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Transcriptional Control of Nitrate Reductase Synthesis in Squash, Cucurbita Maxima

Susan Martino
Loyola University Chicago

TRANSCRIPTIONAL CONTROL OF NITRATE REDUCTASE SYNTHESIS IN SQUASH, CUCURBITA MAXIMA

by

SUSAN JO MARTINO

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VITA

The author, Susan Jo Martino, is the daughter of James and Phyllis (Schofield) Martino. She was born October 2, 1962 in Tipton, Indiana.

Her elementary education was completed in the Hamilton County public schools in Cicero, Indiana. Her secondary education was completed in 1981 at Hamilton Heights High School, Arcadia, Indiana.

In August, 1981, Miss Martino entered Indiana University, receiving the degree of Bachelor of Science in biology in May, 1985. In 1985, while attending Indiana University, she was a member of the Biology Student Association and was chosen Outstanding Senior Biology Student.

In August, 1985, Miss Martino was granted an assistantship in biology at Loyola University of Chicago, enabling her to complete the Master of Science in 1987.

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CHAPTER I

INTRODUCTION

Nitrogen is essential for the growth of healthy plants. Most higher plants obtain nitrogen through the soil in the form of nitrate. Nitrate is transported into the plant and processed into amino acids by means of the nitrate assimilation pathway, unique to higher plants. In the cytoplasm, nitrate is first reduced to nitrite by nitrate reductase. In the chloroplast, nitrite is then converted to ammonium via the enzyme nitrite reductase. Ammonium is then further reduced to glutamate by the enzymes glutamine synthetase and glutamate synthase (1). As for most multi-step biochemical processes, the first step is considered to be rate-limiting. In nitrate assimilation, this step is the conversion of nitrate to nitrite. Therefore, much attention has been focused on nitrate reductase, the enzyme involved in catalyzing this reaction.

Previous studies have identified nitrate and light as factors that enhance nitrate reductase activity in higher plants (2, 3). In contrast,

darkness and reduced forms of nitrate diminish nitrate reductase activity. The changes in nitrate reductase activity have been shown to result from actual changes in protein levels in barley (3), soybeans and squash (unpublished results). However, changes in nitrate reductase activity may also be due to activation/inactivation of the nitrate reductase protein in response to nitrate in Chlorella (4). Such a mechanism of activation/inactivation for nitrate reductase regulation in squash is not supported in the literature.

Little is known concerning the molecular biology of nitrate assimilation in higher plants. This study will focus on the possible molecular events that are involved in the regulation of squash nitrate reductase protein levels in response to various environmental conditions. Changes in the level of nitrate reductase specific mRNA resulting from treatment with various nitrogen sources will be monitored. If increasing mRNA levels correspond to increasing protein levels, control is transcriptional. However, if protein levels increase with increasing activity, without any corresponding increase in mRNA levels, control is at

the level of translation. A better understanding of this regulatory mechanism may eventually lead to the engineering of plants capable of utilizing soil nitrogen more efficiently.

CHAPTER II

REVIEW OF RELATED LITERATURE

Soil nitrogen is a major requirement for the growth of healthy plants. Approximately 2% of a plant's dry weight consists of nitrogen (5). This organic nitrogen is generally derived from either atmospheric nitrogen (N_2) or nitrate (NO_3^-). Most higher plants acquire the majority of their nitrogen from the assimilation of nitrate. The capability to assimilate nitrate is limited to certain bacteria, fungi, algae and higher plants (1). In higher plants, nitrate assimilation occurs primarily in the leaves (6).

The nitrate assimilatory pathway is outlined as follows:

	2e-		6e-			
Nitrate	----->	Nitrite	----->	Ammonium	----->	Glutamate
	nitrate reductase		nitrite reductase		gln synthetase glu synthase	(1)

Nitrate is reduced to ammonium which is then converted into amino acids, the basic components of proteins. Since a large amount of reducing power and energy is

needed to complete this conversion, this pathway is highly regulated to avoid excess energy expenditure when amino acids are available. The first step in this pathway, the reduction of nitrate to nitrite, is regarded as the rate-limiting step in nitrate assimilation (7). Much attention has therefore been given to nitrate reductase, the enzyme catalyzing this step.

Enzymology of Higher Plant Nitrate Reductases

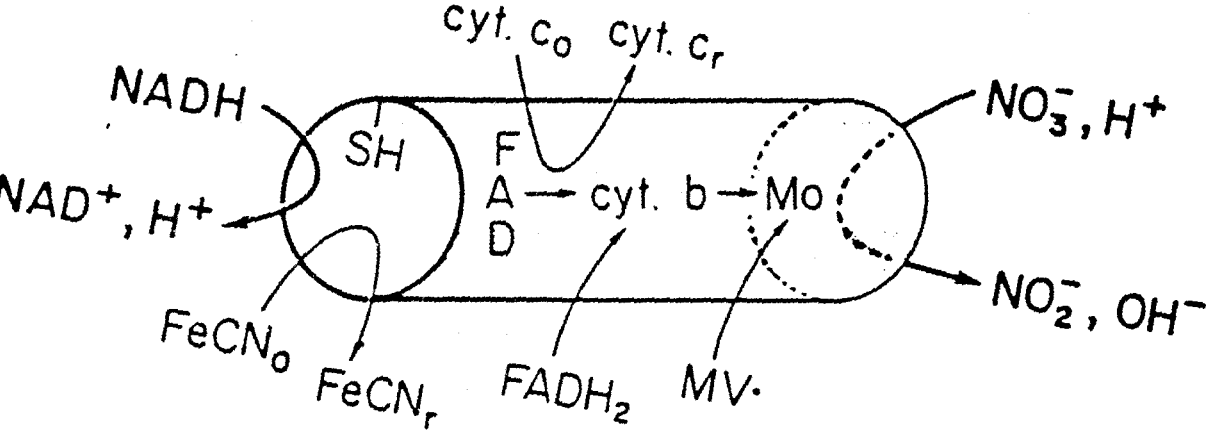
Assimilatory nitrate reductases are soluble, electron-transferring proteins with molecular weights in the range of 200-300 Kd (7, 8, 9). The transfer of electrons is mediated by enzyme-bound heme iron, flavin adenine dinucleotide (FAD), cytochrome b_{557} and a molybdenum-pterin cofactor (10). This electron shuttle system transfers electrons between the physically separated pyridine nucleotide oxidation site and the nitrate reduction site (8,11). The physical arrangement of nitrate reductase is schematized in Figure 1.

Both physiological and nonphysiological activities have been described for nitrate reductase.

FIGURE 1.

Schematic of Nitrate Reductase

Nitrate reductase is depicted as an electron shuttle system with separate active sites for NADH and nitrate reduction. Electrons are donated by the pyridine nucleotide NADH to an enzyme-bound sulfhydryl group (SH). The electrons are further transferred to the nitrate reduction site via flavin adenine dinucleotide (FAD), cytochrome b and molybdenum. The dehydrogenase partial activity is illustrated by the reductions of ferricyanide and cytochrome c. Electrons for nitrate reduction can also be provided from reduced flavins and reduced methyl viologen dyes (8).


cyt. c_o
cyt. c_r
NADH
SH
F
A
D
cyt. b
Mo
NO_3^-, H^+
NAD^+, H^+
NO_2^-, OH^-
$FeCN_o$
$FeCN_r$
$FADH_2$
MV·

The physiological activity involves the reduction of nitrate to nitrite occurring at the molybdenum cofactor site (12). The reducing power for this reaction is generated by a pyridine nucleotide which donates electrons to the catalyst via a specific enzyme sulfhydryl group (12). Electron transfer occurs through FAD and cytochrome b_{557}.

There are also two types of apparent nonphysiological activities. The first is a dehydrogenase activity where nitrate reductase is capable of mediating the pyridine nucleotide-linked reduction of various electron acceptors such as ferricyanide, cytochrome c, dichlorophenolindophenol and other one and two electron acceptors (1). The second type reduces nitrate with electrons provided by viologen dyes, reduced flavins or methylene blue (1, 13).

Studies have shown these partial activities are selectively inhibited. For example, pyridine nucleotide-linked and dehydrogenase reactions are inhibited by sulfhydryl binding agents such as p-hydroxymercuribenzoate (14). Molybdenum-linked reactions are inhibited by metal binding agents, which have no effect on dehydrogenase activities (15).

Various types of antibodies against nitrate reductase also selectively inhibit these partial activities (16, 17, 18). These studies on partial activities have enabled researchers to uncover many details relating to the overall characteristics and functions of nitrate reductase.

Squash nitrate reductase is characterized as a homodimer with a subunit molecular weight of 115,000 daltons (19). Each subunit contains one equivalent of FAD, cytochrome b_{557} and molybdenum-pterin cofactor. The amino acid composition of squash nitrate reductase shows acidic amino acids predominating over basic amino acids. This is reflected in an isoelectric point of 5.7 (19). The enzyme is specific for the pyridine nucleotide NADH as its source of reducing power and has a pH optimum of 7.5 (13). As for most other higher plants, squash nitrate reductase is located in the cytoplasm with the other nitrate assimilatory enzymes being chloroplastic (20, 21).

The Biochemistry of Higher Plant Nitrate Reductase Regulation

Nitrate reductase regulation has been extensively studied in the fungus Neurospora crassa (22). The availability of nitrate assimilatory mutants has

facilitated these efforts. Dunn-Coleman et al. found nitrate reductase activity to be induced by nitrate and repressed by glutamine (23). Furthermore, glutamine was able to repress nitrate reductase activity even when high concentrations of nitrate were present. Other reduced forms of nitrogen, including ammonium and the amino acids glycine, proline and glutamate also had repressing effects on nitrate reductase activity (23).

The regulation of nitrate reductase activity in higher plants is much less understood. Studies similar to those done with N. crassa have determined nitrate reductase activity to be greatly increased upon the addition of exogenous nitrate in several species of higher plants (24-28). However, there are also examples in higher plants of nitrate not increasing nitrate reductase activity (29, 30). Thus, a well defined pattern of nitrate reductase activity regulation in higher plants has yet to emerge.

Studies performed in our laboratory using squash and soybeans have also demonstrated increases in nitrate reductase activity in the presence of nitrate. Upon treatment with glutamine, a reduced form of nitrogen, nitrate reductase activity decreased. Treatments with combinations of nitrate and glutamine

resulted in intermediate nitrate reductase activity. Using Western blotting on squash, it was further shown the changes in nitrate reductase activity were the result of *de novo* nitrate reductase protein synthesis (unpublished results).

Little is known concerning the mechanism by which reduced forms of nitrogen repress nitrate reductase activity. Studies performed by Oaks *et al.* have demonstrated nitrate reductase activity repression upon treatment with various amino acids (31). This response also exists in a tissue-specific manner in cotton where nitrate reductase activity is repressed in roots but not in leaves following the addition of various amino acids (32).

Mechanisms by which nitrate reductase activity is regulated other than the availability of nitrate have also been studied. Since most higher plants store large amounts of nitrate in vacuoles, Barneix *et al.* have examined the role of nitrate flux through plant cells in regulating nitrate reductase activity (33). The uptake of nitrate has also been implicated as a key regulatory step in the overall process of nitrate assimilation. McClure *et al.* have

identified a 31 Kd polypeptide from maize roots which they believe is involved in the active uptake of nitrate (34). Nitrate uptake systems have also been studied in barley seedlings (35) and cultured tobacco cells (36). In both cases it was demonstrated that the addition of nitrate activated the nitrate uptake system. The rate of nitrate reduction was correlated with the rate of uptake following an initial lag period.

Higher plant nitrate reductase mutants have also provided a tool in studying the regulation of nitrate reductase. Mutants which have been generated in plants are of two basic types: deficiencies in the synthesis of the nitrate reductase apoprotein and molybdenum cofactor mutants. Mutants of both types have been characterized in N. tobacum, Datura innoxia, Hordeum vulgare, Pisum sativum and Glycine max (37-41). It is evident from these studies that separate genes exist which code for the nitrate reductase apoprotein and the molybdenum cofactor. The specific mechanisms by which these genes are regulated are poorly understood at the molecular level.

Molecular Biology of Higher Plant Nitrate Assimilation

Recently, much attention has been focused on the molecular aspects of the regulation of nitrate assimilatory enzymes. Somers et al. investigated two possible mechanisms of nitrate reductase regulation: synthesis/degradation and activation/inactivation (3). Barley was grown under various nitrate and illumination conditions. In plants that received no nitrate or light, there was no cross reacting material with anti-nitrate reductase antibodies. Western blots performed on total proteins detected the presence of nitrate reductase only in plants which received both nitrate and light. It was concluded that barley nitrate reductase is regulated by de novo synthesis and degradation of protein, not activation/inactivation of pre-existing protein. Similar results have been attained in our laboratory using squash plants treated with nitrate and glutamine (unpublished results).

Commere et al. performed RNA studies using maize and tobacco (42). Poly (A+) mRNA was isolated from both maize and tobacco and used in a rabbit reticulocyte in-vitro translation system. A protein corresponding to the size of the nitrate reductase subunit was immunoselected with antibodies raised against purified maize nitrate reductase. This study

demonstrated the translatability of nitrate reductase specific mRNA *in-vitro*. However, no studies were performed to examine levels of nitrate reductase specific mRNA in response to exogenous nitrate or reduced forms of nitrogen.

Gupta and Beevers have used *in-vitro* translation and immunoprecipitation procedures to study the regulation of nitrite reductase, the second enzyme in the nitrate assimilation pathway (43). These researchers used mRNA isolated from plants given either nitrate or no nitrate in a wheat germ *in-vitro* translation system. Immunoprecipitation of the *in-vitro* protein products detected a polypeptide slightly larger than nitrite reductase. The size difference was attributed to the presence of a transit sequence. Following *in-vivo* protein labelling, under conditions of added nitrate and light, a protein corresponding to the actual molecular weight of nitrite reductase was immunoprecipitated with anti-nitrite reductase antibodies. The amount of immunoprecipitable protein increased over time corresponding to increases in nitrite reductase activity. These researchers concluded that increases in nitrite reductase activity were the result of increases in nitrite reductase protein.

A partial cDNA of the squash nitrate reductase gene has recently been isolated by Crawford *et al.* (10). The plasmid, pCmc1, contains a 1.2 kb insert corresponding to a portion of the nitrate reductase gene. This clone was identified by immunological screening. Northern blots of poly (A+) RNA from plants receiving nitrate and others not receiving nitrate were probed using this cDNA. Hybridization was seen with a 3.2 kb transcript of RNA isolated from plants that had been treated with nitrate. Hybridization was not seen under conditions of zero added nitrate. These initial studies performed to characterize the clone suggested control of nitrate reductase to be at some level of transcription. Similar studies have been done by Cheng *et al.* (44). These researchers have a partial cDNA clone for nitrate reductase isolated from barley. Northern blotting again showed an increase in nitrate reductase specific mRNA following treatment with nitrate.

CHAPTER III

METHODS

PLANT MATERIAL. Squash seeds (100 g, Cucurbita maxima var Buttercup) were grown in vermiculite for seven days in an environmental growth chamber (25° C and 16 h light / 8 h dark, intensity of 47 W/m^2). The plants were watered with tap water, containing no detectable nitrogen, (data not shown) throughout this seven day period. On day seven, plants were irrigated with a modified Hoagland's solution (45) containing different nitrogen sources (0 mM or 50 mM nitrate and 10 mM glutamine). The concentrations of metabolites used were based on previous studies performed in our laboratory. Harvesting of cotyledons was done 20 hours following this irrigation.

Proteins were extracted by homogenizing the cotyledons in cold buffer containing 100 mM KH_2PO_4 and 1 mM EDTA, pH 7. Nitrate reductase activity was determined colorimetrically as described by Hageman and Reed (46). Protein concentrations were determined using the procedure of Lowry et al. (47) using bovine serum albumin as a standard..

RNA ISOLATIONS. All glassware was baked at 200° C for 2 hours. Solutions were prepared with sterile DEPC-treated distilled deionized water. Cotyledons from squash plants grown as described above were used for RNA isolations. The isolation procedure was that of Dodd *et al.* (48). Cotyledons (10g) were homogenized to a fine powder with a mortar and pestle in liquid nitrogen. The powder was stirred at 4° C for thirty minutes in a buffer containing 10 ml STE (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA at pH 7), 1 ml mercaptoethanol, 1 ml 10% SDS, 10 ml phenol, 0.5 ml 10% bentonite and 10 ml chloroform:pentanol (24:1). After centrifugation at 10,000 rpm for 15 minutes in an SS34 rotor, the aqueous phase was collected and adjusted to 15% ethanol. This was then passed through a Bio-Rad Cellex N-1 column to isolate total single-stranded (ss) RNA. The ssRNA was further fractionated into poly (A+) and poly (A-) RNA by an oligo (dT)-cellulose column as described by Aviv and Leder (49). The RNA samples were denatured with glyoxal and separated on 1% agarose gels (50). After staining with ethidium bromide, the gels were photographed and the approximate sizes of the transcripts determined. Purities and concentrations of the RNA were determined spectrophotometrically by measuring absorbance at 260 and 280 nm.

TWO-DIMENSIONAL GEL ELECTROPHORESIS. Crude protein extracts isolated as per Zivy et al. (51) from 7 day old squash plants treated with either 0 mM nitrate or 50 mM nitrate were used for two-dimensional gel electrophoresis. The procedure followed was as described by O'Farrell (52). Isoelectric focusing gels were poured in glass tubes (150 mm x 1.5 mm inside diameter). The gel mixture consisted of 1.33 ml 30% acrylamide, 2 ml 10% NP-40, 5.5 g urea, 1.97 ml distilled water, 0.4 ml of Ampholines, pH 5 to 7 and 0.1 ml of pH 3 to 10 (total of 2% Ampholines). The mixture was swirled until the urea was completely dissolved and then 10 ul 10% ammonium persulfate and 7 ul TEMED were added. The tubes were filled with a loading needle to a height of 13 cm, overlayed with 8 M urea and allowed to polymerize 1 to 2 hours. After the gels were pre-run to establish the pH gradient, 20 ul of sample suspended in lysis buffer (9.5 M urea, 2% NP-40, 2% Ampholines and 5% mercaptoethanol) was loaded and the gels were run at 400 V for 16 hours. Hyperfocusing of the bands was achieved by running the gels at 800 V for one hour. The gels were then removed from the tubes with an extrusion needle and equilibrated in SDS buffer (10% glycerol, 5%

mercaptoethanol, 2.3% SDS and 0.0625 M Tris-Cl at pH 6.8). The gels were then either frozen at -80 ° C or analyzed in the second dimension by 10 % SDS-PAGE as per Laemmli (53). Following the second dimension, the gels were silver stained as per Morrissey (54).

IN-VIVO TRANSLATION. Cotyledons were harvested from seven day old squash plants and cut into discs with 3 mm diameters. These discs were placed in vials containing 6 ml of either 0 mM KNO_3 or 10 mM KNO_3 and 10 uCi of (^{35}S)-methionine. Vials were kept in either the light or dark. At various time intervals (4 and 8 hrs), samples were removed and quick frozen in liquid nitrogen (43). Proteins were extracted as before and analyzed by 10% SDS-PAGE, fluorography and autoradiography at -80° C.

IMMUNOPRECIPITATION. Levels of nitrate reductase specific protein being synthesized in squash cotyledons under the various conditions were determined by immunoprecipitation using polyclonal anti-nitrate reductase antibodies. The procedure was as described by Gupta and Beevers (43). In-vivo total protein extracts were incubated with 50 ul of antiserum for one hour at room temperature and then overnight at 4° C.

The mixture was incubated with 50 ul of a crude cell suspension of Protein A (10% w/v) from Staphylococcus aureus for one hour at room temperature. Following this incubation, the mixture was pelleted through a 1 M sucrose pad in TNET-M buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, 4 mM methionine at pH 7.5) by spinning for ten minutes at high speed in a microcentrifuge. The resulting precipitate was washed five times with TNT-M buffer (TNET-M buffer minus EDTA) and one time with acetone. The precipitate was resuspended in 50 ul dissociating buffer (20 mM Tris-Cl, 2% SDS, 5% mercaptoethanol, 2 M urea, 10% glycerol, 0.001% bromphenol blue at pH 7.6) and boiled for three minutes. Analysis of the immunoprecipitated proteins was done using 10% SDS-PAGE, fluorography and autoradiography at -80o C.

DOT BLOTS. Dot blots were performed to qualitatively determine the presence or absence of nitrate reductase-specific RNA transcripts. Total poly (A+) RNA samples were incubated 15 minutes at 50° C in 50% DMSO, 1 M deionized glyoxal and 12.5 mM sodium phosphate buffer at pH 6.5. After quick cooling on ice, the samples were dotted onto a positively charged membrane with a sterile capillary tube and air dried

(55). The glyoxal reaction was reversed by placing the membrane in 50 mM NaOH for 15 seconds and then neutralizing the membrane in 0.2 M Tris-Cl, 1X SSC at pH 7.5 for 30 seconds. The membrane was incubated with a radiolabelled probe to localize areas of RNA-DNA hybridizations. The nick translation involved incubating a pUC19 plasmid containing a 1.2 kb cDNA insert of the nitrate reductase gene (10) with DNAse, DNA Pol I and ^{32}P-labelled dCTP for one hour at 15° C. The reaction mixture was then passed through a Sephadex G-50 column to separate the labelled plasmid from unincorporated ^{32}P-labelled dCTP.

The probe was subsequently heat denatured and used for hybridizations. Hybridizations were done in 50% formamide, 4X SSPE (51), 5X Denhardt's (51), 1% SDS, 5% sodium Dextran sulfate, 0.2 mg DNA per ml at 42° C overnight. Washes were performed as follows: 1) Two fifteen minute washes at 45°-50° C in 4X SSPE, 50% formamide, 0.5% SDS, 2) Two rinses at room temperature in 2X SSPE, 0.5% SDS, 3) Two thirty minute washes at 60° C in 0.5X SSPE, 0.5% SDS (10). Blots were mounted on filter paper and autoradiographed at -80° C for 14 days.

MATERIALS. Squash seeds (Cucurbita maxima, var Buttercup) were obtained from W. Atlee Burpee Co., Warminster, PA. The environmental growth chamber was a Biotronette Mark III model designed by Lab Line Instruments, Inc., Melrose Park, IL. Centrifuges used included a Model 235 B Microfuge from Fisher and a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge from Dupont. Radioactivity was measured in a Tri-Carb 300 Liquid Scintillation Counter from Packard Instruments. Phenol, agarose, acrylamide, oligo (dT)-cellulose and the nick translation kit all were obtained from Bethesda Research Laboratories, Gaithersburg, MD. (^{35}S)-methionine was purchased from Amersham. New England Nuclear (Dupont) supplied the ^{32}P-labelled dCTP used in nick translations and the GeneScreen Plus™ for blotting. All other chemicals used were obtained from Sigma as reagent grade or better. Polyclonal anti-nitrate reductase antibodies were a gift from Dr. Wilbur Campbell, Michigan Tech University. The squash nitrate reductase cDNA clone used as a probe was a gift from Dr. Nigel Crawford, Stanford University.

CHAPTER IV

RESULTS

Metabolite Effects on Squash Nitrate Reductase Activity

Initial studies of metabolite effects on nitrate reductase activity in squash cotyledons were done using nitrate, glutamine and a combination of these two metabolites as nitrogen sources. It can be seen in Table I in the presence of 50 mM nitrate, activity levels were increased by approximately 20-fold over the control treatment of 0 mM nitrate. When 10 mM glutamine was provided as the sole nitrogen source, nitrate reductase activity levels were similar to the zero nitrogen control. The combined treatment of 50 mM nitrate/10 mM glutamine resulted in intermediate between 50 mM nitrate and zero nitrogen levels of activity. These results showed that added nitrate alone greatly enhanced nitrate reductase activity whereas the addition of glutamine alone had no effect on activity compared with zero nitrogen controls. The combination of 50 mM nitrate and 10 mM glutamine produced an intermediate level of activity. This

indicated that at 10 mM, glutamine is not capable of completely overriding the inducing effects of nitrate on nitrate reductase activity, but can significantly repress activity. Statistics (t-tests and analysis of variance) performed on similar data previously in our laboratory (unpublished results) have shown these differences in activity to be significant.

Changes in nitrate reductase activity in squash cotyledons over a 24 hour period in response to the addition of 50 mM nitrate at time zero are depicted in Figure 2. This study was conducted on a 16 h light/8 h dark cycle (47 W/m^2). As can be seen, nitrate reductase activity was negligible at time zero and progressively increased over time. A burst in activity was observed when the lights came on. The activity continued to increase until it reached a maximum at 20 hours. Table II lists activities from Figure 2. These results demonstrated the necessity for nitrate to obtain maximal nitrate reductase activity.

Two-Dimensional Gel Electrophoresis

In order to determine if the obvious physiological changes in nitrate reductase activity

corresponded with any gross cellular changes, a series of two-dimensional polyacrylamide gels were performed. The sensitivity of the protein separation based on both isoelectric point and size increases the overall resolution. Figures 3 and 4 are total protein profiles extracted from plants given 0 mM nitrate and 50 mM nitrate, respectfully. Upon close analysis, no significant differences in the two-dimensional profiles were seen between the two treatments. The changes in nitrate reductase activity were not reflected in the overall total proteins that were being synthesized. The actual location of the nitrate reductase protein is currently being studied in our laboratory using Western blotting on two-dimensional total protein profiles. The approximated expected location of nitrate reductase, based on molecular weight and isoelectric point, is indicated by the arrow.

In-Vivo Studies

To begin studying the mechanism by which these metabolites affected nitrate reductase activity, an in-vivo protein labelling experiment was conducted. Figure 5 is an autoradiogram (14 day exposure) of total proteins extracted from cotyledon discs incubated with

(^{35}S)-methionine in either 0 mM nitrate or 10 mM nitrate. When comparing lane **2** with lane **3**, it could be seen that protein synthesis was greatly enhanced by light with little protein banding seen in samples incubated in the dark. Similarly, discs incubated with 10 mM nitrate (lane **2**) also displayed enhanced protein synthesis compared to those incubated in 0 mM nitrate (lane 4). However, no differences could be seen in the major overall protein banding patterns between treatment groups.

When these protein samples were immunoprecipitated with anti-nitrate reductase antibodies, a band corresponding to the size of nitrate reductase was seen only in the sample given both light and nitrate (lane 6, Figure 6) after a four month exposure. Proteins extracted from samples incubated with no nitrate (lane 4) or in darkness (lane 3) did not have any immunoprecipitable nitrate reductase protein. This suggested the increase in nitrate reductase activity, in the presence of nitrate and light, was the result of <u>de novo</u> protein synthesis, although not ruling out the presence of a small quantity of inactive precursor protein. Since this is an <u>in-vivo</u> study, any nitrate reductase that is present

but inactive would not incorporate the radiolabelled methionine and therefore would not be detected by fluorography.

Isolation of Poly (A+) mRNA from Squash Cotyledons

Cotyledons from plants receiving the treatments listed in Table I were used for poly (A+) mRNA isolations as outlined previously. Table III shows the concentrations and purities of the poly (A+) mRNA isolated from these samples. A 260/280 ratio of greater than 1.5 is indicative of a sample relatively free from protein contaminants. The concentrations obtained did not correlate with the corresponding nitrate reductase activity levels for each treatment group (see Table I). The yields obtained are comparable to values of 20 ug poly (A+) RNA/g tissue as reported in the literature (43). Typical RNA patterns on 1% agarose gels are depicted in Figure 7. In all cases (total, poly (A+) and poly (A-) RNAs), few distinct bands could be seen. This is due to the wide variety of transcript sizes present in each sample. When samples from each of the four treatment groups listed in Table I were run on a 1% agarose gel and stained with ethidium bromide, no apparrent differences

existed between treatments and the resulting RNA smears obtained (Figure 8). The mRNA was of various sizes, ranging from approximately 1 kb to 22 kb in length. Again, differences in nitrate reductase activities were not reflected in the patterns or amounts of total poly (A+) mRNA obtained from each group. Since nitrate reductase comprises only 0.05% of total plant proteins (43), the amount of nitrate reductase specific RNA would be 10 ng of the total poly (A+) RNA. This number is based on a concentration of 20 ug poly (A+) RNA. The electrophoretic system used is not sensitive enough to detect any changes occurring in such minute quantities.

Dot Blot Analysis

With the size of the transcript which hybridized to the probe previously established at 3.2 kb (10), a series of dot blots were used as a quick method of qualitatively determining the presence of the nitrate reductase-specific transcript in RNA samples obtained from various treatment groups. An autoradiogram (14 day exposure) of dot blots performed on glyoxal-treated poly (A+) mRNA isolated from the treatment groups listed in Table I is shown in Figure 9. Using the

radiolabelled probe containing the 1.2 kb cDNA insert of the nitrate reductase gene, hybridization was most extensive in the 50 mM nitrate sample (dot 3). The 0 mM nitrate (dot 2) and 10 mM glutamine (dot 5) samples showed negligible amounts of hybridization. The 50 mM nitrate/10 mM glutamine (dot 4) sample hybridized with the probe, but to a somewhat lesser degree than the 50 mM nitrate group. This pattern of hybridization also correlated with the nitrate reductase activities for the various treatments given in Table I. Based on these results, it can be said the increase in nitrate reductase activity is due to an actual increase in the steady state mRNA levels for nitrate reductase. These results indicated the control of nitrate reductase synthesis to be at a level of transcription.

TABLE I.

Data presented in Table I are levels of nitrate reductase activity determined colorimetrically as per Hageman and Reed (47). Samples (approximately 1 g) were harvested in triplicate 20 hours following irrigation with a modified Hoagland's solution containing the various metabolites (46). The activities shown are averages of the individual activities for the three samples in each group. Activities are given in units of nmol/min-mg protein ± standard error of the mean.

TABLE I

METABOLITE CONTROL OF NR ACTIVITY IN WHOLE PLANTS
(Mean ± SEM)

Metabolite	Activity[a]
0 mM KNO_3	0.12 ± 0.14
50 mM KNO_3	2.71 ± 0.17
50 mM KNO_3/ 10 mM Gln	1.87 ± 0.14
10 mM Gln	0.13 ± 0.17

[a]nmoles/min-mg protein

FIGURE 2.

Nitrate Reductase Activity Curve Following Induction with 50 mM Nitrate

Squash plants were grown for 7 days in vermiculite on an 16 h light/8 h dark cycle. On day 7, the plants were irrigated with a modified Hoagland's solution (45) containing 50 mM nitrate. Samples were taken at various time intervals between 0 and 24 hours following irrigation and assayed for nitrate reductase activity. Nitrate reductase activity is negligible at T = 0 showing a peak of activity 20 hours after induction. The data are in units of nmol nitrate reduced/min-mg protein and are plotted as the mean $\pm$ standard error of the mean.

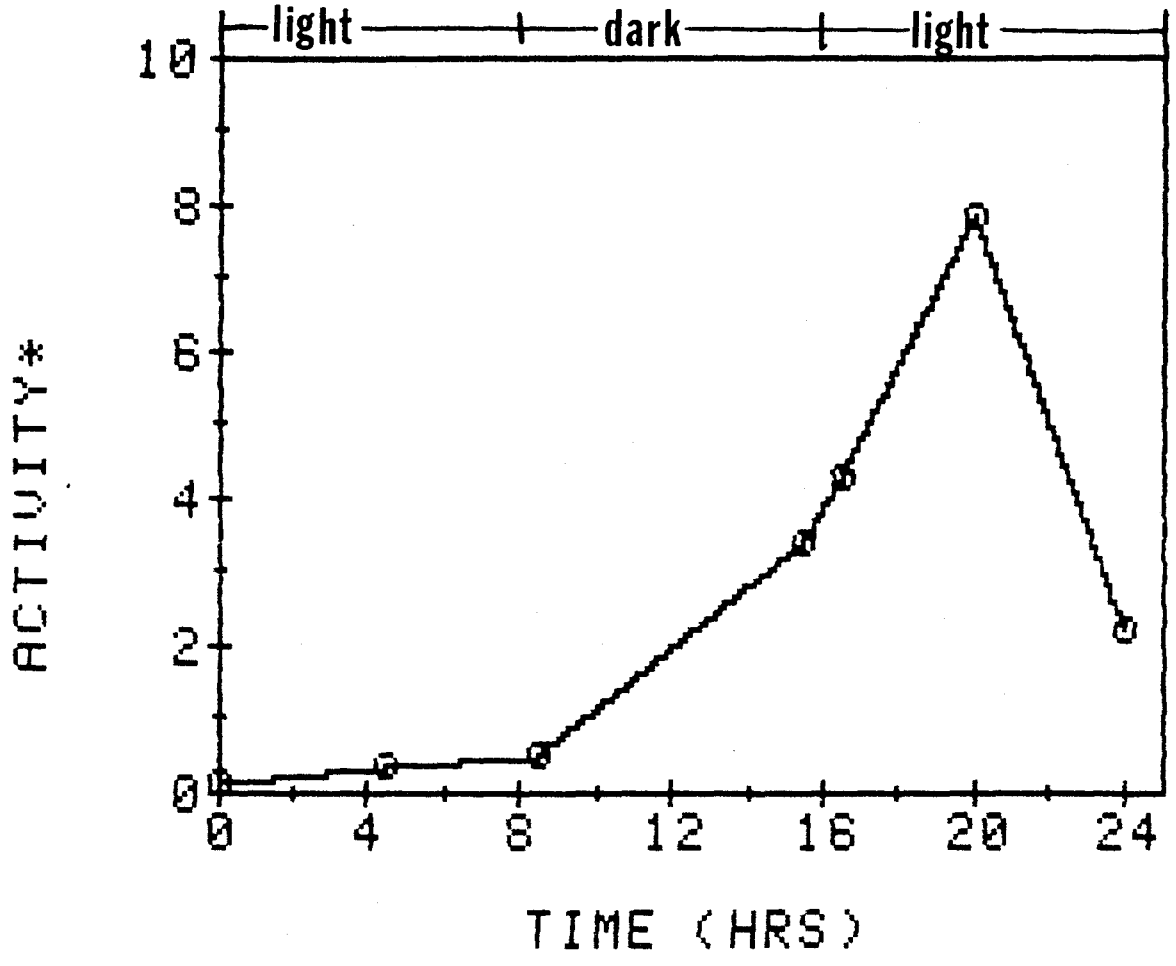

light
dark
light
10
8
6
4
2
0
ACTIVITY*
0
4
8
12
16
20
24
TIME (HRS)

TABLE II.

Data presented in Table II are levels of nitrate reductase activity determined colorimetrically as per Hageman and Reed (47). Samples (approximately 1 g) were harvested in triplicate at various time intervals throughout a 24 hour time interval following irrigation with a modified Hoagland's solution containing 50 mM KNO_3 at T = 0 hours. During the 24 hour period, the plants were kept on a 16 h light/ 8 h dark cycle. The + and - indicate the presence or absence of light at the time of harvesting. Activities are given in units of nmol/min-mg protein $\pm$ standard error of the mean.

Table II

CHANGES IN NITRATE REDUCTASE ACTIVITY WITH TIME FOLLOWING INDUCTION WITH 50 mM NITRATE
(Mean ± SEM)

Time[a]	Lights	Activity[b]
0	+	0.11 ± 0.03
4.5	+	0.35 ± 0.04
8.5	-	0.48 ± 0.07
15.5	-	3.42 ± 0.10
16.5	+	4.26 ± 0.18
20	+	7.80 ± 0.29
24	+	2.18 ± 0.02

[a]Time in hours following induction with 50 mM nitrate at T = 0.
[b]nmol/min-mg protein

FIGURE 3.

Two-Dimensional Electrophoretic Profile of a Non-Induced Total Protein Extract

Samples were extracted from 7 day old squash cotyledons irrigated with a modified Hoagland's solution (45) containing no nitrogen source. The protein extracts (20 ul) were run in the first dimension on 1.5 mm tube gels with a pH gradient of 3 to 10 for isoelectric focusing. The tube gels were run in the second dimension on 10% SDS-PAGE and silver stained as per Morrissey (54). The approximate location of nitrate reductase, based on molecular weight and isoelectric point, is indicated by the arrow.

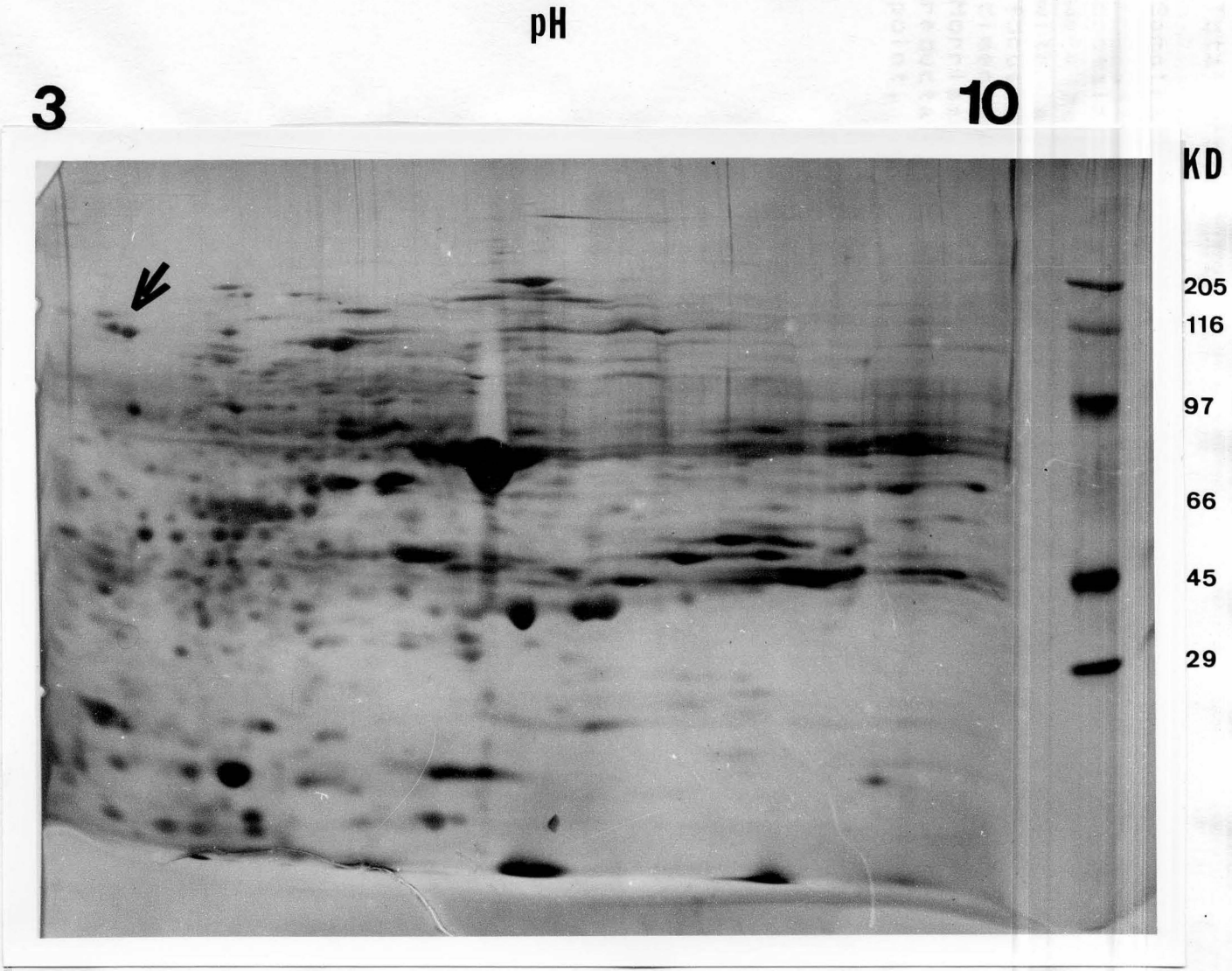

pH
3
10
KD
205
116
97
66
45
29

FIGURE 4.

Two-Dimensional Electrophoretic Profile of an Induced Total Protein Extract

Samples were extracted from 7 day old squash cotyledons irrigated with a modified Hoagland's solution (45) containing 50 mM nitrate. The protein extracts (20 ul) were run in the first dimension on 1.5 mm tube gels with a pH gradients of 3 to 10 for isoelectric focusing. The tube gels were run in the second dimension on 10% SDS-PAGE and silver stained as per Morrissey (54). The approximate location of nitrate reductase, based on molecular weight and isoelectric point, is indicated by the arrow.

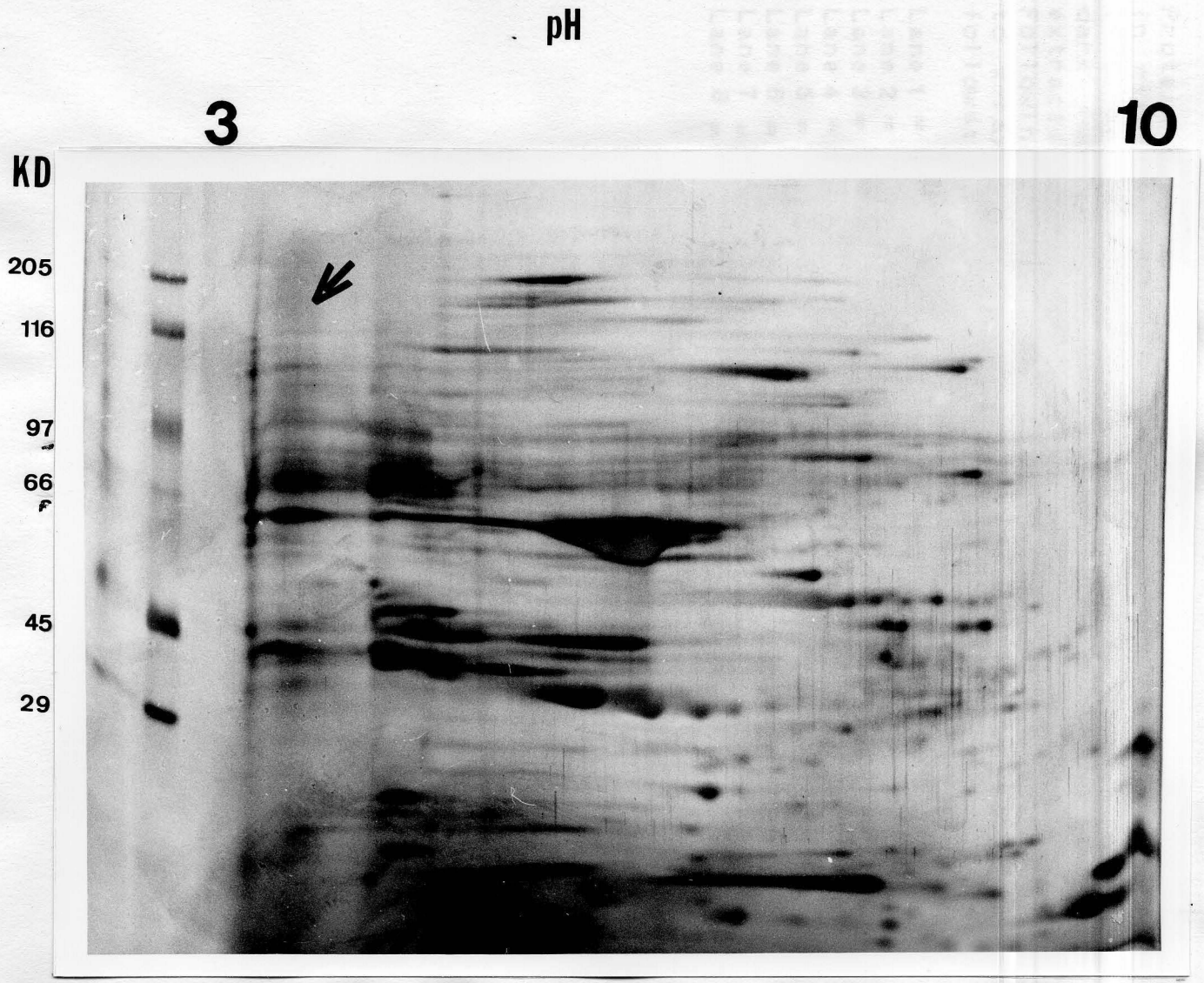

pH
3
10
KD
205
116
97
66
45
29

FIGURE 5.

In-Vivo Labelled Total Proteins

Protein extracts obtained from squash discs incubated in either 0 mM nitrate or 10 mM nitrate and 10 uCi (^{35}S)-methionine which were kept either in the light or dark for 4 and 8 hours (43). The total protein extracts (20 ul) were analyzed on 10% SDS-PAGE (53). Following fluorography, the gels were dried and exposed to x-ray film for 14 days. The treatments are as follows:

Lane 1 = 10 mM nitrate, dark, 8 hrs
Lane 2 = 10 mM nitrate, light, 8 hrs
Lane 3 = 0 mM nitrate, dark, 8 hrs
Lane 4 = 0 mM nitrate, light, 8 hrs
Lane 5 = 10 mM nitrate, dark, 4 hrs
Lane 6 = 10 mM nitrate, light, 4 hrs
Lane 7 = 0 mM nitrate, dark, 4 hrs
Lane 8 = 0 mM nitrate, light, 4 hrs

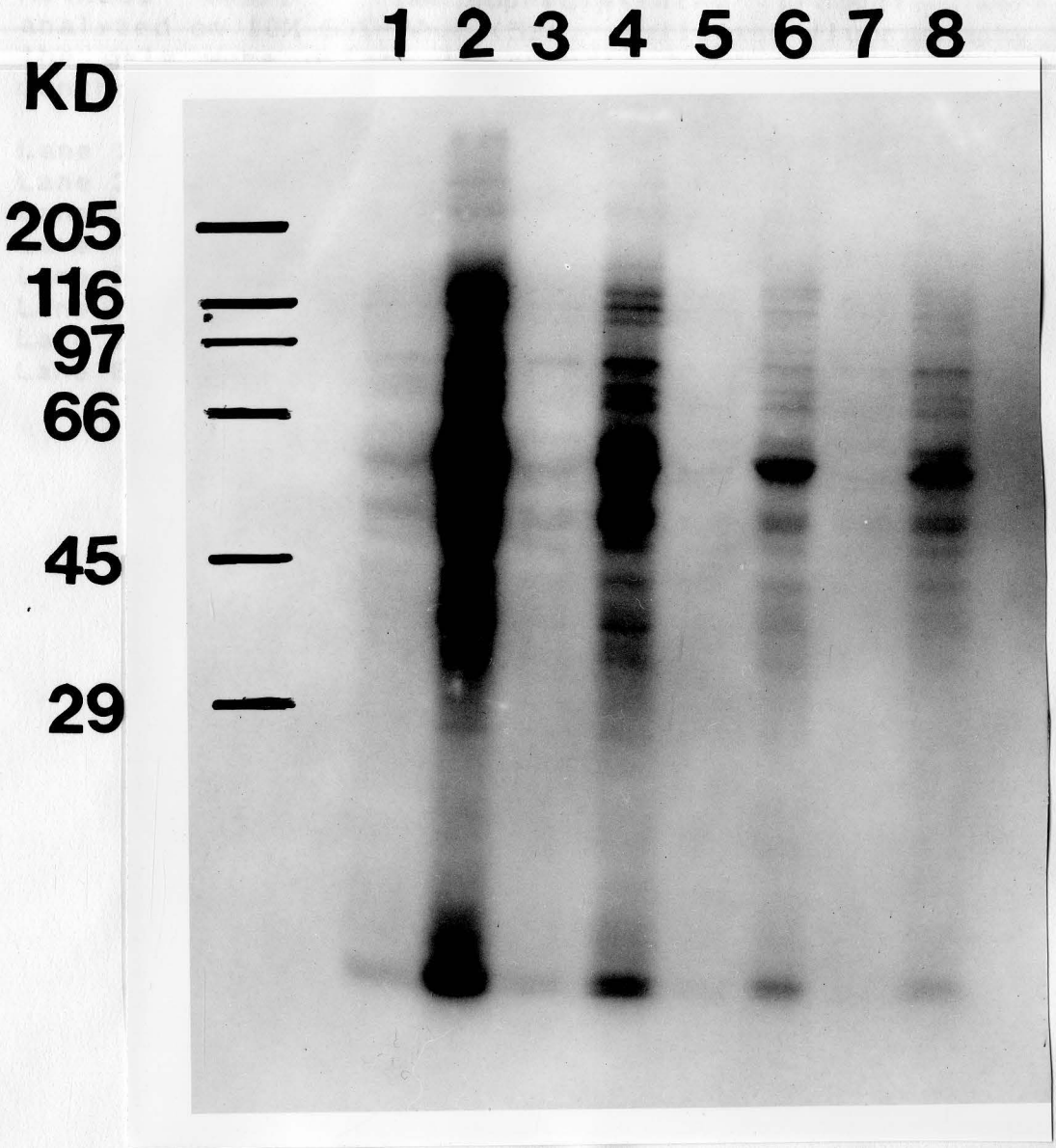

1 2 3 4 5 6 7 8
KD
205
116
97
66
45
29

FIGURE 6.

In-Vivo Labelled Immunoprecipitated Protein Products.

Immunoprecipitations were performed using anti-nitrate reductase antibodies on total proteins obtained from the in-vivo labelling experiment as described in Methods (43). Immunoprecipitated products were analyzed on 10% SDS-PAGE (53). Following fluoragraphy, the gels were dried and exposed to x-ray film for 4 months. Treatments are as follows:

Lane 1 = 10 mM nitrate, dark, 4 hrs
Lane 2 = 10 mM nitrate, light, 4 hrs
Lane 3 = 0 mM nitrate, dark, 4 hrs
Lane 4 = 0 mM nitrate, light, 4 hrs
Lane 5 = 10 mM nitrate, dark, 8 hrs
Lane 6 = 10 mM nitrate, light, 8 hrs
Lane 7 = 0 mM nitrate, dark, 8 hrs
Lane 8 = 0 mM nitrate, light, 8 hrs


1 2 3 4 5 6 7 8

TABLE III.

Concentrations of total poly (A+) RNA isolated from 10 g squash cotyledons for each of the various treatment groups are listed in units of mg/ml. The purity of the RNA sample is determined by an 260/280 absorbance ratio of greater than 1.5 . These values are also listed for each sample.

TABLE III

CONCENTRATION AND 260/280 READINGS OF POLY (A+) RNA ISOLATED FROM SQUASH COTYLEDONS

Metabolite	Concentration[a]	260/280[b]
0 mM KNO_3	3.65	1.6
50 mM KNO_3	5.80	1.9
50 mM KNO_3/ 10 mM Gln	2.00	2.0
10 mM Gln	10.80	1.8

[a] mg/ml
[b] indicator of RNA purity

FIGURE 7.

Agarose Gel of Total, Poly (A+) and Poly (A-) RNA Samples

Glyoxal-treated total, poly (A+) and poly (A-) RNA samples isolated from 7 day old squah cotyledons were run on a 1 % agarose gel. The gel was run at 40 mamps for 5 hours and stained with ethidium bromide overnight. Samples are as follows: lane 1 = poly (A-) RNA; lane 2 = poly (A+) RNA; lane 3 = total RNA. Molecular weight markers were Hind III digested lambda DNA.

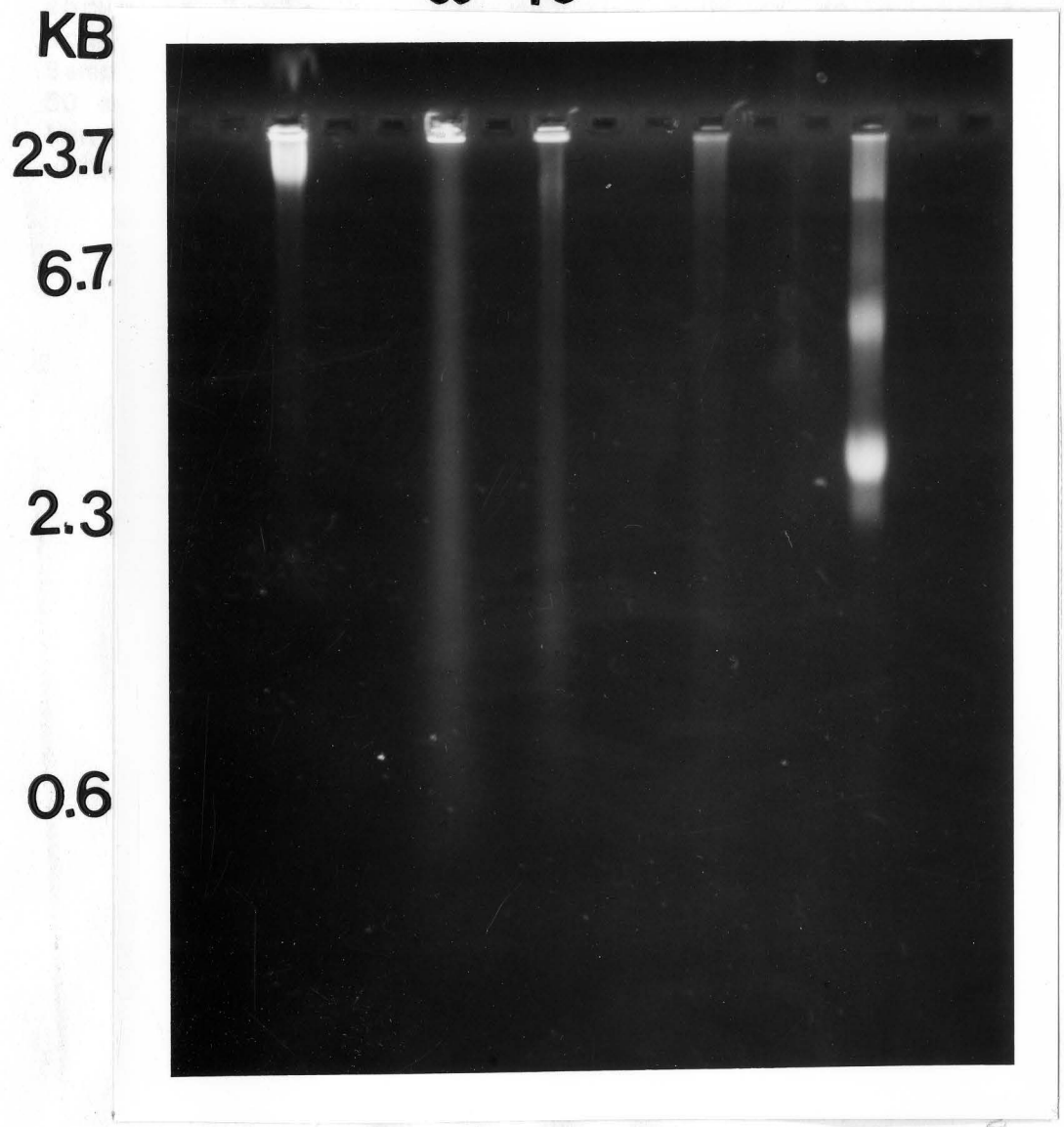

3
2
1
KB
23.7
6.7
2.3
0.6

FIGURE 8.

Agarose Gel of Poly (A+) RNA Isolated from Various Treatment Groups

Glyoxal-treated poly (A+) samples isolated from 7 day old squash cotyledons were run on a 1% agarose gel. The samples were obtained from the treatment groups shown in Table I. The gel was run at 40 mamps for 5 hours and stained with ethidium bromide overnight. Samples are as follows: lane 1 = 0 mM nitrate; lane 2 = 50 mM nitrate; lane 3 = 50 mM nitrate/ 10 mM glutamine; lane 4 = 10 mM glutamine. Molecular weight markers were Hind III digested lambda DNA.

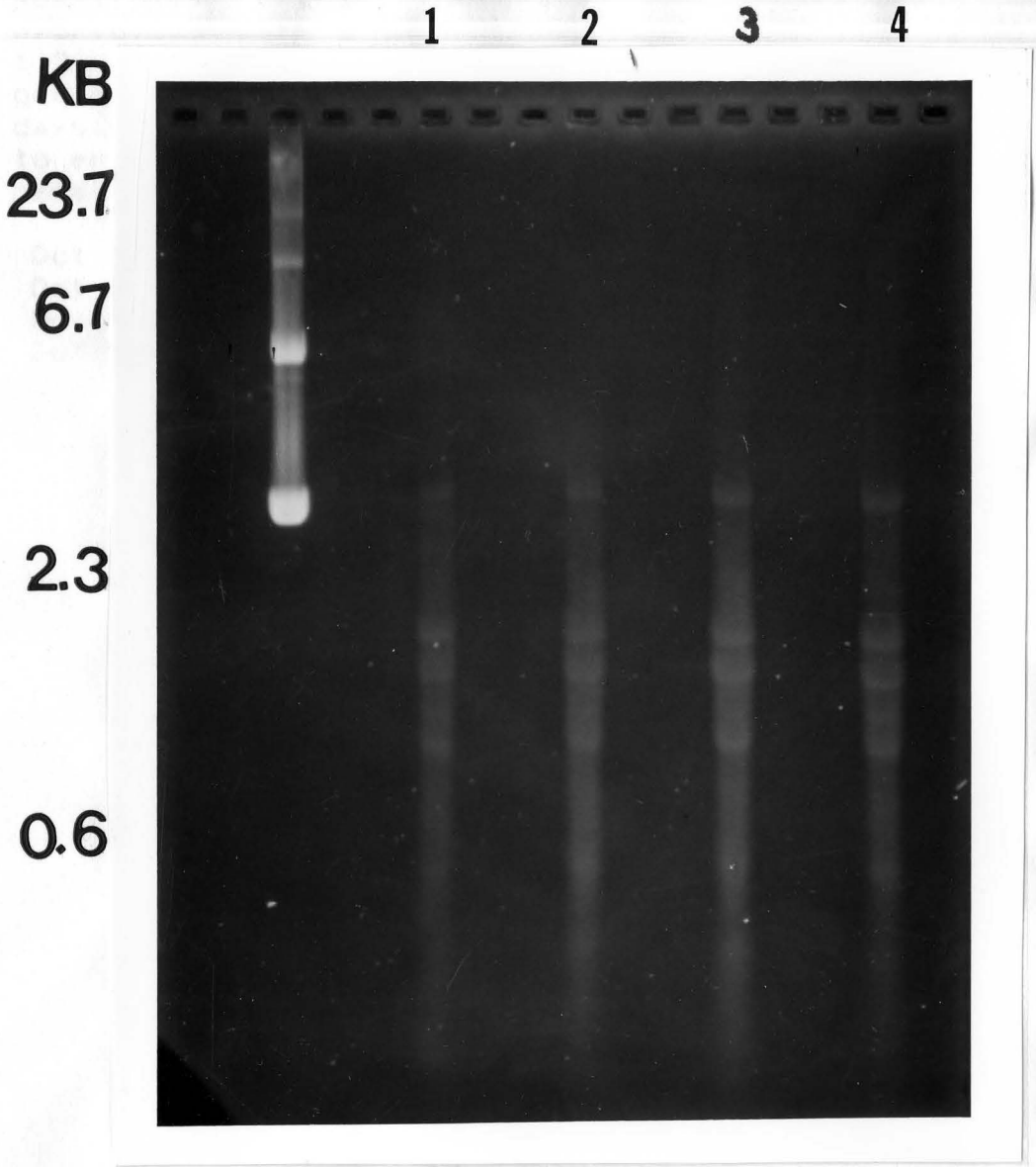
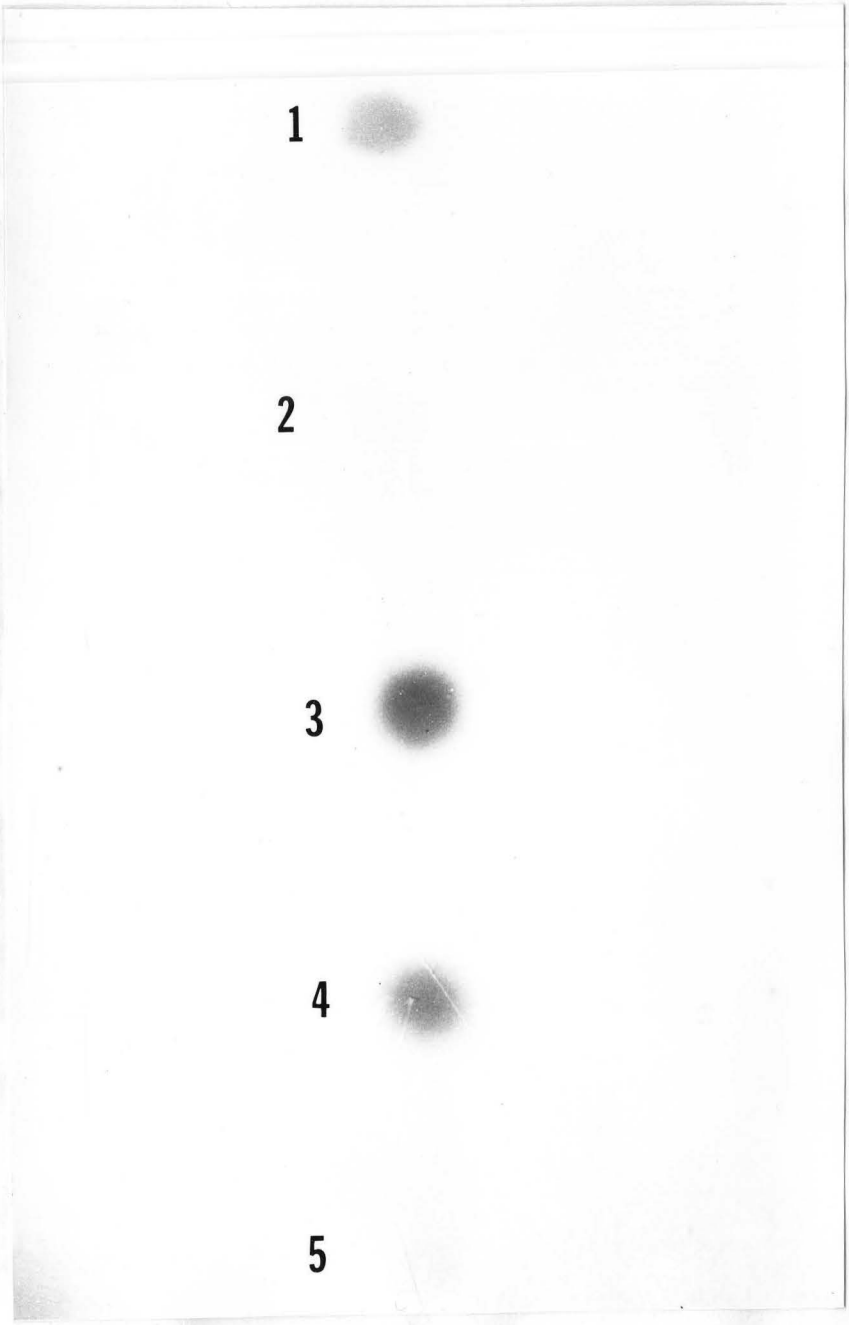

1
2
3
4
KB
23.7
6.7
2.3
0.6

FIGURE 9.

Dot Blots of Poly (A+) RNA Isolated from Various Treatment Groups

Glyoxal-treated poly (A+) RNA (8 ug) was dotted onto a positively charged membrane. The membrane was hybridized with a nick translated probe containing a 1.2 kb cDNA insert of the squash nitrate reductase gene. The membrane was exposed to x-ray film for 14 days. Unlabelled probe (4.4 ug, dot 1) was a control to ensure the hybridization system was functional. The remaining dots are as follows:

Dot 2 = 0 mM nitrate
Dot 3 = 50 mM nitrate
Dot 4 = 50 mM nitrate/10 mM glutamine
Dot 5 = 10 mM glutamine


1
2
3
4
5

CHAPTER V

DISCUSSION

In the presence of nitrate, nitrate reductase activity increased significantly when compared with zero nitrogen controls. Nitrate reductase activity in the presence of glutamine closely resembled the zero nitrogen control. The combination of nitrate and glutamine resulted in intermediate activity between the nitrate and zero nitrogen groups. In 1957, Tang and Wu first observed an increase in nitrate reductase activity in rice seedlings following the addition of nitrate (56). Barley seedlings also show an increase in nitrate reductase activity upon the addition of nitrate (3). Squash nitrate reductase activity has also been shown by others to be enhanced in the presence of nitrate (10). Other studies documenting changes in nitrate reductase activity in response to nitrate have also been reported (24-28). The mechanism(s) of this effect is currently not well understood in higher plants.

Two-dimensional gel electrophoresis has been used

as a tool to detect changes in proteins being synthesized under various conditions (57). However, results from total protein profiles obtained from two-dimensional electrophoresis of samples from the nitrate and zero nitrogen groups did not show any significant changes in the major proteins being synthesized. The marked physiological changes in nitrate reductase activity were not mirrored by any significant changes in the overall protein patterns. These findings suggest the effects of nitrate which lead to increases in nitrate reductase activity are highly specific. The changes caused by nitrate are restricted mainly to factors resulting in fluctuations in nitrate reductase activity. Overall protein synthesis appears not to be affected by the addition of nitrate. However, since nitrate reductase comprises only 0.05% of plant proteins (42), total protein profiles lack the sensitivity to detect any changes which might be specifically occurring in nitrate reductase protein levels.

Immunoprecipitation of radiolabelled <u>in-vivo</u> translation products demonstrated the need for both nitrate and light for the synthesis of nitrate reductase subunit. Gupta and Beevers reported similar

results in pea seedlings for nitrite reductase, the second nitrate assimilatory enzyme (43). The presence of nitrate reductase protein correlated with nitrate reductase activity. Based on these present studies, as well as a series of Western blots performed on squash previously in this laboratory (unpublished results), the induction of nitrate reductase activity, in the presence of added nitrate and light, is controlled by de novo synthesis of the nitrate reductase protein. These results differ from those obtained in Chlorella where evidence for an enzymatically inactive nitrate reductase protein precursor has been reported (4).

The molecular weight of the immunoprecipitated nitrate reductase protein corresponded to the known squash subunit molecular weight of 115 KD (19). Therefore, it appears that nitrate reductase is synthesized as individual subunits which are then paired to form the functional homodimer characteristic of squash nitrate reductase (19). Unlike the situation for the chloroplastic enzyme nitrite reductase (43), nitrate reductase appears not to be synthesized as a larger precursor containing a transit sequence to facilitate translocation. However, an in-vitro translation experiment is needed to eliminate the

possibility of a larger precursor being processed during the in-vivo labelling time intervals. However, since nitrate reductase is not transported out of the cytoplasm (20, 21), the addition of such a transit sequence would not be expected.

Squash plants given various metabolites were used for the isolation of total poly (A+) mRNA. The concentrations of mRNA obtained were comparable to others reported in the literature (42). These concentrations were not a reflection of the corresponding nitrate reductase activities of each sample. The transcripts were of various sizes, ranging from 22 kb to 1 kb in length. When these mRNAs were screened using dot blots for the presence of nitrate reductase-specific transcripts, the pattern of hybridization obtained correlated with nitrate reductase activity levels. Levels of hybridization were highest with RNA isolated from plants given nitrate compared to no hybridization in the samples from zero nitrate and glutamine alone. The combined treatment of nitrate and glutamine resulted in intermediate levels of hybridization between the nitrate and glutamine groups alone. Therefore, changes in nitrate reductase activity levels are the direct

result of changes in the steady state levels of nitrate reductase-specific mRNA.

The size of the transcript which hybridized with the clone used has previously been determined by Crawford et al. (10) to be approximately 3.2 kb in length. A transcript of this size would be sufficient to code for a protein the size of the nitrate reductase subunit. These researchers also found nitrate reductase specific RNA levels to increase following the addition of nitrate compared to samples receiving no nitrate. Nitrate reductase activities and protein levels also correlated with the observed changes in mRNA. Cheng et al. have shown hybridization to a 3.5 kb transcript in barley with a partial nitrate reductase cDNA clone isolated from barley (44). These researchers have also established changes in nitrate reductase activity in response to nitrate correspond with changes in nitrate reductase specific mRNA. The data shown here confirm the earlier findings in squash by Crawford et al. (10). Hybridizations were seen only with RNA isolated from nitrate grown plants. Expansions on this earlier work presented here using dot blots have shown actual steady state levels of

nitrate reductase specific transcripts to change in response to nitrate.

The actual mechanism by which nitrate affects the transcription of nitrate reductase-specific mRNA has yet to be delineated. However, some hypotheses can be suggested. First, some type of repressor protein, as yet unidentified in higher plants, might be associated with the nitrate reductase gene. A similar scenario has been documented in the fungi N. crassa and A. nidulans (22). Upon the addition of nitrate, this repressor protein might undergo a conformational change resulting in displacement, allowing transcription of the nitrate reductase gene to proceed.

Nitrate might also be affecting transport of the nitrate reductase transcript out of the nucleus into the cytoplasm where it is available for translation. In the absence of nitrate, the transcript might be restricted to the nucleus. Upon the addition of nitrate, transport into the cytoplasm might be facilitated and translation proceeds. To examine this possibility, RNA samples isolated from the nucleus could be screened for the presence or absence of nitrate reductase-specific transcripts.

Another possibility for the action of nitrate on nitrate reductase activity might be to increase the transcriptional rate of the nitrate reductase gene in the presence of nitrate. Also, nitrate might also be affecting the stability of the transcripts, allowing more efficient translation to occur. Further studies (run-off transcription assays and transcription inhibition studies) are in order to begin to examine the feasibilities of these suggested hypotheses.

In order to better understand the details of this transcriptional control, the gene for nitrate reductase in higher plants needs to be cloned. Upon isolation of such a genomic clone, various manipulations can be performed to better understand how this gene is expressed and at what key points the various metabolites might be altering this expression. These studies would set the stage for further manipulations to increase the efficiency of nitrate reductase. This gene could then be transferred to other higher plant species that are less efficient at assimilating nitrate.

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APPROVAL SHEET

The thesis submitted by Susan Jo Martino has been read and approved by the following committee:

Dr. John Smarrelli, Jr., Director
Assistant Professor, Biology, Loyola

Dr. Jeffrey Doering,
Associate Professor, Biology, Loyola

Dr. Albert Rotermund,
Associate Professor, Biology, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committe with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

8/20/87
Date

John Smarrelli Jr
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