two of the tall selections from the irradiated lots. All other mutant lines were different from the parent lines and from each other. Certain protein and enzyme bands were missing in the mutant lines. Marked changes in the pattern of peroxidases were observed in short selections. One of the mutant lines showed a new isozyme for LAP.

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TABLE 1

Line	Number of Radiation Exposures	Direction of Selection	$\bar{x}$ height in 8th generation
P-1	None	Tall	77.4
P-2	None	Short	71.4
T-3	Beta (4) Neutrons (1)	Tall	74.8
T-4	Beta (4) Neutrons (2)	Tall	86.0
T-5	Neutrons (1)	Tall	80.3
S-1	Neutrons (1)	Short	53.6
S-2	Beta (4) Neutrons (1)	Short	55.0
S-3	Beta (4) Neutrons (1)	Short	22.0

LEGENDS TO FIGURES

Figure 1. Diagrammatic representation of the electrophoretic pattern of leaf proteins, stainable by amido black, in parental and the mutant types of tobacco. Relative migration distance of each band is indicated at the side. Origin is at the top; the proteins migrated towards the positive electrode. P-1, P-2 are the tall and short selections from unirradiated control. T-3, T-4 and T-5 are the tall and S-1, S-2 and S-3 are the short selections originating from the irradiated parents.

Figure 2. Diagrammatic representation of the electrophoretic pattern of the esterases from leaf extracts of the parental and the mutant types. Other details as for Figure 1.

Figure 3. Diagrammatic representation of the electrophoretic pattern of leucine amino peptidase from leaf extracts of the parental and the mutant types. Other details as for Figure 1.

Figure 4. Diagrammatic representation of the electrophoretic pattern of peroxidases in leaf extracts from the parental and the mutant types. Other details as for Figure 1.

AMIDO BLACK

Figure 1

ESTERASES

Figure 2

LEUCINE AMINOPEPTIDASE

Figure 3

Figure 4