NITRATE REDUCTASE IN MAIZE ROOTS: LOCALIZATION AND MOLECULAR CHARACTERIZATION

Ву

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A Thesis

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McMaster University

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i

NITRATE REDUCTASE IN MAIZE ROOTS

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ABSTRACT

Nitrate reductase (NR) is the first enzyme involved in the pathway of nitrate assimilation in plants. It converts nitrate to nitrite. By including the serine protease inhibitor, chymostatin, in the extraction buffer, NR from maize (Zea mays L.) roots was stabilized in vitro. Contrary to early results, it was found in substantial amounts in the mature regions of the root. Two isozymes of NR were identified, an NADH monospecific form found predominantly in the root tip, and an NAD(P)H bispecific form which was predominant in the mature portion of the roots. Both isozymes were found to reach substantial levels of activity, approximately one-third to one-half the levels found in shoots. The levels of NR activity in both shoots and roots varied with the age of the plants and the conditions of growth. Subsequent purification and biochemical characterization of the two isozymes suggested similarities in the characteristics of the isozymes. However, the NADH form had an exceptionally high K_m for NADH which suggests that the NADH:NR may not be active in the

iii

assimilation of nitrate as it may not be able to compete with other dehydrogenases for reductant.

A partial cDNA clone of root NR was isolated, sequenced and identified as a gene distinct from the gene which codes for NR in maize leaves. The NR in maize roots was affected in a positive manner by nitrate at the levels of activity and transcription. However, it did not appear to be affected by either a diurnal rhythm or directly by light, as was found for leaf NR. Through the use of the tissue print hybridization technique, NR mRNA was found to be expressed throughout the maize root with the exception of the root tip.

In conclusion, NR in maize roots is present in high enough amounts to account for a substantial level of nitrate reduction in the roots, suggesting that maize roots have an important role in the overall metabolism of nitrate in maize.

iv

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V

TABLE OF CONTENTS

.

-

INTRODUCTION	1			
Importance of Nitrate to Crop Plants				
Uptake and Distribution of Nitrate in Higher Plants	2			
The Fate of Nitrogen Within Higher Plants	7			
Nitrate Reductase	12			
a) Biochemistry	12			
i) Isolation and purification	12			
ii) Types of nitrate reductase	14			
b) Regulation	16			
i) Regulation by nitrate	16			
ii) Regulation by light	18			
iii) Regulation by other factors	19			
c) Identification of structural and genetic components involved in the regulation and synthesis of nitrate reductase	22			
i) Mutant analysis	22			
ii) Elucidation of the structure of NR	23			
iii) Identification of genes which code for NR	27			
iv) Molecular regulation of NR	28			
d) Localization	31			
i) Localization of NR	31			

ii) Localization of the reductant supply for NR	35
Chapter 1: Stabilization of Root Nitrate Reductase	38
Introduction	39
Materials and Methods	41
Growth Conditions of Plants	41
a) Agar grown plants	41
b) Hydroponically grown plants	41
Nitrate Reductase Activity Assay	42
Results	44
Storage Effects on Nitrate Reductase Activity in Maize Roots	44
Development of an Extraction Buffer to Stabilize Root NR <i>in vitro</i>	44
Stabilization of Root Nitrate Reductase Activity with Chymostatin and Altered Growth Conditions	46
Inhibition of Maize Root Proteinase (MRP) by Chymostatin	56
Test of Removal of NADH and NADPH from the NR Assay Medium with Oxaloacetate or Alcohol Dehydrogenase and Acetaldehyde	58
Discussion	62
Chapter 2: Purification and Biochemical Characterization of Root Nitrate Reductase	64
Introduction	65
Materials and Methods	67
Growth Conditions of Plants	67

.

.

Extraction and Nitrate Reductase Ass Plant Organs	ays of 67
Preparation of Antiserum	68
Protocol for Partial Purification of Reductase from Maize Roots	Nitrate 68
MRP Activity Assay	72
Calibration of Sephadex G-150 Column	n 73
Results	74
Inhibition of NR Activity by Antiser	rum 74
Induction of NRA in Roots and Shoots with 10mM KNO ₃	s of Maize 74
Induction of NRA in Maize Roots with and Nitrate	h Ammonium 76
Effect of Ammonium and Nitrate on Ma Seedlings	aize 78
<i>In vivo</i> Stability of NAD(P)H:NRA in upon the Removal of Nitrate	Maize 80
Partial Purification of NADH:NR and NAD(P)H:NR from Maize Roots	82
Biochemical Characterization of NAD NAD(P)H:NR from Maize Roots	H:NR and 90
Discussion	102
Chapter 3: Influence of Light/Dark Cycles on Regulation of Nitrate Reductase and Reductase	the 106 d Nitrite
Introduction	107
Materials and Methods	110
Plant Material and Growth Condition	s 110
RNA Isolation and Hybridization	111
Enzyme Assays	112

Results	114				
Effect of Plant Age on NR and NiR Activites	114				
Effect of Altered Light Regimes on NR Activity and mRNA in Shoots	116				
Effect of Different Light Regimes on the Expression of Root FR	119				
Effect of Light Regimes on NiR Activity and mRNA in Shcots	121				
Discussion	124				
Chapter 4: Molecular Characterization of Root Nitrate Reductase	129				
Introduction	130				
Materials and Methods					
Screening of Root cDNA Library	133				
a) λ Plaque Lifts	133				
b) Screening of Plaque Lifts	133				
c) Rescreening of Plaques	135				
Purification of λ DNA	135				
λ DNA Filter Hybridization	136				
Subcloning of λ cDNA Inserts into pUC 18	136				
a) Ligation of the Insert into pUC 18	136				
b) Transformation of Competent Cells with the Ligation Mixture	137				
Sequencing of cDNA Clone	137				
Growth Conditions of Plants	138				
RNA Extraction and RNA Blot Hybridization	138				

x

Extraction of Genomic DNA and DNA Blot Hybridization	139
Results	142
Isolation of a cDNA clone for Maize Root NR	142
Sequencing of p1501	143
Comparison of p1501 to Other Known Sequences of NR	5 143
Induction of Maize Root mRNA by Nitrate	148
Hybridization of Root and Leaf cDNA clones t mRNA from Different Maize Organs	:0 150
Hybridization of Root and Leaf NRs to Separate Genes	153
Discussion	155
Chapter 5: Localization of Nitrate Reductase in Maize Roots	160
Introduction	161
Materials and Methods	165
Growth Conditions of Plants	165
Nitrate Reductase Activity Assay	166
Tissue Preparation for Electron Microscopy	166
Immunocytochemical Labeling	167
a) Antibodies and Labels	167
b) Post-embedding Treatment	168
Preparation of Tissue Prints	169
Staining of Tissue Prints with India Ink	170
Hybridization Procedure	171

.

Results	172
Immunocytochemical Localization of Nitrate Reductase	177
Tissue Printing of Maize Roots	177
Expression of NR mRNA in Maize Roots	178
Discussion	181
GENERAL DISCUSSION	185
LIST OF APPENDICES	192
BIBLIOGRAPHY	238

•

,

,

LIST OF TABLES

• •

Table #		Page
1	Effect of storage on the NADH:NRA of maize roots.	45
2	Effect of the addition of exogenous protein and PMSF to extraction buffer on NADH:NRA in maize roots.	47
3	Effect of changing the ratio of frozen root powder to extraction buffer on NRA.	48
4	Stabilization of root NR with chymostatin.	49
5	Effect of protease inhibitors on NRA in root extracts of agar grown plants.	51
6	Effect of protease inhibitors on NADH:NRA in extracts of hydroponically grown roots.	52
7	Concentration of chymostatin required to stabilize NADH:NRA in maize roots.	55
8	Effect of protease inhibitors and the antibody to MRP on the recovery of nitrate reductase activity from leaves and roots in optimized assays.	57
9	Effect of chymostatin on MRP activity.	59
10	The disappearance of NADH or NADPH with oxaloacetate or alcohol dehydrogenase and acetaldehyde at 340nm.	60
11	Effect of nitrogen source on NRA in segments of maize roots.	79
12	Effect on nitrogen source on the induction of NRA in the roots and shoots of maize.	81

13	Test of purification conditions on the	85
	stability of nitrate reductase acitivity.	

- 14 Test for the presence of MRP activity in 86 ammonium sulfate pellets and supernatants.
- 15 Recovery of NRA from root NR purification 89 protocol.
- 16 Summary of biochemical characteristics of the 99 NADH and NAD(P)H enzymes of maize roots and leaves.
- 17 Analysis of nitrate treated maize tissues for 174 their NRA and for the amount of label observed on plastids in sections of the tissues.
- 18 Survey of K_ms, molecular weights and pH optima 186 of higher plant NRs.

.

2.

LIST OF FIGURES

-

ĩ

2

Figure #		Page
1	Nitrogen metabolism in maize.	8
2	Structural model of higher plant nitrate reductase.	25
3	Disappearance of maize root NRA in vitro over time.	54
4	Inhibition of maize leaf and root NRA by antiserum made against purified leaf NR.	75
5	Time course of induction of NRA in maize roots and shoots with 10mM KNO ₃ .	77
6	In vivo stability of NR in maize root tip and mature root segments.	83
7	Elution profile of NADH and NADPH enzyme activities from the blue Sepharose column.	88
8	pH optima of partially purified NAD(P)H:NR and NADH:NR.	92
9	Calculation of K_m for NADH of the NADH eluted enzyme.	93
10	Calculation of K_m for NO_3^- of the NADH eluted enzyme.	94
11	Calculation of K_m for NO_3^- of the NADPH eluted enzyme.	95
12	Calculation of K_m for either NADH or NADPH of the NADPH eluted enzyme.	96
13	Calculation of K_m for nitrate of the maize leaf enzyme.	97
14	Calculation of K_m for NADH of the maize leaf enzyme.	98

xiv

,

- 15 Calibration curve of Sephadex G-150 column. 101
- 16 Development of (a) nitrate reductase activity 115 and (b) nitrite reductase activity in maize shoots and roots during the first 8 days of growth after germination.
- 17 Variations in the amount of NR activity and 117 mRNA levels in the shoots of 6 d old maize plants either (a) maintained in a 16 h light/ 8 h dark regime (b) transferred at time 0 h to continuous light (c) transferred at time 0 h to continuous darkness.
- 18 Variations in the amount of NR activity and 120 mRNA levels in the roots of 6 d old maize plants either (a) maintained in a 16 h light/ 8 h dark regime (b) transferred at time 0 h continuous light (c) transferred at time 0 h to coninuous darkness.
- 19 Variations in the amount of NiR activity and 122 mRNA levels in the shoots of 6 d old maize plants either (a) maintained in a 16 h light/ 8 h dark regime (b) transferred at time 0 h to continuous darkness or (c) transferred at time 0 h to continuous light.
- 20 Maps of p1501, p1502 and p1503. 144
- 21 Nucleotide and amino acid sequence of the 145 maize root NR cDNA clone.
- 22 Comparison of maize root and shoot NR 146 nucleotide sequences.
- 23 Comparison of maize root NR amino acid 147 sequence to other known NR sequences from higher plants.
- 24 Induction of NR mRNA in maize root tips and 149 mature roots upon the addition of nitrate to the medium.
- 25 Hybridization of root coding region, root 152 non-coding region and leaf NR cDNA clones to mRNA from maize leaves and roots.

26	Southern blot of maize genomic DNA probed with leaf and root NR cDNA clones.	154
27	Electron micrograph of nitrate induced maize root cortical cells labeled with maize NR-specific Ab and GAR IgG-gold.	175
28	Electron micrograph of maize root cortical cells labeled with preimmune serum and Gar IgG-gold.	176
20		

٠

29	Tissue	prints	of	maize	roots.	•	17	9	
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LIST OF ABBREVIATIONS

Ab	antibody
AS	asparagine synthetase
β-Ме	β-Mercaptoethanol
BSA	bovine serum albumin
cv	cultivar
DEP	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
FAD	flavin adenine dinucleotide
GAR	goat anti-rabbit
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
HEPES	N-2-hydroxyethyl piperazine-N'-2
	ethanesulfonic acid
IgG	Immunoglobulin G
IPTG	isopropyl beta-D-thio-galactopyranoside
MES	2-(N-morpholino) ethanesulfonic acid

MOPS	3-(N-morpholino) propanesulfonic acid
MRP	maize root proteinase
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NED	N-1-napthylene-diamine-dihydrochloride
NiR	nitrite reductase
NR	nitrate reductase
NRA	nitrate reductase activity
NR CRM	nitrate reductase cross-reacting material
NRP	nitrate reductase protein
ΟΑΑ	oxaloacetic acid
0.D.	optical density
PMSF	phenyl methyl sulfonyl flouride
PVP	polyvinyl pyrrolidone
RT	room temperature
SDS	sodium dodecyl sulfate
X-Gal	5-bromo-4-chloro-3-indoyl β-D-
	galactopyranoside

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INTRODUCTION

Importance of Nitrate to Crop Plants

In agricultural soils, nitrate is the predominant form of nitrogen available to plants. This is a result of the addition of fertilizers which contain nitrate, and the conversion of ammonia to nitrate by nitrifying bacteria such as <u>Nitrosomas</u> and <u>Nitrobacter</u> species. Ammonium may be formed by the mineralization of organic soil nitrogen or may be added in fertilizers (Wray, 1988). Nitrate can be assimilated by a variety of bacteria, fungi, algae and plants and it has been predicted that these organisms consume more than 10^4 megatons of nitrate each year (Guerrero et al., 1981).

Nitrogen fertilizers have been used extensively in agriculture to increase crop yields. The use of high levels of nitrogen fertilizer has, however, become a subject of enviromental concern. The production of nitrogen fertilizers is not only costly in an economic sense, but it also requires a high input of fossil fuel to make the fertilizers. Once the fertilizers are applied, they can leach out of the soil and accumulate in water supplies, both above and below ground. High concentrations of nitrate act as a pollutant, upsetting the natural chemical balance of water which may result in massive growths of algae and aquatic flowering plants. The nitrate may also be converted to nitrite which is thought to have carcinogenic properties when found in foods. Finally, nitrate may be converted by the process of denitrification in the soils to nitrous oxides, a form of air pollution. Nitrous oxides are a component of acid rain and are involved in reactions which lead to the production of secondary pollutants such as aldehydes and peroxyacytnitrates, each of which is harmful to both plant and animal life forms (Smith, 1980). Thus, the studies determining how nitrate is assimilated into plants is of ecological, economic, agricultural and possibly of political importance as well.

Uptake and Distribution of Nitrate in Higher Plants

The mechanism by which nitrate is taken up by plants is poorly characterized at the biochemical level. Most of the information available has been generated by studies using either unlabeled NO_3^- or labeled ${}^{15}NO_3^-$ (Morgan <u>et al.</u>, 1973), ${}^{13}NO_3^-$ (Oscarson <u>et al.</u>, 1987) and the nitrate analogue ${}^{36}ClO_3^-$ (Deane-Drummond and Glass, 1982). The process of uptake has been proposed to be mediated by at least two systems in the roots, one of which is constitutive while the other is inducible by nitrate. The constitutive

system was identified by studies with $^{13}NO_3$ in barley which showed that the K_m for nitrate uptake was lower in plants which had been grown in the presence of ammonium without nitrate when compared to that measured for plants which had been exposed to nitrate during the experimental period (Lee and Drew, 1986). The inducible system is characterized by the presence of a lag period after the addition of nitrate, before maximal rates of nitrate uptake occur (Jackson et al., 1986). Inhibitors of RNA and protein synthesis significantly reduce the rate of nitrate uptake, suggesting that a protein component is synthesized when nitrate is added to the system (Rao and Rains, 1976). It is possible to inhibit uptake with uncouplers of oxidative phosphorylation and low temperature (Rao and Rains, 1976; Clarkson and Warner, 1979). This, plus the fact that both systems work against an electrochemical potential gradient, suggests that these processes are mediated by a carrier which requires metabolic energy (Wray, 1988).

Attempts have been made to identify mutants with defective uptake systems (Oostindier-Braaksma and Feenstra, 1972, 1973; Wallsgrove, 1987). A mutant called B1 was identified in <u>Arabadopsis</u>, which appears to be altered in a protein(s) which is involved in the regulation of the 'inducible' or second system of nitrate uptake (Doddema and

Telkamp, 1979). Wallsgrove (1987) mutagenized barley with azide and was able to generate mutants which demonstrated either low or intermediate levels of nitrate uptake. By using functional complementation analysis of these mutants it may be possible to identify some of the genes and subsequently the proteins involved in the process of nitrate uptake, however, to date, none of the protein components involved in the nitrate uptake system have been clearly identified (for review see Jackson <u>et al</u>., 1986; Wray, 1988).

The enzyme nitrate reductase (NR), the first enzyme involved in the assimilation of nitrate into plants, is also thought by some workers, to be one of the protein components involved in the uptake of nitrate into roots. Butz and Jackson (1977) proposed that NR associated with the plasma membrane might be part of a complex which transports and reduces nitrate. Ward <u>et al</u>. (1988) found that fragments of anti-NR IgG made from purified <u>Chlorella</u> NR inhibited up to 90% of the uptake of nitrate into barley seedlings. They suggested that either NR or an antigenically related protein which was involved in the transport of nitrate was reacting with the antibody. They found that 4% of total measurable NRA in root cells was associated with the plasmalemma. These results support the theory of Butz and Jackson (1977).

The idea of NR being involved in the uptake of nitrate has been brought into question however, by the elegant experiments of Warner and Huffaker (1989). These workers used barley mutants which had lesions in the structural genes of both the NADH:NR and the NAD(P)H:NR isozymes. There appear to be only two genes which code for NR in barley (Warner <u>et al</u>., 1987). It was found that the level of either of the NRs in the plants had no effect on the kinetics of nitrate uptake into the plants, suggesting therefore, that NR does not have a role in the uptake of nitrate.

Once nitrate is taken up into a plant root it can be transported to the shoot or it can remain in the root where it is either stored as nitrate or reduced (Oaks, 1986). Techniques for measuring the level of nitrate reduction in roots were pioneered by Pate (1973). By measuring the nitrogen components in the xylem sap, he and others were able to show that the level of reduction of nitrate in the roots varied with different species of plants and with the age of the plants (Wallace and Pate, 1965; Pate,1973; Andrews, 1986; Wallace, 1986). However, Rufty <u>et al.</u> (1982) showed that the distribution of nitrogen within a plant is a complex process. Using ¹⁵NO₃⁻, they found that very little of the reduced nitrogen in soybean xylem sap contained ¹⁵N

whereas ${}^{15}\mathrm{NO_3}$ was a prominent constituent. They proposed that nitrate which had been reduced in the leaf, was transferred to the root tissues and then recycled to the top of the plant. Attempts to measure the capacity of roots to reduce nitrogen have also been made using ${}^{15}\mathrm{NO_3}$ as a tracer. Gojon <u>et al</u>. (1986) using these techniques, showed that during the induction process 70% of nitrate reduction within maize plants was carried out in the roots. Upon reaching a steady-state level of reduction, roots were responsible for 27% of the total reduction. This suggests that roots are an important component of overall nitrate reduction in maize.

The intracellular distribution of nitrate within higher plants has been discussed in terms of nitrate pools. These pools have been identified as a metabolic pool and a non-metabolic or storage pool (Ferrari <u>et al.</u>, 1973; Aslam and Oaks, 1976; Guerrero <u>et al.</u>, 1981: Oaks, 1986). The metabolic pool contains the nitrate wnich is available both for reduction and for translocation to the shoot. It may be important in regulating the level of nitrate reduction in a particular part of the plant (Oaks, 1979, 1986). The nonmetabolic pool is probably located in the cell vacuole as this is the area which contains the majority of the nitrate within the cell (Martinoia <u>et al</u>., 1980; Granstedt and Huffaker, 1982). When high levels of nitrate have been

administered to the plant, the vacuolar nitrate can be released to the cytoplasm when the plants are exposed to light (Aslam and Oaks, 1976). A mechanism which can control the flux of nitrate through the tonoplast membrane has not yet been clearly identified (Wray, 1988). Randall and Sze (1986) identified a proton-translocating ATPase, and Wagner and Mulready (1983) found a pyrophosphatase in the tonoplast membrane. Neither of these have been shown to be associated with nitrate transport (Wray, 1988). However, a study by McClure et al. (1987) identified a 31kD protein, associated with tonoplast membranes, which increased quantitatively upon the addition of nitrate to maize roots. They proposed that this protein may be a component of the nitrate transport mechanism. As with the uptake of nitrate into plants, the mechanisms which mediate movement of either nitrate or the components of its reduction within and between cells, have proven difficult to identify.

The Fate of Nitrogen Within Higher Plants

A schematic diagram for the reduction of nitrate in higher plants is shown in Figure 1. When nitrate enters a cell it can be reduced to nitrite by the enzyme nitrate reductase (NR, EC 1.6.6.1) (Figure 1, step 1). Nitrate reductase is a complex, multi-centre redox enzyme which can

Figure 1. Nitrogen metabolism in maize. The relative importance of a particular step is highlighted by the width of the arrow pointing to the step. The numbers signify particular steps or enzymes within the pathway as folllows: 1) nitrate reductase 2) nitrite reductase 3) reduction of a ferredoxin-like protein by pyridine nucleotide reductase 4) glutamine synthetase 5) glutamate synthase 6) glutamate dehydrogenase. This figure is adapted from Oaks (1986).



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use NADH, NADPH or both as a reductant, dependant on which isoform of the enzyme is present in a particular tissue or species (Beevers and Hageman, 1969). It is a substrate inducible enzyme (Tang and Wu, 1957) and it also appears to require light for its induction (Hageman and Flescher, 1960). It is thought to be localized in the cytosol of higher plants. This idea has been the subject of intensive investigation, which will be discussed in a later section of this chapter (Grant <u>et al.</u>, 1970; Dalling <u>et al.</u>, 1972; Vaughn <u>et al.</u>, 1984; Kamachi <u>et al.</u>, 1987; Vaughn and Campbell, 1988).

The nitrite formed by NR is transported to plastids where it is reduced to ammonium by the enzyme nitrite reductase (NiR, EC 1.7.7.1) (Figure 1, step 2) (Paneque <u>et</u> <u>al</u>., 1963, Miflin, 1974; Oaks and Hirel, 1985; Bowsher <u>et</u> <u>al</u>., 1989). This reaction uses ferredoxin in chloroplasts (Hucklesby <u>et al</u>., 1972) and a ferredoxin-like protein (Suzuki <u>et al</u>., 1985) in root plastids (Emes and Bowsher, 1991), as a source of reductant. In roots this ferredoxinlike protein is thought to be reduced in turn by a pyridine nucleotide reductase (Suzuki <u>et al</u>., 1985; Emes and Bowsher, 1991) (Figure 1, step 3). As with NR, NiR appears to require nitrate and light for its synthesis (Gupta and Beevers, 1984; Rajeskhar and Oelmuller, 1987; Back <u>et al</u>.,

1988). This enzyme has been shown to be encoded by a nuclear gene which contains a transit peptide for targeting the enzyme to plastids (Back <u>et al</u>., 1988).

The ammonia generated from the NiR reaction appears to be predominantly converted into glutamine by the action of GS (glutamine synthetase, EC 6.3.1.2) (Figure 1, step 4; Miflin and Lea, 1980). Glutamine synthetase requires glutamate and ATP in addition to the ammonium to make glutamine. This reaction can occur in the plastids or in the cytoplasm as different isoforms of the enzyme have been found in these locations (Emes and Fowler, 1983; McNally and Hirel, 1983; Vezina and Langois, 1989; Vezina <u>et al.</u>, 1987).

Glutamate synthase (GOGAT, EC 1.4.7.1) (Figure 1, step 5) is thought to mediate the regeneration of glutamate. The enzyme GOGAT uses glutamine, α -ketoglutarate and either reduced ferredoxin, NADH or NADPH as an electron donor to form two molecules of glutamate. One or both of the glutamate molecules, depending on demand, can be used for the regeneration of glutamine, hence the GS-GOGAT pathway. Evidence suggests that GOGAT is localized in the plastids (Miflin, 1974; Lee, 1980; Suzuki <u>et al.</u>, 1981). Asparagine synthetase (AS, EC 6.3.5.4) may use either the ammonia generated directly from the action of NiR, or the amide nitrogen of glutamine to form asparagine (Figure 1, slashed arrow) (Rognes, 1975; Guerrero <u>et al</u>., 1981; Oaks and Hirel, 1985; Oaks, 1986). In maize, glutamine is the major reduced form of nitrogen. In most temperate legumes, however, asparagine is the major form of reduced nitrogen transported to the shoots as demonstrated by the high levels of these compounds measured in the xylem sap (Pate, 1973; Andrews, 1986).

Glutamate dehydrogenase (GDH, EC 1.4.1.2) is the last major enzyme potentially involved in the assimilation of nitrogen into plants. It uses α -ketoglutarate, ammonium and either NADH or NADPH as a reductant supply to form glutamate (Figure 1, step 6). Glutamate dehydrogenase is found in the mitochondria of both roots and leaves (Suzuki et al., 1981; Oaks and Hirel, 1985) and an NADPH form has been found in chloroplasts (for pertinent references see Stewart et al., 1980). The function of GDH in higher plants is not understood (Miflin and Lea, 1977, 1980; Stewart et al., 1980; Oaks and Hirel, 1985; Yamaya and Oaks, 1987). The remaining portion of this introduction will concentrate on the enzyme nitrate reductase as this is the enzyme on which my thesis is based.

Nitrate Reductase

a) **Biochemistry**

i) Isolation and purification

Nitrate reductase was first described by Evans and Nason in 1953. Much of the interest in this enzyme is related to its substrate induciblility i.e. the addition of nitrate resulted in the appearance of nitrate reductase activity (NRA) (Tang and Wu, 1957). As a result it has been considered to be the limiting step in the reduction of nitrate (Tang and Wu, 1957; Beevers and Hageman; 1969). Since its discovery many attempts have been made to extract and isolate NR in vitro so that its biochemical properties could be examined. Unfortunately, NR has generally been found to be unstable in <u>vitro</u> (Wallace and Oaks, 1985). The instability of NRs has been linked to the presence of inactivating proteins in a variety of species which appear to act preferentially on NR in vitro either by cleaving the enzymes or by binding to them (Oaks et al., 1972; Wallace, 1973; Yamaya and Ohira, 1977; Jolly and Tolbert, 1978; Sorger et al., 1978; Yamaya et al., 1980a,b; Miller and Huffaker, 1981; Hamano et al., 1984). It is not known if these inactivators have a role in regulating NR in vivo.

Using blue dextran-Sepharose affinity chromatography, Solomonson (1975), was able to purify a stable form of NR from the green alga Chlorella. Campbell (1976) subsequently used affinity chromatography to separate different isozymes of NR from soybean leaves. With the development of high pH buffers and the use of inhibitors (Wallace, 1975; Kuo et al., 1982) it has now been possible to stabilize this enzyme in vitro from many species and subsequently, to purify the enzyme. Nitrate reductases from a variety of species have now been purified using the improved extraction techniques and improved affinity columns of blue sepharose or more recently with immunoaffinity columns made with monoclonal antibodies to NR (Fido and Notton, 1984; Nakagawa et al., 1984,1985; Harker et al., 1986; Fido, 1987; Oji <u>et</u> <u>al</u>., 1988; Moureaux <u>et</u> <u>al</u>., 1989). In each case the conditions of the purification required modification for the particular tissue being studied (Redinbaugh and Campbell, 1981; Fido and Notton, 1984; Nakagawa <u>et</u> <u>al</u>., 1984, 1985; Harker <u>et</u> <u>al</u>., 1986; Oji <u>et</u> al., 1988). The purification of NR has allowed for the biochemical characterization of different isozymes of NR, both within and between species, and the development of NRspecific antibodies. With antibodies it has been possible to study the regulation of the enzyme at the protein level

and more recently to identify cDNA clones of the enzyme. These topics will be discussed in more detail in later sections.

ii) Types of nitrate reductase

When Evans and Nason made their discovery in 1953, they described a bispecific NR in soybean leaves which could use either NADH or NADPH as reductant. Subsequently, two other isozymes of NR were found to be present in soybean leaves, an NADH:NR which uses only NADH as reductant and a second bispecific isozyme which differs from the other two forms in that its activity is constitutively expressed and it has a different pH optimum (Jolly <u>et al</u>., 1976; Dean and Harper, 1988). Dean and Harper (1988) have suggested that the NAD(P)H:NR constitutive enzyme is involved in the production of nitric oxide and nitrogen dioxide in the leaflets of soybean plants. However, the role of constitutive forms of NR in the reduction of nitrate or in other functions is still uncertain.

Since the discovery of the first soybean NR it has been found that NADH:NR is the most common form of NR in higher plants. Some plants have been identified which appear to contain only NADH:NR such as <u>Vicia faba</u> (Hewitt and Notton, 1980) and <u>Nicotiana plumbagnifolia</u> (Gabard <u>et</u> al., 1987) or which contain only NAD(P)H:NR for example, Erythrina senegalensis (Stewart and Orebamjo, 1979). Some crop plants appear to contain both NADH:NR and NAD(P)H:NR (Beevers and Hageman, 1980; Srivastava, 1980; Guerrero et al., 1981; Campbell and Smarelli, 1986). When NAD(P)H:NR has been identified in a particular crop plant organ, NADH:NR has also been found in the same organ. Other parts of the same plant however, may contain only the NADH:NR form. For example, in maize, biochemical data suggested that both the scutellum and roots contain distinct isozymes of NADH:NR and NAD(P)H:NR. Only NADH:NRA has been measured in the leaves (Redinbaugh and Campbell, 1981; Campbell, 1988). However, Sorger et al. (1986), using lines of maize which expressed varied levels of NADH:NRA and NAD(P)H:NRA were able to show through crosses that the ratio of NADH:NR in the leaf and scutellum remained constant but the ratio of the leaf NADH:NR to the NAD(P)H:NR in the scutellum varied, suggesting a genetic link between the two NADH:NRs that did not exist with the NAD(P)H:NR. A possible genetic link was also found for the NAD(P)H:NRs in the root and in the scutellum as lines with low levels of NAD(P)H:NR in one of the tissues also had low levels in the other tissue.

Barley is an interesting case because, like maize, only NADH:NR is measurable in the leaves of wild type

plants. However, if the structural gene (nar1) for this enzyme is mutated such that the gene is no longer expressed (nar1a mutant), NAD(P)H:NRA is measurable in its place and is sufficient to let the plants grow on nitrate as the only nitrogen source (Dailey et al., 1982; Harker et al., 1986). In the roots of wild type plants, both NADH:NRA and NAD(P)H:NRA are measurable. Using genetic crosses of plants mutated in either the nar1 locus or plants which upon mutation did not express NAD(P)H:NR, it was possible to identify the nar7 locus which appears to be the structural gene for the NAD(P)H:NR (Warner <u>et al.</u>, 1987). This locus does not appear to be linked to the nar1 locus. The NADH:NR and the NAD(P)H:NR found in the roots and leaves in either the wild type or the mutants appear to come from these two loci (Dailey et al., 1982; Warner et al., 1987). It is still not known why the NAD(P)H:NR is expressed in leaf tissues when the NADH:NR isozyme is not, or how this differential expression is controlled.

b) <u>Regulation</u>

i) Regulation by nitrate

The effect of nitrate on NR in higher plants has been well documented (Beevers and Hageman, 1969; Filner <u>et</u> <u>al.</u>, 1969). Zielke and Filner (1971), used density
labelling techniques to show that the appearance of NRA in cultured tobacco cells was due to <u>de novo</u> synthesis of the NR protein. When nitrate is removed from plants, NRA declines rapidly (Oaks <u>et al.</u>, 1972; Aslam and Oaks, 1976). With the production of NR-specific antibodies to this enzyme, it has been possible to show that this induction of activity upon the addition of nitrate and the decline upon its removal occurs at the level of the NR protein (NRP), as measured by the appearance and disappearance of NR crossreacting material (NR CRM). Nitrate reductase activity in higher plants is therefore regulated at least in part by the synthesis and degradation of NRP (Zielke and Filner, 1971; Somers <u>et al.</u>, 1983; Remmler and Campbell, 1986).

The isolation of antibodies to NR has made it possible to obtain cDNA clones of NR (Cheng <u>et al.</u>, 1986; Crawford <u>et al.</u>, 1986; Calza <u>et al.</u>, 1987). Using these clones it has been possible to show that nitrate has an effect at the level of transcription. Cheng <u>et al.</u> (1986) showed that the addition of nitrate to barley caused an increase in NRA, NRP and translatable NR mRNA. This was supported by the work of Crawford <u>et al.</u> (1986) who showed an increase in NR mRNA upon the addition of nitrate to squash cotyledons, which coincided with the appearance of NRA and NRP in the organ. These results have been repeated

using other species such as tobacco (Calza <u>et al</u>., 1987), tomato (Galangau <u>et al</u>., 1988) and maize (Gowri and Campbell, 1989).

ii) Regulation by light

The regulation of NR by light is another area which has received much attention. Jones and Sheard (1972) first demonstrated that NRA could be induced by phytochrome in pea seedlings. Phytochrome has since been shown to elicit a reponse in a variety of other species (for a review see Duke and Duke, 1984). This response, however, is seen only with etiolated tissue as demonstrated by Melzer et al. (1989). Red and blue light were found to cause an increase in the level of NR mRNA in etiolated barley seedlings. Alternatively, if they grew seedlings in the light and then transferred them to dark and added nitrate to the system, low levels of NRA and NR mRNA could be detected. If the seedlings were subsequently exposed to white light, an increase in both NRA and NR mRNA was observed, however, red, far red or blue light had no effect. In green tissues, light which is not acting through phytochrome is having an effect at some other level, for example reductant supply, nitrate uptake or the translocation of nitrate (Duke and Duke, 1984; Melzer <u>et</u> <u>al</u>., 1989; Rufty <u>et</u> <u>al</u>., 1989). The

expression or enhancement of NRA by any form of light is dependant on the presence of nitrate in the system (Sharma and Sopory, 1984). Light has also been implicated in the regulation of NR through a circadian rhythm (Galangau <u>et</u> <u>al</u>., 1988). In tomato and tobacco, NRA, NRP and NR mRNA fluctuate, throughout the light/dark cycle of the plant, though not in parallel. The levels of these parameters increase before the onset of light and decrease during the light period. The involvement of light in this process could be due to a role in 'setting' the circadian rhythm or in controlling the reductant or nitrate supply.

iii) Regulation by other factors

The expression of NR appears to be regulated by a variety of other environmental and internal factors such as nutrient conditions of the plant and seedling age (Beevers and Hageman, 1980). The effect of ammonium on NR has been investigated, but the results have not always been simple to interpret. In plant cell cultures, the addition of ammonium to the medium usually results in increased NRA (Beevers and Hageman, 1980; Srivastava, 1980). In whole plants, this has generally been found to be the case, with a few exceptions (Mohanty and Fletcher, 1976; Oaks <u>et al</u>., 1977; Oaks, 1979; Mengel <u>et al</u>., 1983; Rajeskhar and Mohr, 1986). For

example, in maize shoots the addition of ammonium alone did not lead to the appearance of either NRP or NRA but when added in addition to nitrate, Oaks and Long (1990) found NRA and NRP levels to be the same as with nitrate alone. Remmler and Campbell (1986) and Oaks et al. (1977) found that the addition of ammonium and nitrate to maize seedlings led to an enhancement of NRA and NRP in the shoots over levels attained with nitrate alone. These results could be influenced , however, by seedling age, pH and composition of the medium, growth conditions and the actual levels of ammonium and nitrate used (Radin, 1975; Oaks et al., 1977; Beevers and Hageman, 1980). Radin (1975), using cotton roots, found that if plants were fed 100mM nitrate, then concentrations of 3mM ammonium or less did not affect the NRA of the tissue. However, if only 3mM nitrate was fed to the plants, then any amount of ammonium added to the system caused an inhibition of NRA. Even within an organ, ammonium has been found to have different effects. In maize roots. the addition of both nitrate and ammonium inhibited NRA in the mature portion of the roots, but caused an increase in the root tips. The ammonium did not appear to have an effect on the levels of nitrate within the roots, as compared to levels in nitrate grown roots, but an increase in ammonium was found in both segments of the roots (Oaks et

<u>al</u>., 1977). The difficulties found in studying the effects of ammonium on NR may be due, at least in part, to problems encountered in controlling the levels of nitrate and ammonium, particularily in leaf tissues.

Another component which has been studied for its ability to regulate NR is glutamine, an end product of the nitrate assimilatory pathway. Much of this research has been done with tissue cultures as it is easier to control the concentration and form of the nutrients added to the cells. They also provide a more uniform system for study. Oaks (1974) found that if cells were grown with 20mM glutamine very little NRA was detected following transfer to media containing 25mM nitrate and 10mM glutamine. This suggests that glutamine was repressing the initial induction of NRA by nitrate. When Nelson et al. (1984) grew soybean cotyledon cultures with glutamine as the sole nitrogen source and then transferred them to 25mM nitrate and 2mM ammonium, the NRA increased dramatically. The level of NRA reached was not affected by the addition of 10mM glutamine to the nitrogen sources as in the previous example. Curtis and Smarrelli (1987) also using soybean callus or suspension cells found again that cells grown on glutamine and then transferred to nitrate and glutamine had increased NRA. When they grew the cells on nitrate, however, and then

transferred the cells to a media which contained glutamine, a decrease in NRA was observed. These results were supported by a study by Langendorfer <u>et al</u>. (1988) using whole squash plants. In addition, glutamine appeared to reduce NRA by decreasing both the steady-state levels of NRP and the concentration of nitrate within the plant cells. Oaks <u>et al</u>. (1977) also found that glutamine reduced NRA and the concentration of nitrate in maize roots suggesting that the action of glutamine in the regulation of NR is complex.

c) <u>Identification of structural and genetic components</u> <u>involved in the regulation and synthesis of nitrate</u> <u>reductase</u>.

i) Mutant analysis

The first approach taken to determine the loci involved in the regulation of NR and the synthesis of the apoprotein was to identify mutants. The most popular screening strategy has been to isolate mutants which are unable to convert chlorate (an analogue of nitrate) to chlorite (Oostindier-Braaksma and Feenstra, 1972). The presence of chlorite in the plant is lethal and therefore only chlorate-resistant mutants (or NR-defective) are able to survive the screening. Mutants have been isolated from a variety of species including Nicotiana tabacum (Muller and Grafe, 1978; Evola, 1983a,b), N. plumbagnifolia (Marton et <u>al</u>., 1982a,b; Negrutiu <u>et al</u>., 1983; Gabard <u>et al</u>., 1987), Hordeum vulgare (Tokarev and Shumny, 1977; Kleinhofs et al., 1980,1985; Wray et al., 1985), Petunia hybrida (Steffen and Scheider, 1984), Pisum sativum (Feenstra and Jacobsen, 1980; Warner et al., 1982) and Glycine max (Streit et al., 1985,1987). These mutants are either defective in the apoprotein of NR or are defective in at least one of six genes involved in the synthesis of the molybdenum cofactor. The identification of these mutants and the isolation of both cDNA and genomic clones for NR from a variety of species by recombinant DNA techniques (Cheng et al., 1986, 1988; Crawford et al., 1986; Calza et al., 1987; Daniel-Vedele et al., 1989; Gowri and Campbell, 1989; Vaucheret et al., 1989) has facilitated the study of the structure of NR, the identification of loci within a species and the molecular regulation of NR.

ii) Elucidation of the structure of NR

Through the use of amino acid sequences derived from the DNA sequences of NR clones, mutant analysis and limited action proteolysis, it has been possible to delineate the domains of NR. Nitrate reductase is a multicentre, redox enzyme which contains three cofactors. These are a molybdenum cofactor, flavin adenine dinucleotide (FAD) and cytochrome b5 each of which delineates a functional domain separated from each other by a hinge region (Figure 2). The functional domains have been defined by comparison of the NR amino acid sequences to sequences of proteins of known function. The heme binding region showed homology to the cytochrome b5 superfamily (Le and Lederer, 1983; Calza et al., 1987), the molybdenum-pterin-binding domain was found to be homologous to the domain of similar function of rat liver sulfite oxidase and the FAD/NADH domain showed homology to human erythrocyte cytochrome b5 reductase (Calza et al., 1987; Crawford et al., 1988; Daniel-Vedele et al., 1989; Vaucheret et al., 1989). All of the NRs sequenced thus far show a high degree of homology at the amino acid level, particularily in the regions of the functional domains, suggesting that NR is a conserved protein (Vaucheret <u>et al</u>., 1989).

The hinge regions have been found to contain sites which are senstive to proteolytic cleavage. It has been suggested that the cleavage of NR by specific proteinases may be important in the regulation of NRA <u>in vivo</u> (Guerrero <u>et al.</u>, 1981). The hinge regions and the N-terminus of the protein are the least conserved and the most hydrophillic

Figure 2. Structural model of higher plant nitrate reductase as developed by Solomonson <u>et al</u>. (1986) and Kubo <u>et al</u>. (1988) (figure taken from Caboche and Rouze, 1990).



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portions of NR (Campbell and Kinghorn, 1990). If NR is digested with a limited action proteinase the ability of NR to reduce nitrate by using NADH is lost. Two fragments are generated each of which contains partial activities as shown in Figure 2. One of the fragments contains the molybdenumpterin and can use artificial electron donors to reduce nitrate, suggesting that this is the region of the protein which is active in reducing nitrate. The second fragment contains the FAD portion of the protein and is capable of using NADH to reduce ferricyanide, suggesting that this is the part of the protein which oxidizes the NADH (Kubo <u>et</u> <u>al.</u>, 1988; Solomonson and Barber, 1989).

These observations have been supported by the study of NR mutants (for review see Caboche and Rouze, 1990). Within the structural gene for NR at least four complementatic: groups have been identified. The particular mutation in NR determines how many of the partial activities of the enzyme can still function. A study was carried out with twenty-two <u>N</u>. <u>plumbagnifolia</u> mutants which determined that complementation only occured <u>in vivo</u> between mutants from different complementation groups i.e. the mutations were in different domains of the protein. This was also demonstrated <u>in vitro</u> by the addition of a protein extract from one mutait to the extract from another. The ability of

mutations to complement each other in this manner suggest that electrons can be transferred between functional domains of different subunits on either the same or different molecules (Caboche and Rouze, 1990).

iii) Identification of genes which code for NR

The identification of loci for NR by the analysis of mutants together with recombinant DNA techniques is making it possible to identify the number of genes or isozymes within a single species or between species. The two systems which have best characterized in this respect are tobacco and barley. In N. plumbagnifolia, a diploid species, only one locus was identified by mutational analysis (Gabard et al., 1987). In N. tabacum, an allotetraploid plant with genomes derived from N. sylvestris and N. tomentosiformis, two unlinked loci were identified, each of which is assumed to have come from the two progenitor species. However there does not appear to be any heterogeneity of the NR in \underline{N} . tabacum as determined by isoelectric focusing (Muller, 1983). That only one of the NR genes is being expressed has been confirmed by using the cDNA clone isolated by Calza et al. (1987). In barley, as discussed earlier, there appears to be one locus for the NADH:NR gene, nar1, as demonstrated by genomic crosses (Melzer et al., 1989 and references

therein). The NAD(P)H:NR isozyme appears to be linked to the nar7 locus, a locus which is not linked to nar1 (Warner <u>et al.</u>, 1987). This gene has now been cloned (A. Kleinhofs, personal communication). Cheng <u>et al</u>. (1988) has identified two genomic clones from <u>Arabadopsis thaliana</u> but the expression of these two genes has not been reported in detail.

iv) Molecular regulation of NR

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It might have been expected that mutants would have been discovered in higher plants which had defects in the post-translational processing of NR or in its regulation, but this has not been the case. It may be that mutations in the post-translational processing of NR are lethal. The insertion of the cofactors into NR is probably common to many enzymes and disruption of this process could therefore, have many effects (Wray, 1988). The lack of regulatory mutants is somewhat surprising as it has been possible to find regulatory mutants in both Neurospora and Aspergillus (for reviews see Cove, 1979; Marzluf, 1981; Marzluf and Fu, 1990). In <u>Neurospora</u> crassa, for example, using both mutational analysis and recombinant DNA techniques it has been possible to identify many of the genes involved in the synthesis and regulation of NR and NiR. In Neurospora, the

apoproteins for NR and NiR are synthesized by nit-3 and nit-6 respectively. The genes which have a role in the synthesis and assembly of the molybdenum cofactor are called nit-1, nit-7, nit-8 and nit-9 (Tomsett and Garett, 1980; Marzluf, 1981). The genes which carry out these functions have also been identified in higher plants as described In Neurospora, however, the regulatory genes nitearlier. 2, nit-4 and nmr have been discovered and at least partially characterized by Marzluf and Fu (1990). The genes nit-2 and nit-4 are thought to encode regulatory proteins which act in a positive manner for control of the NR and NiR structural The gene nit-2, appears to be a major regulatory genes. enzyme which is required for the synthesis of many of the structural genes involved in nitrogen metabolism (Stewart and Vollmer, 1986; Fu and Marzluf, 1987). The gene nit-4 appears to be specific for NR and NiR and is thought to have a role in the induction of these two enzymes by nitrate (Marzluf, 1977, 1981). A mutation in either leads to the absence of either NR or NiR (Marzluf, 1981). The third regulatory gene identified, nmr, appears to act in a negative manner and is thought to be important in nitrogen repression (Dunn-Coleman et al., 1981; Debusk and Ogilvie, 1984). In <u>Neurospora</u>, glutamine is responsible for nitrogen repression (Premakumar et al., 1979; 1980). The synthesis

of NR and NiR requires induction by nitrate together with nitrogen derepression. It has been proposed that glutamine might act by binding to one of the regulatory proteins such as the *nit-2* or *nmr* products.

The regulation of *nit-3* at the level of transcription was demonstrated by the translation of mRNA which had accumulated in vitro in experiments where the synthesis of the enzyme was blocked (Premakumar et al., 1979, 1980) and subsequently by RNA blot hybridizations probed with a cDNA clone of nit-3 (Fu and Marzluf, 1987). No nit-3 mRNA was detectable in wild type cells which had not been induced with nitrate or were subjected to nitrogen repression. When the cells were derepressed and nitrate added, high levels of nit-3 mRNA were measurable. When nit-2 or nit-4 mutants were tested, nit-3 mRNA was not found under any conditions, suggesting that these two genes act at the level of transcription (Marzluf and Fu, 1990). In nmr mutant strains, nit-3 mRNA was found to be expressed to a small extent under conditions of nitrogen repression. This suggests that the nmr gene product acts in a negative manner (Marzluf and Fu, 1990).

One of the areas of interest in the studies of fungi genetics has been to determine if NR has an autoregulatory role. Strains have been found which are mutated in the *nit*- 3 gene that express nit-3 mRNA constitutively whether nitrate has been added to the system or not. This suggests that NRP is involved in the regulation of nit-3 at the level of transcription. It was proposed that NRP might bind with the nit-4 gene product and thereby block the activation of the nit-3 and nit-6 genes. With the recent cloning of the nit-4 gene it will be possible to investigate this proposal (Marzluf and Fu, 1990).

Analagous mutants have not yet been found in higher plants. With the isolation of genomic clones, for example in <u>Arabadopsis</u> as isolated by Cheng <u>et al</u>. (1988), it should be possible to identify cis-acting regulatory DNA sequences in the 5' upstream portion of the gene by using gene transfer systems. The isolation of the trans-acting regulatory proteins may prove to be a somewhat more difficult task, based on the experience with mutational analysis studies.

d) Localization

i) Localization of NR

It is generally believed that in higher plant cells, NR is localized in the cytoplasm. Using sucrose density gradients to try to biochemically separate organelles from the cytosol, NR was found to be localized in the cytosolic fractions of leaves and roots of a variety of genera (Grant et al., 1970; Dalling et al., 1972; Oaks and Gadal, 1979; Suzuki et al., 1981). Miflin (1970), using similar techniques found barley root NRA to migrate with NiRA in a particulate fraction of the separation. This result, however, was later shown to be an artifact of bacterial contamination (Blevins et al., 1976). More recently with the development of antibodies to NR, immunocytochemical techniques have been used to investigate the question of localization. The use of these techniques should eliminate the artifacts encountered in biochemical studies associated with the breakage of cells. Roldan et al. (1982) found NR in the tonoplast membranes and in the cell wall-plasmalemma region of <u>Neurospora</u>. This group suggested that in Neurospora, NR may have a function in the absorption of nitrate. In green algae, NR was labeled on the pyrenoid region of the chloroplast (Lopez-Ruiz et al., 1985a,b). In higher plants, Vaughn et al. (1984) labeled NR predominantly in the cytoplasm of norflurazon treated squash cotyledons, however, there was a small amount found associated with organelles tentatively identified as plastids. A nontreated sample was not included in their study as a control. Although norflurazon caused a super-induction of NRA in their system, it is not known how this induction occurs or

what effect it might have on the localization of the NR thereby making it difficult to interpret these results. It is also interesting to note that when Rajeskhar and Mohr (1986) used one-half the concentration of norflurazon used by Vaughn <u>et al</u>. (1984), on mustard seedlings, their plastids were photooxidatively damaged and bleached, with no NRA detectable. This was supported by the results of Deanne-Drummond and Johnson (1980) who found barley leaves bleached when treated with norflurazon and again did not contain any NRA. Vaughn <u>et al</u>. (1984) did not appear to have any of these bleaching effects in their tissue, however, there may be differences due to the modes of application of the norflurazon and the species studied.

Kamachi <u>et al</u>. (1987), using antibody made against pure spinach NR, found label in the stroma of chloroplasts in spinach leaves. However, an important control, an uninduced sample i.e. tissue which had not been exposed to nitrate, was not included in this study. The most recent study in higher plants was done by Vaughn and Campbell (1988) who found NR in the cytoplasm of mesophyll cells of maize leaf tissue. This agreed with the results of Harel <u>et</u> <u>al</u>. (1977) who found NR only in the mesophyll cells of maize leaves upon the physical separation of the bundle sheath and mesophyll cells. The differences in these results could be

due to a variety of factors. These studies have been done on different species, of different physiological ages which have been grown under a variety of regimes. Any of these parameters could influence the types and distribution of NR within the plant (Solomonson and Barber, 1990). The most likely source of differences between the results, however, is the use of antibodies in these studies. The purity of an antibody preparation and the conditions used for labeling can significantly affect the results (Solomonson and Barber, 1990). Also, the particular type of antibody being used can also make a difference. Gowri and Campbell (1989) found that their antibody to NR also reacted with NAD(P)⁺:glyceraldehyde-3-phosphate dehydrogenase. Epitopes which react to other plant proteins may be present in a preparation of antibodies made against NR because of the conservation of the functional domains in NR which are similar to other plant proteins (Solomonson and Barber, 1990). It is therefore important to ascertain that the antibody used has a high titer, and a high degree of specificity for NR only.

The localization of NR in the cytosol as originally determined by biochemical studies, is supported by the absence of a transit sequence in cDNA and genomic clones. Nitrate reductase is a nuclear gene which would require such

a sequence to facilitate transport of the NR protein into a plastid (Cheng <u>et al</u>., 1986,1988; Crawford <u>et al</u>., 1986; Calza <u>et al</u>., 1987).

ii) Localization of the reductant supply for NR

The question of where the reductant for NR originates is tied to the question of localization of the enzyme. As the general belief has been that NR is in the cytosol, theories have been proposed to explain the supply of reductant to the cytosol. In photosynthetic tissues, it has been proposed that NADH is provided from either the chloroplast via a malate shuttle (House and Anderson, 1980), the reactions of glycolysis in the cytosol (Mann et al., 1978) or from a malate shuttle from the mitochondria (Woo and Canvin, 1980; Naik and Nicholas, 1986). House and Anderson (1980) found that pea chloroplasts could reduce nitrate to ammonia when NR, NADH and light were added. They could also reduce nitrate if NAD-specific malate dehydrogenase, oxaloacetate, NAD, NR, nitrate and light were added. They suggested that NADH could therefore be supplied to the cytosol by a malate/oxaloacetate shuttle in the light.

Two main theories have been have been proposed to account for the supply of reductant in roots. The first is via the pentose phosphate pathway, present in both the cytosol and the plastids, which would generate NADPH directly into the cytosol from the oxidation of glucose-6phosphate and 6-phosphogluconate. Alternatively mitochondrial malate dehydrogenase could provide NADH to the cytosol via a shuttle mechanism as described above for shoots. Neither of these pathways has been proven to be the correct pathway (for reviews see Lee, 1980; Emes and Bowsher, 1991).

This thesis documents the study of the enzyme nitrate reductase in maize roots. Maize was chosen for this project for two reasons, it is an economically important plant, and it has been the object of many studies on nitrogen metabolism. Very little was known about how nitrate reductase functions in the roots of higher plants. The majority of studies have focused on NR in leaves because of easy access of that organ. Since the roots are the first part of the plant to have contact with nitrate and they are a non-photosynthetic organ, the enzyme(s) might be expected to be under different regulatory controls than in the leaf. In this study NR in maize roots was stabilized with the protease inhibitor chymostatin. Two isozymes of NR were found within this organ and these were identified, purified and biochemically characterized. A partial cDNA clone of root NR was isolated, sequenced and identified as a gene distinct from the gene which codes for NR in maize leaves. The regulation of NR was investigated with respect to light and nitrate. The localization of NRP in cortical root cells was attempted by using immunocytochemical techniques. Finally, the tissue blotting technique was used to examine the localization of the expression of NR in roots.

Chapter 1: Stabilization of Root Nitrate Reductase

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Introduction

Nitrate reductase from a variety of plant species is unstable in vitro (Wallace and Oaks, 1985). This instability has often been attributed to inactivating proteins. Protein inhibitors of NR have been characterized in <u>Neurospora</u> (Sorger et al., 1978), rice cells (Yamaya and Ohira, 1977), soybean leaves (Miller and Huffaker, 1981; Jolly and Tolbert, 1978) and barley leaves (Hamano et al., 1984). In maize roots, a protease referred to as MRP (maize root proteinase), has been found to selectively inactivate NR (Wallace, 1973, 1975; Yamaya <u>et al</u>., 1980a,b). This protease was originally purified by Wallace (1974). It was found to have a molecular weight of 54,000 to 75,000 and to be inhibited by PMSF, suggesting that it is a serine protease. MRP is the main protease in the mature portion of the root (Wallace and Shannon, 1981). It appears to be present but inactive in the root tip (Wallace, 1973) and in leaves (V.J. Goodfellow, personal communication). NR, on the other hand, has been found to be reasonably stable and to have higher recoverable activities in root tips and leaves relative to mature root regions (Oaks <u>et al</u>., 1972; Wallace, 1973; Aslam and Oaks, 1976; Wallace and Shannon,

1981; Nakagawa <u>et al.</u>, 1984). MRP is stable over a greater pH and temperature range than is NR. Thus the low levels of extractable NR activity and the apparent short half-life of NR in mature regions of the maize root (Oaks <u>et al.</u>, 1972) could be an artifact of MRP activity. It could account for the <u>in vitro</u> instability of NR in maize roots.

A variety of methods have been used to try to stabilize maize root NR <u>in</u> <u>vitro</u> (Wallace, 1974, 1975). In particular, the addition of PMSF and/or casein (3%) to the extraction buffer has been found to confer some stability to the enzyme. We have identified a protease inhibitor, chymostatin, which effectively stabilizes NR in maize roots. Chymostatin is a low molecular weight compound which has been isolated from culture filtrates of Streptomyces species (Umezawa et al., 1970; Tatsuta et al., 1973; Umezawa, 1976). It has been found to inhibit many serine proteases, including chymotrypsin, and a few cysteine proteases. In our extracts prepared with chymostatin, higher levels of NR activity are found in mature root regions than in root tips. An NAD(P)H bispecific form of NR is found to be dominant in the mature regions of the root, whereas an NADH requiring NR is the major form in the root tip.

Materials and Methods

Growth Conditions of Plants

a) Agar grown plants

Maize kernels (Zea mays cv W64A x W182E) were germinated in petri plates (15cm diam.) containing 1% (w/v) agar made up in 1/10 Hoagland's solution modified to contain 10mM KNO₃. The plates were placed in a growth chamber without a light supplement for 48 hours at 28°C. The 3cm long roots were dried with paper towels and cut into 2 segments, a 1cm root tip segment and a mature segment consisting of the remaining 2cm next to the kernel. These segments were frozen in liquid N₂, ground to a fine powder with a mortar and pestle and kept at -70° C for up to 3 days. b) Hydroponically grown plants

Plants were germinated in the same manner as described above with the exception that KNO_3 was omitted from the agar. The seedlings were then transferred to aerated hydroponic boxes (10x20x8cm) which contained 1L of 1/10 Hoagland's solution. Plants were grown with either continuous light or with a 16 hour light/ 8 hour dark cycle at 28°C. A supplement of 10mM KNO₃ was added where required. Plants were harvested as described above but roots were cut into three consecutive segments, a 1cm tip, a segment consisting of the adjacent 2cm of the root and a third segment that began 3cm from the tip and included the rest of the root up to the kernel.

Nitrate Reductase Activity Assay

The following is an optimized version of the NR assay. All assays were performed in this manner unless otherwise stated. Tissue samples were extracted at 4°C with a high pH Tris-HCl buffer developed by Kuo et al. (1982). The extraction buffer included Tris-HCl (25mM, pH 8.5), EDTA (1mM), FAD (20uM), BSA (1% w/v), dithiothreitol (1mM) and cysteine (10mM). Leupeptin (10uM) was added to the buffer used to extract leaf material and chymostatin (10uM dissolved in DMSO) was usually added to extract root material. When comparisons were made of different concentrations of chymostatin, DMSO alone was added to the extraction buffer of the control roots. One gram of frozen powder was ground with 4mL of extraction buffer in a mortar. The extracts were centrifuged at 10,000 x g for 20 min at 4°C, filtered through Miracloth (Calbiochem) and kept on ice until measured. The assay mixture consisted of 0.2mL Hepes buffer (0.65M, pH 7.0), 0.2mL KNO3 (0.1M) and 0.2mL extract.

When NADPH activity was to be measured 0.1mL OAA (2.64mg/mL H_2O) was also added to the reaction mixture. Water was added to bring the volume of the mixture up to 1.4mL. The reaction was started with the addition of 0.1mL NADH (3.6mg/mL) and/or 0.1mL NADPH (4.2mg/mL) both of which were made up in 0.04M KPO₄, pH 7.0. The mixture was incubated at 28°C for 20 min. The reaction was stopped with the addition of 0.1mL alcohol dehydrogenase (0.5mg/mL 0.1M KPO4, pH 7.0) and 0.1mL 2% (v/v) acetaldehyde. After 2 mins, 1mL of 1% (w/v) sulfanilamide in 1N HCL and 1mL of 0.01% (w/v) NED in water were added to produce a colour reaction with the NO₂⁻ produced by the assay. After 30 mins the samples were measured spectrophotometrically at 540nm. One unit of NR is defined here as the amount of NR which catalyzes the formation of 1umole of NO_2^- per hour. One unit of MRP is equivalent to the amount of MRP required to inactivate 1 unit of NR per hour.

Results

Storage Effects on Nitrate Reductase Activity in Maize Roots

The first problem to be addressed in the stabilization of maize root NR was that of storage of the roots. It was discovered early on in this project that maize roots, ground or intact, could not be stored at -70°C for an indefinate period of time and still retain NRA as had been previously found for leaf powders. The results of a test of the effect of storage at -70°C on NRA are shown in Table 1. Because NRA dropped significantly 2 days after harvest, all of the following assays were performed within the first two days of harvest.

Development of an Extraction Buffer to Stabilize Root NR in vitro

High levels of stable NRA from maize leaves can be extracted using the high pH buffer developed by Kuo <u>et al</u>. (1982). This buffer was used as a starting point for developing a buffer for extracting NR from maize roots. In the first series of experiments protein concentration and type were altered, with or without the addition of PMSF to the buffer. Both BSA and casein have been found to be Table 1. Effect of Storage on the NADH:NRA of Maize Roots.

No. of days after freezing	Relative act: 1cm	ivity (%) / : 2cm	root segment 3cm
0	100.0ª	10.4	15.5
1	117.2	7.5	14.7
· 2	74.5	0.7	0.0
-	60.1	2.5	0.8

Root segments were frozen in liquid N_2 , ground to a fine powder and stored at -70°C for the appropriate length of time. The extraction buffer contained 5mM PMSF in addition to the components described in "Materials and Methods". For each gram of frozen powder, 1mL of extraction buffer was used.

* 100.0(%) = 652nmoles NO₂ produced/h/GFW

effective at stabilizing NR in extracts (Schrader <u>et al</u>., 1974; Wallace, 1975; Kuo <u>et al</u>., 1982). PMSF is a commonly used protease inhibitor and was already known to inhibit the action of MRP (Shannon and Wallace, 1979). The results of this experiment are shown in Table 2. An addition of 1% BSA without PMSF to the buffer gave the highest activity.

The next part of the extraction procedure to be altered was the ratio of frozen root powder to extraction buffer (Table 3). Though a ratio of 1/5 conferred the highest activity, a ratio of 1/4 was chosen for its higher reproducibility of results.

Stabilization of Root Nitrate Reductase Activity with Chymostatin and Altered Growth Conditions

Maize was grown under two different conditions. The first, which had been used in all of the previous experiments, was on agar plates containing 10mM KNO₃ for 48 hours without a light supplement. At this age the roots were approximately 3cm long. When the roots were cut into segments and assayed for NR activity, it was found that the majority of the extractable activity was in the root tip (Table 4). This activity appeared to be predominantly from an NADH monospecific form of NR. A small amount of NAD(P)H bispecific activity was also present. Table 2. Effect of the Addition of Exogenous Protein and PMSF to Extraction Buffer on NADH:NRA in Maize Roots.

Relative NRA (%)/ Additions to the extraction buffer segment of root Protein PMSF 1cm 2cm 3cm 1% BSA 100.0 5.7 5.4 1% BSA 68.7 ÷ 5.0 1.8 1% BSA (propan-2-ol)^a + 52.0 1.4 0.0 2% BSA 77.7 5.1 1.1 ---3% BSA 58.7 5.4 7.7 + 3.2 3% BSA 60.7 3.0 ----3% casein 52.5 0.0 0.0 + 3% casein 38.0 2.8 1.1 no protein added 59.6 0.0 0.0 + no protein added -88.9 3.0 3.0

This table is a compilation of sets of experimental data, each of which contained a treatment of 1% BSA and no PMSF. This treatment was made equal to 100.0% in each experiment so that the experiments could be compared. The extractions were performed with 1mL of extraction buffer for each gram of frozen powder.

^a Propan-2-ol was used to dissolve the PMSF in this treatment only. Methanol was used in all other treatments.

Table 3. Effect of Changing the Ratio of Frozen Root Powder to Extraction Buffer on NRA.

Frozen root powder	Extraction buffer	Amount BSA in buffer	Relative	NRA/Segment of root	
(g)	(mL)	(१)	1 cm	2cm	Зст
1	1	1	74.8	3.9	2.9
1	2	1	83.6	6.5	4.8
1	3	1	92.9	3.9	8.4
1	4	1	160.0	5.1	8.4
1	5	1	110.7	7.6	10.0
1	3	3	98.0	4.9	3.7
1	4	3	80.0	1.1	3.5

This table represents 3 sets of data, each of which contained a 1/4 ratio of powder to buffer. In each set the 1/4 result was set equal to 100.0 so that the experiments could be compared. Table 4. Stabilization of Root NR with Chymostatin.

Treatment	NRA	$(\mu moles NO_2^{-})$	produced/h/GFW)
	1cm	2-3cm	upper portion
Agar grown	plants		
NADH	1.26 ±0.01	0.17 ±0.03	
NADPH	0.26 ±0.02	0.03 ±0.01	
NADH+NADPH	1.29 ±0.01	0.09 ±0.04	

Hydroponically grown plants

NADH	2.42	$\pm 0.07(22)^{a}$	2.08	±0.04(28)	2.13	±0.15(15)
NADPH	0.90	±0.02(15)	2.08	±0.06(30)	2.55	±0.12(23)
NADH+NADPH	2.04	±0.03 (3)	2.31	±0.08(30)	2.59	±0.01(23)

Hydroponically grown plants + 10µM chymostatin

NADH 1.96 ± 0.01 (4) 3.08 ± 0.06 (5) 4.26 ± 0.10 (0) NADPH 1.17 ± 0 (46) 2.62 ± 0.32 (7) 4.25 $\pm 0.10(16)$ NADH+NADPH 1.72 ± 0.10 (1) 3.44 ± 0.02 (1) 4.58 $\pm 0.27(10)$ Plants were grown with 10mM KNO₃, harvested and assayed as described in "Materials and Methods". The initial activities are represented in the first column under each root section heading.

^a The values in parentheses represent the percent loss of activity after 2 h on ice.

Alternatively, the kernels were germinated on agar plates without a light supplement in the absence of KNO₃. After 48 hours seedlings were transferred to an aerated hydroponic system containing 1/10 Hoagland's solution and 10mM KNO₃. This system was placed in continuous light for a 24 hour period before the plants were harvested. When NR activity (NRA) was measured in these roots, it was found that the NADH:NRA was very similar in all segments of the root. Also, the NRA measured in the root tips of the hydroponically grown plants was approximately double the activity found in root tips of the agar grown plants (Table 4). Higher levels of NADPH:NRA were found in the mature regions of the root relative to the root tip region. Because the NADH and NADPH:NRAs were not additive, the NADPH:NRA in the mature root segments is likely to be from a bispecific form of the enzyme.

A number of different protease inhibitors were tested in our system (Table 5 and Table 6). One of these, chymostatin, greatly improved the stability of the NR obtained from the mature regions of the root (Table 4 and Table 6). When this inhibitor was used, NR activity was found to be higher in mature root segments than in root tips. When the extracts with chymostatin were left on ice for 2 hours essentially all of the activity was recovered.

Table 5. Effect of Protease Inhibitors on NRA in Root Extracts of Agar Grown Plants.

Additions to buffer	N	RA
	1cm	2-3cm
no additions	100.0 ^ª	8.9
10mM ε aminocaproic acid	74.1	22.7
100mM ε aminocaproic acid	88.2	18.8
5mM o-phenathroline ^{bc}	54.5	1.8
0.1mM ethylmalemide ^b	95.0	6.3
10μM leupeptin	73.1	10.2
0.25% soybean trypsin inhibitor ^b	65.0	9.9
0.1mM p-chloromercuribenzoic acid ^{bc}	92.6	8.5
0.1gm PVPP	12.9	2.9
5mM EDTA	93.6	10.2

^a 100.0 is equivalent to 2.16 μ moles NO₂⁻ produced/h/GFW. ^{b,c} The concentration of these inhibitors was obtained from ^b Hamano <u>et al</u>. (1983) or from ^c Wallace (1974).
Table 6. Effect of Protease Inhibitors on NADH:NRA in Extracts of Hydroponically Grown Roots.

Additions to buffer NRA 1 cm 2-3cm 100.0^a no additions 108.9 0.5mM N-BA^{bc} (α -N-benzoyl-L-arginine) 90.6 105.4 97.4 10.0mM N-BA 119.4 0.05mM TLCK^{bc} (N-a-p-tosyl-L-lysine 72.0 117.3 chloromethyl ketone) 1.0mM TLCK 109.3 123.7 2.5mM iodoacetamide^b 69.8 46.5 90.9 0.5mg/mL chymostatin 166.8 2.7TIU/mL aprotinin 91.6 101.9 0.4mM p-NH₂-PMSF (p-amidinophenyl-85.3 104.0 methyl-sulfonyl flouride) 0.2mM PPACK (D-phenylalanyl-L-prolyl-95.9 87.0 L-arginine) 78.4 79.3 1.0mM isatoic anhydride 1mM pepstatin 90.4 76.2 104.2 10.0mM N-carbobenzoxy-L-phenylalanyl-91.2 L-alanine

^a100.0 = 1.744 μ moles NO₂⁻ produced/h/GFW ^{b,c} The concentration of these inhibitors was obtained from ^b Hamano <u>et al</u>. (1983) or from ^c Wallace (1974). Without chymostatin, approximately 25% of the activity was lost over that period (Table 4). Over a longer period of time (Figure 3), it can be seen that the NRA in the extract prepared from root tips is significantly more stable than the extract prepared from mature root segments. Both are stable enough under these conditions that assays can be performed reproducibly in the first few hours after extraction.

The optimal concentration of chymostatin required to stabilize root NRA was determined for the 1cm tip and 2-3cm mature root segment of hydroponically grown plants. The NRA of the 1cm tip assayed without chymostatin was set at 100.0. All other activities are relative to this value. The addition of DMSO alone to the extraction buffer had little effect on NRA (Table 7). Additions of chymostatin had little effect on the NRA recovered from root tip segments. In the mature regions of the root, however, a large enhancement of NRA was seen. The highest activity was recovered when 10µM chymostatin was included in the extraction buffer. Consequently this concentration was used routinely in preparations of root NR.

Using the optimized conditions for growing and extracting NR from maize roots, a re-evaluation of the effect of the major proteases as well as a test of the

Figure 3. Disappearance of maize root NRA *in vitro* over time. Extracts were prepared as described in "Materials and Methods" of induced maize root tips and mature root segments. These extracts were kept on ice and measured at intervals for NRA.

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NRA (µmoles NO_ produced/h/GFW)

Table 7. Concentration of Chymostatin Required to Stabilize NADH:NRA in Maize Roots.

Treatment	Relative NADH:NRA	
	1 cm	2-3cm
Regular buffer	100 ^a	94
+ DMSO alone	95	99
+ 1.0mM chymostatin	110	150
+ 0.1mM chymostatin	100	161
+ 0.01mM chymostatin	107	242
+ 0.005mM chymostatin	127	183

After germination, maize seedlings were grown in a hydroponic system for 24h with 10mM KNO₃. The roots were harvested and assayed as described in "Materials and Methods".

^aThe relative value of 100.0 is equivalent to 1.05 μ moles NO₂-produced/h/GFW.

effect of the antibody to MRP on extracts of leaves and/or roots was performed (Table 8). Leupeptin was found to increase activity only in maize leaves while chymostatin again was found to be the most efficient inhibitor for increasing the recovery of NRA in roots though PMSF was more effective under these conditions than it had been in previous trials (Table 2). The addition of the antibody to MRP appeared to have little effect on the recovery of NRA at the concentration used in this experiment.

Inhibition of Maize Root Proteinase (MRP) by Chymostatin

Purified preparations of maize leaf NR and maize root MRP (Yamaya <u>et al</u>., 1980b; Wallace and Shannon, 1981; Poulle <u>et al</u>., 1987) were used to examine the effect of chymostatin on MRP activity. Test tubes were set up containing 0.2mL water, 25µL pure NR, 10µL MRP and 1µL of chymostatin at the required dilution. A range of chymostatin concentrations from 10 nanomoles to 0.01 picomoles were tested. Control tubes contained water, NR and either 10µL MRP and 1µL DMSO or just 1µL DMSO. The tubes were mixed and incubated at 28°C for 15 min. At this time the other components required for the NR assay were added and the tubes incubated for a further 10 min. Colour development reagents were added and the results were Table 8. Effect of Protease Inhibitors and the Antibody to MRP on the Recovery of Nitrate Reductase Activity from Leaves and Roots in Optimized Assays.

Relative NADH:NRA

Treatment	leaf	root tip	mature root
			segments
control	100 ±0(4.67)) ^a 100 ±0(1.34)	100 ±3.43(1.34)
+ leupeptin	121 ±2.81	70 ±4.62	106 ±3.49

+	PMSF		89 ±0.96	150 ±0.29
+	chymostatin	99 ±0	100 ±0.12	170 ±0.34
+	Ab to MRP ^b		99 ±0.23	111 ±0.01

^a The values in brackets represent the actual value of NRA in umoles NO_2^- produced h^{-1} GFW⁻¹.

^b A 1/100 dilution of antibody to MRP was added to the extraction buffer before the tissue was added for extraction.

measured spectrophotometrically. From this experiment it was found that 10 picomoles of chymostatin were required to inactivate 1.5 units of MRP (Table 9). Wallace (1974) required 7.5 millimoles of PMSF to inhibit 1.5 units of MRP. Shannon and Wallace (1979) used the inhibition of MRP by PMSF, a known serine protease inhibitor, to show that MRP was a serine protease. The inhibition of MRP by chymostatin supports this evidence as chymostatin is also an inhibitor of serine proteases.

Test of Removal of NADH and NADPH from the NR Assay Medium with Oxaloacetate or Alcohol Dehydrogenase and Acetaldehyde

There are two potential problems in our NR assay procedure with respect to the pyridine nucleotide reductants that are used to catalyze the NR reaction. The first is that NADPH may be converted to NADH by endogenous phosphatases therefore giving an over-estimation of NADPH:NR activity. This problem can be eliminated by adding oxaloacetic acid (OAA) to the assay mixture before addition of the extract. The addition of this chemical enables endogenous NADH:malate dehydrogenase to remove any NADH formed by endogenous phosphatases. Table 10 shows that OAA removes NADH efficiently from the assay mixture without affecting the NADPH concentration. The second problem

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Table 9. Effect of Chymostatin on MRP Activity.

Amount of chymo	statin N	IRA (μ moles NO ₂	produced/h)
pmoles	NR	NR+MRP	NR+MRP+chymostatin
10	0.53 ±0	0.16 ±0.01	0.51 ±0
1	0.55 ±0	0.19 ±0.01	0.18 ±0.01

Purified preparations of NADH:NR from maize leaves and MRP from maize roots were incubated with or without chymostatin and then assayed for the remaining NRA. DMSO was present in all treatments.

Table 10. The Disappearance of NADH or NADPH with Oxaloacetate or Alcohol Dehydrogenase and Acetaldehyde at 340nm.

Reductant	Absorbance at 340nm			
	Omin	0.5min	1min	4min
NADH	1.15			
+ OAA			0	
NADPH	1.20			
+ OAA			1.20	
NADH	1.76	· · · · · · · · · · · · · · · · · · ·		
+ ADH + Acet.		0.34	0.06	0
NADPH	1.91			
+ ADH + Acet.		1.21	0.65	0

Mixtures were made up as if for a normal NR assay as described in "Materials and Methods" including 0.2mL of a root extract. The absorbance at 340nm was measured after the addition of the reductant and then over a period of time once either the OAA or the ADH and acetaldehyde were added. encountered with NADH and NADPH is that they interfere with the colour reaction used to measure NRA quantitatively on a spectrophotometer. The addition of alcohol dehydrogenase and acetaldehyde solve this problem by oxidizing both pyridine nucleotides as shown in Table 10.

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Discussion

The presence of MRP has made the study of NR in maize roots difficult. Early studies with maize roots showed that NRA was highest in the root tip segments. The mature root (all of the root excluding the 1cm tip) was found to contain very little NRA. In experiments designed to look at the turnover of NR, the enzyme in mature segments had a much higher rate of turnover than the tip enzyme (Oaks et al., 1972; Wallace, 1973; Wallace, 1975; Aslam and Oaks, 1976). Our results with the agar grown roots confirm these earlier findings. The use of a hydroponic system greatly increased NRA throughout the root, particularily in mature root segments. This may have been the result of both an increase in NO3 availability to the plant in a hydroponic system as compared to an agar plate, and the increased age of the plants. Enzyme stability remained a problem in extracts from the mature segments of these roots. The optimization of the extraction conditions and the addition of chymostatin increased the recovered levels of NRA in the mature root, and stabilized the activity over at least a 2 hour period. Although the effect of MRP on NR in vivo is not known, the specific cleavage of NR by MRP in vitro is well

documented (Wallace, 1973,1974,1975; Yamaya <u>et al</u>., 1980a,b; Batt and Wallace, 1983; Solomonson <u>et al</u>., 1984). Thus the inhibition of MRP by chymostatin probably explains the increased stability of NR in root extracts.

Two forms of NR in maize root crude extracts were first identified by Redinbaugh and Campbell (1981). We also appear to have found the same forms, a stable NADH form and a more labile NAD(P)H form. The NADH form of NR was predominant in the root tip whereas the mature portion contained predominantly the bispecific form. The high levels of root NRA suggest the importance of maize roots in nitrate reduction. Pate (1973) suggested that under steady state conditions appproximately one-third of the nitrogen shipped to the shoots from maize roots was reduced. The results of Gojon <u>et al</u>. (1986) using $({}^{15}N)NO_3^{-1}$ agreed with this figure. However during induction they found that the roots were carrying out 70% of the whole plant nitrate Earlier results with maize root extracts (Oaks reduction. et al., 1972; Wallace, 1975) indicated that there were very low levels of NR in maize roots making interpretation of these in vivo results difficult. With the addition of chymostatin, however, it appears that roots do have the capacity to carry out substantial levels of nitrate reduction.

Chapter 2: Purification and Biochemical Characterization of Root Nitrate Reductase

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Introduction

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Nitrate reductase was first shown to be a substrate inducible enzyme by Tang and Wu (1957). Since that time the response of NRA to nitrate has been well documented in higher plants (Beevers and Hageman, 1969; Filner <u>et al.</u>, 1969). Once a plant has been exposed to nitrate there appears to be a characteristic lag period, followed by a period of linear increase and the establishment of steady state in NRA levels of both roots and shoots of maize (Oaks <u>et al.</u>, 1972; Aslam and Oaks, 1976; Remmler and Campbell, 1986). Upon removal of nitrate from the system, the activity declines (Oaks <u>et al.</u>, 1972; Aslam and Oaks, 1976).

There have been conflicting reports in the literature with respect to the effect of ammonium on the expression of NRA (Beevers and Hageman, 1972; Ullrich, 1987). In lower plants, ammonium appears to have an inhibitory effect on NRA (for review see Guerrero <u>et al.</u>, 1981). In higher plants however, an activation of NRA has been more commonly observed than its inhibition (Mohanty and Fletcher, 1976; Oaks <u>et al.</u>, 1977, 1979; Oaks, 1979; Mengel <u>et al.</u>, 1983; Rajeskhar and Mohr, 1986). In general, the in

depth studies which have been done on algae have not been repeated in higher plants.

Nitrate reductase is a complex and labile enzyme. It took approximately thirty years from the time that it was discovered before it was purified to homogeneity. Solomonson (1975) was the first to use an affinity column in the purification of NR. In this instance he used blue dextran-sepharose to purify <u>Chlorella</u> NR to homogeneity. The ability to purify NR to homogeneity has allowed for the separation of different forms of NR from a single species, for example NADH:NR and NAD(P)H:NR forms, and the biochemical characterization of these enzymes (Jolly et al., 1975; Redinbaugh and Campbell, 1981; Harker et al., 1986;). Antibodies have also been made to pure NRs which have facilitated studies at the level of the NR protein (Somers et al., 1983; Crawford et al., 1986, Cheng et al., 1986; Remmler and Campbell, 1986; Oaks et al., 1988) and allowed for the isolation of cDNA clones of NR (Cheng et al., 1986; Crawford <u>et al</u>., 1986; Calza <u>et al</u>., 1987).

Some aspects of nitrate and ammonium regulation of NR in roots will be examined in this chapter. The development of a protocol for the partial purification of maize root NADK:NR and NAD(P)H:NR will be presented and the biochemical characterization of the two enzymes discussed.

Materials and Methods

Growth Conditions of Plants

Maize seedlings were grown hydroponically as described in Chapter 1. Ke als were germinated in petri plates containing 1% agar and 1/10 Hoagland's solution for 2 days in the dark at 28°C and then transferred to hydroponic pots containing 1/10 Hoagland's and the nitrogen supplements specified in the individual experiments. The seedlings were grown in a 16h light/ 8h dark cycle at 28°C until harvested. Shoots were harvested to include all of the plant material above the coleoptile. In these plants, the shoots consisted of leaves ranging from 7-15cm in height dependent on the particular growing conditions. Roots were either harvested whole or cut up into segments. Both organs were frozen, ground in liquid N_2 and stored at -70°C until required.

Extraction and Nitrate Reductase Assays of Plant Organs

The extraction and assay procedures for plants organs are documented in Chapter 1. Chymostatin was used in all root extracts and leupeptin was used in all shoot extracts.

Preparation of Antiserum

Crude antiserum that had been made against purified leaf NADH:NR (Poulle <u>et al</u>., 1987) was used. After the blue Sepharose affinity step the NR was further purified by passage over a DEAE-cellulose column followed by native gel electrophoresis before injection into the rabbits (for details see Poulle <u>et al</u>., 1987). The antiserum was precipitated by 45% saturation with ammonium sulfate at 4° C for 30 min. It was then centrifuged at 10,000 x g for 15 min and the pellet was washed twice with 1.75M ammonium sulfate. The pellet was resuspended in a volume of 10mM KPO₄ (pH 8.0) equivalent to the initial volume of the crude antiserum, and then dialyzed overnight against the same buffer.

<u>Protocol for Partial Purification of Nitrate Reductase from</u> <u>Maize Roots</u>

The following is the optimized version of the protocol that was developed to partially purify nitrate reductase from maize roots.

There are two buffers that are required for this protocol:

- a) Extraction buffer:
- 250mM Tris-HCl pH 8.5
- 1mM EDTA pH 8.5
- 10mM cysteine
- 1mM dithiothreitol
- 20µM flavin adenine dinucleotide
- 1% bovine serum albumin (fraction V)
- 10µM chymostatin
- Note: cysteine should be added just prior to use of this buffer
 - chymostatin should be dissolved in DMSO at a concentration of 2.5mg/mL before addition to this buffer just prior to use
- b) Pellet buffer:
- 250mM Tris-HCl pH 8.5
- 1mM EDTA
- 10µM chymostatin
- 20µM flavin adenine dinucleotide
- 10% glycerol
- 1mM β-mercaptoethanol
- Note: Tris, glycerol and water should all be chilled to 4°C either separately or together a day before required.

- all buffers and extracts should be kept at 4°C throughout the entire purification.

Maize seedlings were grown as described earlier. The plants were kept for two days in hydroponic pots with continuous 10mM KNO₃, after transfer from the agar plates. Whole roots were harvested after 4-6 hours into the light period of the second day. The frozen root powder was kept in the -70° C freezer until extracted, usually only a period of up to 4 days.

Day 1. The frozen root pcwder (50g) was added to 200mL of cold extraction buffer in a Waring blender. The slurry was ground in the blender for 2-3 min until it resembled a smooth paste. This slurry was strained through miracloth (which required some squeezing to remove the liquid) and then spun in Oakridge tubes at 8,740 x g for 20 min in a JA 20 rotor of a Beckman J2-21 centrifuge at 4°C. The supernatant was again passed through miracloth to remove any fine particulate matter. The volume of the extract was measured and adjusted to contain 10% glycerol and 1mM β mercaptoethanol. Ammonium sulfate was added to the extract to bring it up to a concentration of 45% saturation and then left to mix at 4°C for 1 hour. The solution was spun at 14,600 x g for 30 min in Oakridge tubes and the supernatant was discarded. Approximately 12mL of pellet buffer was used to resuspend the ammonium sulfate precipitate. This suspension was transferred to dialysis tubing and dialyzed against 1L of pellet buffer for 2 hours.

During this period fresh blue Sepharose (bed volume of approximately 15mL) was equilibrated with the pellet buffer. Before equilibration with the pellet buffer the blue Sepharose (3.5g) was soaked with water, approximately 200mL for 15 min, and then washed with water (200mL for each gram of Sepharose) as described in the Pharmacia Affinity Chromatography booklet.

After the 2 hour period the extract was removed from the dialysis tubing and mixed with the equilibrated blue Sepharose (remove as much buffer as possible before adding the extract) for 2.5 - 3 hours at 4°C with gentle shaking. The blue Sepharose and extract were then loaded onto a column (10cm x 1.7cm i.d.) and washed with pellet buffer, under the force of gravity, until the column was packed and the column eluent was clear. The column could then be sealed and left at 4°C overnight.

Day 2. The column was connected to a fraction collector and a peristaltic pump running at 15mL/h and collecting 200 drops/ tube (3.5mL). Each tube contained 0.1mL of 1M KNO₃. Pellet buffer (15mL) was first washed through the column, followed by 40mL of 150 μ M NADPH (dissolved in pellet buffer) to elute the first peak of NR. The column was then washed with 20mL of pellet buffer. Forty mL of 100 μ M NADH (in pellet buffer) was run onto the column to elute the second peak of NR. The eluted NRs were used immediately for pH optima, kinetic studies or column chromatography or they were dialyzed overnight against glycerol for later use. The glycerol dialyzed NRs could be kept at -20°C for a period of at least 2 weeks.

MRP Activity Assay

MRP activity was assayed by using 50μ L of a pure NR from maize leaves (Poulle <u>et al.</u>, 1987) as a substrate. An aliquot of 200 μ L from the fraction to be tested for MRP activity was added to the NR and incubated at 28°C for 15 min. A control was also set up with the NR and the same buffer that the tested fraction was in. After the 15 min incubation, 200 μ L of 0.1M KNO₃, 200 μ L of 0.65M HEPES (pH 7.0), and 100 μ L of NADH (3.6mg/mL in 0.04M KPO₄, pH 7.0) were added to the mixtures. These mixtures were then left for a further 15 min at 28°C before 1mL of 1% (w/v) sulfamilamide in 1N HCl and 1mL of 0.01% (w/v) NED in water were added to produce a colour reaction which could be read on the spectrophotometer at 540nm.

Calibration of Sephadex G-150 Column

A 500mL Sephadex G-150 column (100cm x 2.6cm i.d.) was made according to the protocols of the Pharmacia Gel Filtration Handbook. It was equilibrated with pellet buffer (recipe above except that no chymostatin was included in the buffer) for 36 hours. The column was run at 15mL/h and 300 drops were collected per fraction. The following proteins were used to standardize the column. Each mixture of proteins was dissolved in 10mL of pellet buffer.

Mixture 1.	catalase	MW 240,000	10mg/mL
	bovine serum albumin	MW 68,000	10mg/mL
	cytochrome c	MW 12,400	5mg/mL
Mixture 2.	gamma globulin	MW 160,000	10mg/mL
	ovalbumin	MW 45,000	10mg/mL
	cytochrome c	MW 12,400	5mg/mL

The two mixtures were applied to the column separately. Fractions were collected and assayed by using BioRad protein reagent to identify the protein peaks as follows. Aliquots of 50µL were taken from each fraction and added to 300µL of water and 200µL of BioRad reagent. The tubes were well mixed and measured for absorbance at 595nm in the spectrophotometer.

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Results

Inhibition of NR Activity by Antiserum

Antiserum made against maize leaf NADH:NP or preimmune serum was added in a series of dilutions to crude extracts from maize shoots and roots. The results are shown in Figure 4. The shoot extract was used undiluted and at a dilution of 1 part crude extract to 3 parts extraction buffer. Root activities were measured with either NADH or NADPH.

Figure 4 shows that both root and shoot activities are inhibited in a similar manner by the antiserum. This suggests that all of the enzymes, including the NADPH form, have epitopes which are recognized by the antiserum. The preimmune serum had no effect on the NRA of either roots or shoots.

Induction on NRA in Roots and Shoots of Maize with 10mM KNO3

Maize kernels were germinated on agar plates and transferred to a hydroponic system after two days as decribed in "Materials and Methods". The seedlings were grown for a further 4 days in 1/10 Hoagland's without a nitrogen supplement at 28° C with a 16 h light/ 8 h dark Figure 4. Inhibition of maize leaf and root NRA by antiserum made against purified leaf NR. Crude extracts were prepared from 6-day-old shoots or whole roots which had been grown hydroponically with 10mM KNO₃. To 1mL of crude extract 1mL of diluted antiserum was added. The antiserum was brought up to 1mL with 10mM KPO₄, pH 8.0. The extract /antiserum mixture was left for 30 min on ice and then assayed for NRA. Shoot extracts were used both undiluted and at 1/4 strength and measured for NADH:NRA. Root extracts were measured for their activity with either NADH or NADPH. Preimmune serum had no effect on any of the enzyme activities (data not shown). The NRA measurements of each extract after 30 min on ice withouc any antiserum was set to 100.0 and all other activities set relative to it.



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cycle. On the 6th day after germination, the hydroponic medium was adjusted to contain a final concentration of 10mM KNO_3 2 hours after the lights came on. Samples of the plants were harvested just before the addition of KNO_3 and then at 30 min, 1 h, 2 h, 4 h, 10 h, 24 h, 48 h and 72 h after this addition. The results are shown in Figure 5. The 24 h, 48 h and 72 h results are not included. Activities for these three readings were approximately the same as that for 10 hours from the same organ. It can be seen that the roots and shoots show quite different patterns of induction. After a short lag, the shoot NRA increased sharply up to a peak around 4 hours and then began to slowly decline. The root NRA, on the other hand, appeared to increase in a slow, steady manner over the entire period measured. Both NADH and NADPH activities showed similar patterns of induction and reached substantial levels of activity with respect to the peak leaf activity.

Induction of NRA in Maize Roots with Ammonium and Nitrate

The effects of ammonium and nitrate were examined on 3-day-old roots which had been grown hydroponically in 1/10 Hoagland's solution containing a supplement of either 10mM NH₄Cl, 10mM NH₄NO₃ or 10mM KNO₃ for 24 hours with continuous

Figure 5. Time course of induction of NRA in maize roots and shoots with 10mM KNO₃. Maize kernels were germinated on agar plates and transferred to an aerated hydroponic tank with 1/10 Hoagland's solution after two days as described in "Materials and Methods". On the 6th day after planting, shoots above the coleoptile and whole roots of an aliquot of plants were harvested 2 hours into the light period. At this time the medium in the tank was adjusted to contain a final concentration of 10mM KNO₃. Shoots and roots were then sampled at intervals during the next 10 hours.



light. The roots were cut up into a 1cm tip segment, a 2-3cm segment which contained the next 2cm from the tip, and an upper portion which consisted of the rest of the root up to the kernel. With NHACL, the highest activity was in the root tip with decreasing amounts being found towards the kernel (Table 11). When both ammonium and nitrate were applied this same trend of high activity in the tip was found again, but at higher levels of NRA compared to the samples treated with ammonium alone. This trend switched when nitrate alone was added to the plants and the highest level of NRA was now in the mature portion of the root. The influence of ammonium on the regulation of NR in higher plants is still somewhat controversial. It is interesting that the different segments of the root are affected in a different manner by the two nitrogen sources. It appears that ammonium may be inhibiting the induction of NRA in mature root segments.

Effect of Ammonium and Nitrate on Maize Seedlings

In this experiment the effect of ammonium and nitrate was examined on older seedlings. Plants were grown hydroponically for 5 days as described in "Materials and Methods". On the morning of the 7th day after germination, 4 hours into the light period, the growth medium was

Table 11. Effect of Nitrogen Source on NRA in Segments of Maize Roots.

Nitrogen Source	NADH:NRA (μ moles NO ₂ produced/h/GFV		
	1 cm	2-3cm	upper
10mM NH4Cl	0.98 ±0.05	0.26 ±0	0.17 ±.01
10mM NH4NO3	3.02 ±0.05	1.94 ±0.02	1.20 ±0.03
10mM KNO3	1.78 ±0.01	1.83 ±0.04	2.51 ±0

Maize kernels were germinated on agar plates as described in "Materials and Methods" without any nitrogen in the medium. The seedlings were then transferred to hydroponic pots containing 1/10 Hoagland's and the appropriate nitrogen supplement for 24 hours in continuous light before harvest.

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adjusted to contain either $5mM \ KNO_3$, $5mM \ NH_4Cl$ or both. A set of control plants did not receive any nitrogen supplement. After 6 hours of induction the shoots above the coleoptile and the whole roots were harvested.

Both organs appear to respond in a similar manner to the addition of the nitrogen supplements (Table 12). Without nitrogen there was a small amount of activity in the leaves and none detectable in the roots. This may reflect some bacterial action in the media as this was not a sterile system, or may be the result of nitrate supplied via the endosperm (Oaks <u>et al.</u>, 1988). The addition of nitrate to the system caused a 6-8 fold increase in the NRA recovered in both organs. When ammonium was added in addition to the nitrate a slight increase in activity was found. Ammonium alone had only a slight effect. Overall, it does not appear that ammonium has much of an effect on NRA in this system.

<u>In vivo stability of NAD(P)H:NRA in Maize Roots Upon the</u> <u>Removal of Nitrate</u>

Germinated maize seedlings were transferred to a hydroponic system as described in "Materials and Methods". The growth media contained 1/10 Hoagland's solution and 10mM KNO₃. After 24 hours the plants were transferred to hydroponic containers without a nitrogen supplement. Table 12. Effect of Nitrogen Source on the Induction of NRA in the Roots and Shoots of Maize.

NRA (µmoles NO_2^- prcduced/h/GFW) Nitrogen Source Shoot Root NADPH NADH 0.83 ±0.03 0.61 ±0.01 1.46 ±0.03 5mM KNO3 5mM KNO₃ + NH₄Cl 0.95 ±0.01 0.84 ±0.01 1.51 ±0.04 0.11 ±0.01 0.06 ±0 0.19 ±0 5mM NH₄Cl 0.11 ± 0.02 0 No Nitrogen 0

Maize seedlings were germinated on agar plates and transferred to hydroponic pots as described in "Materials and Methods". On the 5th day (4 hours into the light period) the appropriate nitrogen source was added to the medium. Shoots above the coleoptile and whole roots were harvested 6 hours later. A group of plants was harvested into 1cm root tip and upper mature root segments at the time of transfer, and then again at 3 and 6 hours after the transfer. The NADH:NRA and NADPH:NRA levels that were measured are shown in Figure 6. It can be seen that the enzymes in the root tip, particularily the NADPH:NRA, disappear more quickly *in vivo* than those in the mature root. This may suggest a higher level of turnover in the tip, but it could also be due to the dilution of the induced cells with new cells produced during the period after the nitrate was removed.

<u>Partial Purification of NADH:NR and NAD(P)PH:NR from Maize</u> <u>Roots</u>

A method for separating and partially purifiying the two forms of NR in maize was developed by using the protocol for the purification of maize leaf NR developed by Poulle <u>et</u> <u>al</u>. (1987) and the extraction requirements in Chapter 1 as a starting point. The optimized procedure can be found in "Materials and Methods" of this chapter. The initial extraction of the root tissue was done essentially as described in Chapter 1 for crude extracts, except that the concentration of Tris-HCl was increased from 25mM to 250mM. This was done to help protect the enzymes from pH shock when they were precipitated with ammonium sulfate.

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Figure 6. In vivo stability of NR in maize root tip and mature root segments. Seedlings were grown on agar for 48 hours before transfer to a hydroponic system containing 10mM KNO₃ as described in "Materials and Methods". Roots were induced for 24 hours before transfer to a minus KNO₃ hydroponic system. Samples were collected at 0, 3 and 6 hours after the removal of KNO₃ and measured for NADH (0) and NADPH (\bullet) activties. Initial values (in µmoles NO₂⁻ produced/h/GFW) were 1.65 for the root tip NADH:NR, 0.93 for NAD(P)H:NR and 1.30 for the mature root NADH:NR and 1.28 for NAD(P)H:NR.

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Poulle <u>et al</u>. (1987) used leupeptin as a protease inhibitor in this initial step. Chymostatin at a concentration of 10 μ M was used throughout the root purification procedure as an inhibitor. After the initial extraction, aliquots of the extract were adjusted to either 1% BSA or 10% glycerol and 1mM β -Me or to all three and left on ice for 2, 24 or 48 hours. The aliquots were then measured for activity remaining. Based on these results (Table 13), it was decided that the initial extract should be adjusted to 10% glycerol and 1mM β -Me.

A series of ammonium sulfate fractions were tested to determine which would be the most effective at recovering NRA. As can be seen in Table 13 the 45% fraction gave the highest recovery of NRA. When these same fractions (i.e. pellets and supernatants) were tested for the presence of MRP activity, by addition of aliquots of the pellets and supernatants to pure NR and then monitoring the disappearance of NRA (Table 14), most of the MRP was found in the supernatants. As the ammonium sulfate concentration increased, however, more MRP activity was brought down in the pellet. The 45% pellet appeared to contain little MRP activity and may explain why the NR was so much more stable in this fraction.

Table 13. Test of Purification Conditions on the Stability of Nitrate Reductase Activity

Time	elapsed	at	4°C
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Sample	Initial NADH	Reading NADPH	2 h NADH	ours NADPH	24 ho NADH N	ours XADPH	48 hou NADH NA	rs DPH
Relative units								
control extract	1716	1689	2094	1344	744	0	0	0
+1% BSA	2310	1536	1836	960	750	0	0	0
+10% glycerc +β-Me	ol 2232	1560	1950	1278	1230	246	0	0
+10% glycero +β-Me +1%BSA	ol 2166	1659	2022	1278	1140	210	0	0
supernatant 0 - 45% cut	0	0.						~~~~~~
supernatant 0 - 60 % c ut	0	0						
supenatant 0 - 80% cut	0	0						
pellet ^a 0 - 45% cut	766	547	623	332	103	24	40	4
pellet 0 - 60% cut	430	268	379	299				
pellet 0 - 80% cut	263	260	259	194				
after 2 hours of dialysis ^b								
0 - 45% cut			323	252	210	80	105	18
0 - 60% cut			273	164				
0 - 80% cut			205	205				

note: the values in each section, denoted by the dashed lines are relative to the values within that section only. ^{a,b} The pellets were redissolved in pellet buffer as described in "Materials and Methods" and then dialyzed against the same buffer. Table 14. Test for the Presence of MRP Activity in Ammonium Sulfate Pellets and Supernatants

Sample NRA (Relative units)

Control 1106

	Supernatant	Pellet
0 - 45% fraction	236	1059
0 - 60% fraction	195	788
0 - 80% fraction	152	48

Aliquots of the different supernatants and pellets were added to pure NR and incubated for 15 min at 28°C as described in "Materials and Methods". The activity of the NRA remaining after the incubation period was measured. The values can be compared to the control value i.e. pure NR incubated with only buffer, to determine how active the MRP was in a particular sample. The need for dialysis of the ammonium sulfate pellet before mixing with the extract was tested. It appeared (Table 13) that 2 hours of dialysis against pellet buffer has a significant effect on the future stability of the enzyme and was therefore incorporated into the protocol.

Blue Sepharose columns have greatly facilitated the purification of NR (Poulle et al., 1987). The use of this column allowed for effective separation of the two forms of NR in maize roots as shown in Figure 7. By first eluting the column with 150µM NADPH, the NAD(P)H:NR was displaced from the column without any apparent contamination of NADH:NR. The more stable NADH:NR could then be eluted with 100µM NADH again without any detectable cross-contamination from the other form. The recoveries using the optimized protocol are shown in Table 15. The elution step from the column appears to be the step in which the greatest loss of activity is found. This could be due to the length of time that the enzymes were on the column, as they were left overnight before elution, or it could be due to the inability to efficiently elute the enzyme protein off the column. When higher concentrations of NADH or NADPH were used (data not shown), the eluted activities were not higher and problems were encountered in obtaining an efficient separation of the two enzymes. Another point that appeared

Figure 7. Elution profile of NADH and NADPH enzyme activities from the blue Sepharose column. After equilibration of the blue Sepharose column with pellet buffer, 40mL of 150µM NADPH was applied to the column at the point denoted by the arrow on the figure. Each fraction was measured for NRA with NADH or NADPH. These activities are plotted. After the 1st peak was eluted, the column was washed with pellet buffer and the 40mL of 100µM NADH was applied. The activities found for this peak are plotted.



Relative Units

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Table 15. Recovery of NRA from Root NR Purification Protocol.

Stage of		NADH	NADPH		
Purification	Relative Units	<pre>% Recovery</pre>	Relative Units	<pre>% Recovery</pre>	
Initial Extract	2808	100	1536	100	
Ammonium sulfate supernatant	0	0	0	0	
Ammonium sulfate precipitate	2084	74	1124	73	
After 2 hours of dialysis	1898	68	1039	68	
Eluent from blue Sepharose column	0	0	0	0	
Eluted with NADPH	73	3	89	6	
Eluted with NADH	248	9	0	0	

to be important in recovering the highest level of activity from the column was in the washing of the blue Sepharose once the column was loaded. If the column was not washed immediately with pellet buffer until the eluate was clear very little activity could be recovered from the column the next day. Though this protocol did not give very high recoveries of the root NRs, it did allow for a good separation of the two forms with sufficient recoveries to test the enzymes for their biochemical properties.

Biochemical Characterization of NADH:NR and NAD(P)H:NR from Maize Roots

Partially purified NADH:NR and NAD(P)H:NR were tested for the following properties; pH optimum, K_m for NO₃, K_m for NADH or NADPH and native molecular weight. All tests were done immediately after elution of the enzyme from the column, without dialysis against glycerol. Two separate purifications of the enzymes were analyzed. A purified preparation of maize leaf NR (prepared by V.J. Goodfellow) was also tested for its K_m for NO₃, K_m for NADH and its native molecular weight.

The pH optima of the enzymes was determined by performing a standard NR assay, as described in "Materials and Methods" of Chapter 1 with the exception that instead of

using HEPES buffer, the assay mixture was made to contain a final concentration of 0.1M KPO₄ at pHs from 6.0 to 8.0 in increments of 0.5. A variety of different buffers were tried in the assay including a range of HEPES buffers, a mixed buffer containg 50mM MOPS, MES, PO_4^- and TRIS (Redinbaugh and Campbell, 1981) and overlapping HEPES, MOPS and MES buffers, all of which gave similar results, but the PO_4^- buffer was found to give the sharpest peak in activities. The results in Figure 8 show that both enzymes had a pH optimum of 7.0.

The determination of K_s for the two enzymes were done using standard NR assays with either a range of NO3 (0.1, 0.25, 0.33, 0.5 and 1mM), NADH or NADPH (0.005, 0.007, 0.01, 0.013, 0.02 and 0.04mM) concentrations. The Lineweaver-Burk plots used for determination of the K_ms were plotted (Figures 9-14) and the Kms determined (Table 16). The NADH:NR enzyme has a lower K_m for NO_3^- than the NAD(P)H:NR. The value for the leaf enzyme was similar to the value for NADH:NR. All three values were well within the range of values normally found in the literature. The K for NADH of the NADH:NR enzyme was very high and questions the ability of this enzyme to function in vivo when using NADH as the electron donor. The Kms of the NAD(P)H:NR enzyme for NADH and NADPH were approximately 15µM and the value for the leaf

Figure 8. pH optima of partially purified NAD(P)H:NR and NADH:NR. Each of the purified enzymes was assayed in a standard assay mixture as described in "Materials and Methods" with the exception that HEPES buffer was omitted and substituted with KPO_4 buffers at the appropriate pH. The final concentration of the KPO_4 in the assay mixture was 0.1M. The NAD(P)H enzyme was measured for both/NADH and NADPH:NRA as shown in (a). Only NADH:NRA was measured for the NADH enzyme (b).

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Figure 9. Calculation of K_m for NADH of the NADH eluted enzyme. NRA (v) was measured for a range of NADH concentrations (s). The results are presented as a Lineweaver-Burk plot.

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r = 0.977

y intercept = 0.104

slope = 0.099



Figure 10. Calculation of K_m for NO_3^- of the NADH eluted enzyme. NRA (v) was measured for a range of $NO_3^$ concentrations (s). The results are presented as a Lineweaver-Burk plot. r = 0.995

y intercept = 1.560

slope = 0.156



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Figure 11. Calculation of K_m for NO_3^- of the NADPH eluted enzyme. NRA (v) was measured for a range of $NO_3^$ concentrations (s). The results are presented as a Lineweaver-Burk plot.

r = 0.995

y intercept = 7.180

slope = 1.804



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Figure 12. Calculation of K_m for either NADH or NADPH of the NADPH eluted enzyme. NRA (v) was measured for a range of reductant concentrations (s). The results are presented as a Lineweaver-Burk plot.

- with NADH	- with NADPH
r = 0.998	r = 0.984
y intercept = 8.03	y intercept = 4.98
slope = 0.124	slope = 0.072



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Figure 13. Calculation of K_m for nitrate of the maize leaf enzyme. NRA (v) was measured for a range of nitrate concentrations (s). The results are presented as (a) a Lineweaver-Burk plot or (b) a Hanes plot (Note: the Hanes plot was used in this calculation to adjust for the similar NRA values generated when using the Lineweaver-Burk plot).

Lineweaver-Burk plot	Hanes plot		
r = 0.994	r = 0.981		
y intercept = 0.336	y intercept = 0.041		
slope = 0.536	slope = .373		





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Figure 14. Calculation of K_m for NADH of the maize leaf enzyme. NRA (v) was measured for a range of reductant concentrations (s). The results are presented as a Lineweaver-Burk plot.

r = 0.992

y intercept = 0.980

slope = 0.013

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Table 16. Summary of Biochemical Characteristics of the NADH and NAD(P)H Enzymes of Maize Roots and Leaves.

	Ro	Leaf		
Parameter	NADH Enzyme	NAD(P)H Enzyme	NADH Enzyme	
pH optimum	7.0	7.0	_ 	
Km for NO3	100µM	251µM	109μM	
Km for NADH	943µM	15.5μM	13.3µM	
Km for NADPH		14.5µM		
Molecular Weight	263,000	263,000	263,000	

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enzyme for NADH was 13µM.

A Sephadex G-150 column was used to determine the native molecular weight of the two root enzymes and to compare them to leaf NR. The column was calibrated as described in "Materials and Methods". The calibration curve is shown in Figure 15. Once eluted from the blue Sepharose column, the NAD(P)H:NR was loaded onto the G-150 column immediately. Twenty-fours later the NADH enzyme was loaded onto the column. Pure leaf NR was loaded another 24 hours later. Each fraction was tested for NR activity and the fraction volume measured. All three enzymes were found to come off at the same volume suggesting that each of the enzymes has a very similar molecular weight. When this value was compared to the calibration curve (Figure 15) it gave a molecular weight of approximately 263,000. Figure 15. Calibration curve of Sephadex G-150 column. Two mixtures of standard proteins were run over a Sephadex G-150 column. The log of their molecular weights versus the volume in which their protein peak was found are plotted. The volume at which the activities of the purified preparations of shoot NADH:NR and root NADH:NR and NAD(P)H:NR peaked, is also marked.

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Discussion

In 1953 Evans and Nason discovered a form of NR in soybean leaves that was capable of using either NADH or NADPH as a reductant supply. Since that time it has become apparent that the most common form of NR in higher plants is actually the monospecific form of NR, NADH:NR. Two forms of NR in maize roots were first identified by Redinbaugh and Campbell (1981). They identified these as a stable NADH:NR form and a more labile NAD(P)H:NR enzyme. I have also found these two forms in maize roots. Though corn leaves do not contain a bispecific form of the enzyme, the NR isolated from this tissue appears to have epitopes that are similar to both forms of the root enzyme as determined by antiserum inhibition tests. Unlike my results, NAD(P)H forms of NR have been found to be immunologically different from their monospecific counterparts in other species. For example, mutant barley leaves which lack the usual NADH:NR but contain an NAD(P)H form of NR in its place are not inhibited to the same extent by antibodies made to the wild-type NR (Dailey et al., 1982; Harker et al., 1986).

The induction curves for NRA in maize roots and shoots upon the addition of nitrate are similar to those

found in the past for other inducible NRs. There is an initial lag period, followed by a period of linear increase until a steady-state level of activity is reached (Oaks et al., 1972; Aslam and Oaks, 1976; Remmler and Campbell, 1986). Activities in the roots do not increase as quickly as in shoots and appear to reach only approximately one-half of the activity, on a per gram fresh weight basis, that the leaf NR reaches. When nitrate was removed from the system there was a rapid loss of the bispecific enzyme relative to the NADH:NR in root tips in vivo. Both forms of NR in the root tip decreased more rapidly than in the mature portion of the root. This is in contrast to results of Oaks et al. (1972) where NR in the mature roots, a tissue which is not rapidly producing new cells, appeared to turnover much more quickly than in the root tips. The difference in these results appears to be due to the hydroponic growing conditions and to the addition of chymostatin to the extraction buffer.

The effect of ammonium additions on maize roots is difficult to explain, but a clear description is possible. With whole roots and shoots, a mild enhancement of NRA was found when ammonium was added to the system. This has often been seen by others (Mohanty and Fletcher, 1976; Oaks <u>et</u> <u>al.</u>, 1977, 1979; Oaks, 1979; Rajeskhar and Mohr, 1986).

However, the ammonium and nitrate appear to have different effects on the individual root segments depending on whether they were applied alone or together (Table 11). Oaks <u>et al</u>. (1977) found that when nitrate and ammonium were applied together, the nitrate level in the tissue was unaffected but the ammonium level increased. As of yet it is still unknown how either of these compounds interact with NR in higher plants, and whether this interaction is indirect or direct.

The ability to separate the two forms of root NR allowed us to examine some of the biochemical properties of the two enzymes. The most interestng result of these experiments was that the NADH:NR form of the enzyme was found to have a very high K_m for NADH, while the values for the NAD(P)H:NR enzyme fell within the range obtained from other species (1.5-68µM) (Table 16). If we assume that both enzymes could function in vivo, then the NAD(P)H:NR isozyme would have an advantage because it can use either NADH or NAD(P)H as reductant. The NADPH could be generated by the pentose phosphate pathway directly into the cytosol making it easily accessible for use by NR (Emes and Bowsher, 1991). The other biochemical properties of the enzymes, the K_s for nitrate and the pH optima, are similar to those found for other species and the maize leaf enzyme. Determining the native molecular weight of NRs has always been a problem.

problem. The method used for determination of the molecular weight of NR appears to have a large influence on the result (Redinbaugh and Campbell, 1981; Nakagawa <u>et al.</u>, 1985). Our result shows that both of the root enzymes and the leaf enzyme have the same apparent molecular weight, 263,000, which falls approximately midway between the reported values which range from 190,000 to 300,000 (Jolly <u>et al.</u>, 1976; Redinbaugh and Campbell, 1981; Kuo <u>et al.</u>, 1982; Oji <u>et al.</u>, 1988). Overall, the two forms of NR in maize roots appear to have many similarities, but they do have some distinct differences with respect to reductant use, <u>in vivo</u> stability and K_ms for nitrate and reductants which identify them as separate enzymes.

Chapter 3: Influence of Light/Dark Cycles on the Regulation of Nitrate Reductase and Nitrite Reductase

Note: The work in this chapter was done in collaboration with Dr. Caroline Bowsher.

Introduction

In higher plants, the principal source of nitrogen under normal field conditions is nitrate. Depending on the concentration of nitrate in the medium and the plant species, a portion of the nitrate is transported via the xylem sap to the leaves (Andrews, 1986). In both roots and leaves, nitrate is either stored in the vacuoles or reduced to nitrite by NR. Nitrite is further reduced to ammonium by the enzyme NiR. The ammonium is then used primarily in the synthesis of glutamine. Subsequently, the amide nitrogen of glutamine is used in the synthesis of glutamate in a reduction mediated by glutamate synthase. The αNH_2N of glutamate is used in the synthesis of many amino acids via transaminase reactions (Miflin and Lea, 1980).

It has been known for some time that both nitrate and light are required for the synthesis of NR (Beevers and Hageman, 1980; Somers <u>et al.</u>, 1983) and NiR (Gupta and Beevers, 1984) proteins. Recently with the cloning of the NR (Cheng <u>et al.</u>, 1986; Crawford <u>et al.</u>, 1986; Calza <u>et al.</u>, 1987; Galangau <u>et al.</u>, 1988) and NiR (Back <u>et al.</u>, 1988; Lahners <u>et al.</u>, 1988) genes, it has been possible to

demonstrate that the induction by nitrate occurs at the level of transcription.

The induction of NR by a phytochrome response was first demonstrated by Jones and Sheard (1972) in pea seedlings and has since been shown to occur in a number of other plant genera (Duke and Duke, 1984). The expression of NR and NiR in photosynthetic tissue is not affected by light in the absence of nitrate. However, upon the addition of nitrate to plants grown in the dark, treatment with red light increased levels by 78% and 51% for NR and NiR respectively (Sharma and Sopory, 1984). This increase is reversible by far red light, which suggests that phytochrome is involved at some level in controlling the expression of these genes. However, the phytochrome response is only seen with etiolated plants, suggesting that light has an affect at some other level (Melzer et al., 1989). For instance, light may have an affect on the level of reductant, uptake of nitrate, transfer of nitrate to the xylem and the release of nitrate from vacuoles in the leaves (Duke and Duke, 1984; Rufty et al., 1989).

In this chapter the results from a study on the expression of NR and NiR genes in maize seedlings grown in the presence of constant nitrate are reported. The amount of enzyme activity and the mRNA levels were analyzed in both

roots and shoots at 4 h intervals during a 48 h period to determine the variability in their expression levels during the course of the day. The expression of these genes is shown to be affected by the light regime utilized. However, light does not appear to have a direct effect on the expression of NR and NiR.

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Materials and Methods

Plant Material and Growth Conditions

Maize kernels (Zea mays cv W64A x W182E) were grown in a growth cabinet without a light supplement at 28°C for 2 d in large petri plates containing 1% agar. Seedlings were then inserted into slits cut into foam and grown hydroponically by floating the foam on the surface of 20L of medium. The medium consisted of 1/10 strength Hoagland's solution modified to contain a final concentration of 10 mM KNO3. Circulation and aeration in the tanks was provided by a submersible pump. The plants were grown under a 16 h light/8 h dark regime for a further 4 d. At this point one of three light regimes was adopted: (i) the light/dark regime continued (ii) continuous light or (iii) continuous The growth chamber contained flourescent and darkness. incandescent light bulbs which emitted a fluence rate of 250 μ mol m⁻² s⁻¹ at the level of the plants.

Plants were harvested by the random removal of 25 plants per sample from the tank. Shoots and roots were excised separately, frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Samples were stored at -70° C for further analysis.

RNA Isolation and Hybridization

Total RNA was extracted from 3g of frozen shoot or root powder by a procedure derived from Lahners et al. (1988, see Appendix 2). For RNA blot hybridization 30 µg of total leaf RNA and 20 µg of total root RNA were denatured with formaldehyde and subjected to electrophoresis through a 1.2 % agarose gel which contained 2.2 M formaldehyde, and the RNA was blotted onto nitrocellulose (Maniatis et al., 1982; see Appendices 4 and 5). The probe used for the hybridization was either an NiR cDNA insert from the plasmid pCIB808 (Lahners et al., 1988), an NR cDNA insert from the plasmid pCIB831, or a wheat rRNA cDNA clone. In all cases, DNA probes were radiolabelled with $[\alpha - {}^{32}P]dCTP$ (Amersham) using a nick translation kit (Amersham International) to a specific activity of 1 to 4 x 10^8 cpm/µg (see Appendices 12 and 13 for protocols). The filters were prehybridized and hybridized with a radioactively labelled denatured DNA probe according to Maniatis et al. (1982). Filters were washed, dried, and subjected to autoradiography on Kodak XOM films as previously described (Lahners et al., 1988). The blot was reprobed with a wheat rRNA cDNA clone to confirm that equal amounts of RNA were loaded on each lane (data not shown). To allow reprobing of the filters with the

different probes, filters were allowed to decay and then rehybridized with a nick translated probe.

Autoradiograms of different exposure times were scanned with a Bio-Rad 620 video densitometer. Relative amounts of mRNAs were determined by peak area measurements.

Enzyme Assays

Frozen samples were ground at a ratio of 1 g frozen plant material to 4 mL extraction buffer and assayed for NR activity as described in Chapter 1. NiR was assayed in the same extract by the method of Losada and Paneque (1971). The following reagents were required for this assay: 0.6mL 150mM Tris-HCL (pH 8.0) 0.1mL 50mM Methyl Viologen 75mg NaHCO₃ + 75mg Nadithionite mixed gently in a volume of 3mL of deionized water 0.1mL 0.1mL 10mM NaNO₂ Extract 0.1mL

All of the above solutions were added together except the NaNO₂ (the solution should be blue). The reaction was started by the addition of the NaNO₂. After 10-30 min incubation at 25° C, the reaction was stopped by vortexing the mixture at high speed until the methyl viologen was completely oxidized (colourless solution). Aliquots of the

solution (0.1mL) were added to 0.9mL of water. Two mL of a mixture of 1mL of 1% sulfanilamide in 1N HCl and 1mL of 0.01% NED in water were added and the colour reaction was measured on a spectrophotometer at 540nm. Control tubes contained no enzyme preparation and were incubated along with the other tubes. In addition tubes which contained all of the ingredients were measured at 0 time in order to assess the interference by the extract alone. All assays were carried out in duplicate.

Results

Effect of Plant Age on NR and NiR Activities

There is some evidence that seedling age affects the expression of NR (Srivastava et al., 1976; Long and Oaks, In order to determine the effect of seedling age on 1990). NR and NiR enzyme activity levels, seedlings were germinated on plates and then transferred to a hydroponic system with a 16 h light/8 h dark cycle. Twenty-four hours prior to harvest the growth medium was adjusted to a final concentration of 10 mM KNO_3 by addition of a stock solution of KNO3 to the known volume of medium. For the following 8 days plant material was harvested 7 hours into the light period. Roots and shoots were assayed separately for both NR and NiR activity. Both of these activities were found to be highest at either day 5 or 6 in both organs, however the effect was much more dramatic in the shoots (Figure 16). Based on these results, the sampling period for subsequent experiments was started on day 6. Any alterations in the light regime were also done at this time.

Figure 16. Development of (a) nitrate reductase activity and (b) nitrite reductase activity in maize shoots (•) and roots (•) during the first 8 days of growth after germination. Plants were grown in 1L pots on 0.1 strength Hoaglands nutrient solution and induced with 10 mM KNO₃ 24h prior to harvesting as described in "Results" (representative data from two experiments).





Effect of Altered Light Regimes on NR Activity and mRNA in Shoots

NR activity and mRNA levels were measured in hydroponically-grown maize shoots over the course of the 16 h light/8 h dark cycle. In seedlings grown under these light conditions, NR activity was found to vary considerably during the time-course (Figure 17a). Maximal activity was attained 4 to 8 h into the light period and then declined gradually. As expected, given the fact that seedling age affects the level of activity (Figure 16), the peak activity was lower on the second day in comparison with the first. Total RNA was probed for the level of NR mRNA by RNA blot hybridization. Results for mRNA production and NRA were parallel (Figure 17a). Two aspects of these results should be noted. The first is that the fluctuations of both the enzyme activity and the mRNA are not necessarily directly correlated with the onset of the light and dark periods. Both the NRA and the mRNA level start to drop approximately half-way through the light cycle. On the other hand, they do begin to increase again with the onset of the light cycle. The second point is that the fluctuation of NRA with seedling age is correlated with the level of NR mRNA.

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Figure 17. Variations in the amount of NR activity (•) and mRNA levels (•) in the shoots of 6 d old maize plants either (a) maintained in a 16 h light/8 h dark regime (b) transferred at time 0 h to continuous light (c) transferred at time 0 h to continuous darkness. The light conditions are indicated in each figure by an open bar (light period) and closed bar (dark period) (experiments repeated twice and representative data shown).



Therefore, it appears that these differences in NRA are primarily due to differences in the level of NR mRNA present.

To help delineate the role of light in controlling expression of these genes, maize seedlings were grown under a 16 h light/8 h dark regime and then were switched into either continuous light or continuous darkness. Plants placed into continuous light on day 6 had the same initial pattern for NRA on the first day of the experiment as was seen for plants grown under the 16 h light/8 h dark cycle. This was expected, since the light conditions during this time period are the same for the two experiments. However, the time in which NR activity was low in these plants was shortened considerably in the absence of a dark period (Figure 17b). Furthermore, while plants grown under 16 h light/8 h dark conditions had far less NRA in the second 24 h period (Figure 17a), the plants grown in continuous light had an NRA peak on the second day equal to that found for the first. At the same time, the maximum level of mRNA present on the second day was increased four-fold under these conditions, when compared to plants grown on a 16 h light/8 h dark cycle (Figure 17a,b).

To further test the effect of different light regimes, maize seedlings were grown under a normal 16 h light/8 h dark cycle until day 6. At this time, the plants were maintained in continuous darkness, with samples removed and assayed for the following 48 h. There was a marked effect on the expression of NR under continuous darkness (Figure 17c). As expected, the initial activity was the same as in the previous experiments, given that the initial time-point is equivalent in each case. Afterwards NRA did not increase nearly as rapidly, with the activity being 4fold less after 4 hours than was found for plants grown in the light. By 12 hours no NRA was detectable. The level of NR mRNA in these plants was also found to decline, with none detectable at the 8 hour time-point. Therefore, the decrease in NR activity is concurrent with the decrease in the level of NR mRNA.

Effect of Different Light Regimes on the Expression of Root

In maize roots both NADH and NAD(P)H:NR activities are expressed as shown in Chapter 1. Since their pattern of expression was similar (except that the NADH activity values were approximately two-fold greater than the NAD(P)H:NRA values) only NADH activities were plotted (Figure 18). Unlike the shoots, roots grown either under a light/dark or a continuous light treatment showed little if any variation

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Figure 18. Variations in the amount of NR activity (\bullet) and mRNA levels (\circ) in the roots of 6 d old maize plants either (a) maintained in a 16 h light/8 h dark regime (b) transferred at time 0 h to continuous light (c) transferred at time 0 h to continuous darkness. The light conditions are indicated as for Fig. 2 (experiments repeated twice and representative data shown)



in the NR activity. The level of NR mRNA does vary somewhat through the course of the first 24 h after day 6. It decreases slightly during the initial part of the light period and then reaches a peak after the lights were on for 12 h. These variations in mRNA do not appear to have a noticeable effect on the level of NRA. When the plants were grown in continuous dark, there was a slow decrease in NRA. However, unlike the results found for shoots, the activity did not decrease nearly as quickly and was still present at the end of the experimental period.

Effect of Light Regimes on NiR Activity and mRNA in Shoots

The cycling of shoot NiR enzyme activity was not as obvious as was seen for NR in shoots under any of the experimental conditions used. For example, when plants were grown under a 16 h light/8 h dark regime the activity slowly decreased during the experimental period, with only minor fluctuations detected (Figure 19a). However, the level of NiR mRNA did show considerable diurnal cycling. The pattern of mRNA expression was almost exactly the same as that found for the shoot NR mRNA (Figure 19a), although the level of NiR mRNA started to increase during the dark period to a considerably greater extent than did the NR mRNA. Figure 19. Variations in the amount of NiR activity (•) and mRNA levels (•) in the shoots of 6 d old maize plants either (a) maintained in a 16 h light/8 h dark regime (b) transferred at time 0 h to continuous darkness or (c) transferred at time 0 h to continuous light. The light conditions are indicated as previously described in Fig. 2 (experiments repeated twice and representative data shown).

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Conditions with continuous light gave results similar to thosee found with the light/dark conditions (Figure 19c).

When plants were grown in continuous darkness, the level of NiRA actually did not decrease any more rapidly than in the light (Figure 19b). In contrast, the level of NiR mRNA is eventually affected, decreasing to an undetectable level at 36 h (Figure 19b). This decrease is considerably slower than that found for the NR mRNA. There is a slight increase in the level of the mRNA at the same time as was seen in the plants grown with a 16 h light/8 h dark cycle. However, it is of too small an amplitude to conclude that the NiR mRNA levels do indeed follow a diurnal rhythm even in the absence of light. The NiR mRNA in roots showed a similar pattern of expression to that found in shoots, however, there were difficulties in reproducing the NiRA values and therefore the data is not presented.

Discussion

A variety of environmental and developmental stimuli regulate nitrate assimilatory genes. The primary signal is the presence of nitrate, which stimulates the transcription of both NR (Cheng <u>et al.</u>, 1986; Crawford <u>et al.</u>, 1986; Calza <u>et al.</u>, 1987; Galangau <u>et al.</u>, 1988) and NiR (Back <u>et al.</u>, 1988; Lahners <u>et al.</u>, 1988) genes and the subsequent production of protein. In the presence of nitrate, the expression of these genes is also modulated by light conditions, diurnal cycle, nutrient conditions and seedling age (Beevers and Hageman, 1980; Srivastava, 1980). However, the mechanisms by which these factors modulate expression and their physiological role in determining the appropriate level of enzyme activity remain unknown.

There are obviously great differences in the expression of these genes in maize depending on seedling age. Between days 4 and 5, the levels of NR and NiR differ substantially in our system. The seedling is no doubt going through considerable physiological change, since it is beginning to rely less on catabolized endosperm protein and more on exogenous sources of nitrogen (Oaks, 1983). Srivastava et al. (1976) observed that in maize, NRA is low in young

leaves when they are functioning as a sink and importing nutrients. As the leaves mature and export rather than import nutrients, there is an alteration in the control of NR production. Nitrate reductase activity reaches a maximum just as the leaves approach maximum size and then declines in older leaves (Srivastava et al., 1976). Although the present work does not study this phenomenon in individual leaves, the age variability in the overall shoot activity levels is apparently due to differences in the level of mRNA. For example, the NR mRNA level on day 7 is considerably lower than on day 6 (Figure 17a) which corresponds to the decline in enzyme activity between these two time points. The mechanism of suppression of the expression of these genes early in seedling development and after the peak activity levels are reached is unknown.

The level of maize shoot NRA varies considerably over the course of a day. This oscillation has been reported for a variety of species (Srivastava, 1980 and references therein). The level of maize NR mRNA in the shoot was found to increase and decline at the same time as the activity. However, the amount of NR mRNA stays fairly constant at around one-third maximal level during the dark period while NRA remains low until the light period commences (Figure 16a). Furthermore, in the constant light experiment, there

appears to be a super-induction of the NR mRNA with peaks at twice the level of that found for the first day, while the activity maximum for the two days is the same. Therefore, while the amount of NRA is in general correlated with the amount of mRNA, there may be other control mechanisms at work.

The shoot NR mRNA and activity decreases rapidly in continuous darkness. This is in agreement with previous work on NR (Remmler and Campbell, 1986) and implies that light is required for NR gene transcription or mRNA stability. Deng et al. (1990) observed substantial decreases in NR protein and NRA in tobacco leaves under similar conditions, although the protein and NRA did not vary in parallel. However, in contrast to our results, levels of tobacco NR mRNA continued to display rhythmic oscillations in the dark and did not completely disappear in leaves until subjected to darkness for 56 hours. There was very little variability of NRA and mRNA levels during a light/dark or continuous light regime in roots. This is possibly due to the fact that non-photosynthetic tissue may obtain reducing power for these enzymes from either photosynthate translocated to the roots or from reserves maintained in the root itself. Furthermore, between 6 and 8 days of age ample seed reserves of carbohydrates are

probably still available to help sustain root growth and supply reductant. The slow decline in root NRA and mRNA caused by continuous darkness might reflect the depletion of these sources of reducing power.

NiR is a more stable protein than NR (Beevers and Hageman, 1969), which may explain the minimal effects of the light treatments on the activity of this enzyme. Interestingly, the shoot NiR mRNA continued to show a diurnal rhythm even in continuous darkness until 44 hours after the removal of the light when it was no longer measurable. Therefore, there was little correlation between the level of NiR mRNA present and the enzyme activity. Post-transcriptional events must be important for maintaining a sufficient level of this enzyme. In roots, NiR mRNA levels varied considerably more than NR mRNA, showing a similar rhythm to that seen in shoots. NR and NiR are found in the cytosol and plastids respectively (Beevers and Hageman, 1980). Since NiR requires post-translational processing, this may account for the differences observed.

A variety of plant activities are controlled by the cyclic alternation of day and night. Expression of a series of nuclear genes coding for chloroplast proteins has been shown to be stimulated by light and to undergo diurnal oscillations (Guiliano <u>et al.</u>, 1988; Otto <u>et al.</u>, 1988;

Paulson and Bogorad, 1988). In some cases, this diurnal rhythm is maintained in the absence of light, although this is not a universal attribute of all of these genes. In studies of both tobacco and tomato (Galangau <u>et al</u>., 1988), it was found that NR also maintained this circadian rhythm in the dark. In maize, although NR mRNA levels clearly are not maintained in the dark, NiR mRNA levels are more stable and there is some maintenance of the circadian rhythm in the dark.

Although, it is clear that the light regime can have a considerable effect on the expression of NR and NiR, the biochemical mechanism behind this type of regulation is unknown. However, given that the diurnal fluctuations do not necessarily correspond to the light/dark cycle, it seems unlikely that light itself directly regulates mRNA levels during the diurnal cycle. Either mRNA synthesis or translation might also be influenced by any one of a variety of factors, including the level of reductant present or the amount of reduced carbon available.

Chapter 4: Molecular Characterization of Root Nitrate Reductase

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Introduction

The majority of information available on NR and its role in nitrate assimilation in higher plants has come from biochemical and physiological studies. One of the main areas of study has focused on the effect of nitrate on NR. For example, Tang and Wu (1957) first showed that nitrate induced the appearance of NRA in rice seedlings. Subsequently, Zielke and Filner (1971), using density labeling techniques, were able to show that the appearance of NR upon the addition of nitrate to cultured tobacco cells was the result of <u>de</u> novo synthesis of the protein. This result was proven more directly by Somers et al. (1983) who showed that the increase in NRA observed when nitrate was added to barley seedlings was correlated with an increase in the appearance of a 110kD protein which cross-reacted with a polyclonal antibody that had been made against purified barley NR. Cheng et al. (1986) utilizing a barley NR cDNA clone were able to demonstrate a correlation between the nitrate induced synthesis of NRP and an increase in the level of NR mRNA.

NR has been identified as a complex, multicentre redox enzyme with different isozymes which utilize either NADH or NAD(P)H or both as reductant. The presence of these isozymes varies between individual plant organs and different species (for reviews see: Beevers and Hageman, 1980; Srivastava, 1980; Guerrero et al., 1981; Campbell and Smarrelli, 1986). However, little is known about the molecular mechanisms involved in the regulation of NR and nitrate metabolism. One of the approaches being used to try to understand this regulation is to identify and isolate genes involved in nitrate metabolism using recombinant DNA technology (for review see Wray, 1988). Using either antibodies made to purified proteins, or synthetic oligonucleotides derived from amino acid sequencing of a purified protein, cDNA clones of barley NR (Cheng et al., 1986), squash NR (Crawford et al., 1986), tobacco NR (Calza et al., 1987) and spinach NiR (Back et al., 1988) have been isolated. These clones have subsequently been used to identify genomic clones and cDNA clones from other genera (Cheng et al., 1988; Lahners et al., 1988; Daniel-Vedele et al., 1989; Gowri and Campbell, 1989; Shiraishi et al., 1990; Vaucheret et al., 1989). With the availability of cDNA and genomic clones the regulation of gene expression can now be examined directly at the molecular level. Cloned sequences can also be compared to other proteins with known sequences to try to determine functional groups within the enzyme.

In this chapter, the isolation of a partial cDNA clone for maize root NR is presented. This clone was sequenced and compared to other known NR sequences. The expression of NR mRNA upon the addition of nitrate and its appearance in different organs was also examined.

Materials and Methods

Screening of Root cDNA Library

a) λ Plaque Lifts

A cDNA library in λ gt11 had been made from root mRNA isolated from nitrate induced maize roots by Caroline Bowsher using an <u>In Vitrogen</u> kit. When the library was originally tested it was estimated that it contained approximately 20,000 plague forming units (pfu).

The library was plated for screening by adsorbing the entire library to 500µL of an overnight culture of ¥1090 cells for 20-30 min at 37°C. The mixture was then added to 9mL of YT top agarose (0.8%) which had been cooled to approximately 55°C, gently mixed and quickly poured onto a fresh, dry 150mm YT plate. The plate was incubated overnight at 37°C. The next day, plaque lifts were performed as described in Appendix 11.

b) Screening of Plaque Lifts

Filters were prehybridized for at least 3 hours at 65°C in plastic bags with 10mL of RNA Prehybridization buffer.

RNA Prehybridization Buffer:

3X SSC 5X Denhardt's Solution 20mM Tris-HCl, pH 7.0 0.1% SDS 2mM EDTA

During the period when the filters were being prehybridized, the probe was prepared. A partial cDNA maize leaf NR clone, pCIB831, that had been isolated at CIBA-GEIGY by Steven Rothstein's group, was used to probe the root library. pCIB831 was digested with Eco RI and the NR cDNA insert isolated from a low melt gel as described in Appendix 12. This fragment was nick translated using the BRL Nick Translation System (described in Appendix 13) and $(\alpha^{32}P)$ dCTP (Amersham) to a specific activity of 1-4 X 10^8 cpm/µg. The probe was boiled for 10 min and then cooled on ice for 10 min just prior to addition to the hybridization mixture.

After the prehybridization was complete, the plaque lifts were removed to a fresh bag and 10mL of fresh RNA Prehybridization buffer was added. The probe was added and the bag sealed, and placed at 55°C overnight. On the following day the filters were washed first with 2X 500mL of 6X SSC for 15 min each at room temperature with gentle shaking. Then with 2X 500mL of 6X SSC to 65°C for 10 min

with shaking. The filters were air-dried on Whatman 3MM paper and then exposed to Kodak OMAT-AR film for the required period of time.

c) Rescreening of Plaques

After the autoradiograms were developed, the films were matched to the plate using the original markings from the plaque lifts. Any signals that appeared on both replicates were picked with the large end of a pasteur pipette and placed in 1 mL of λ buffer with 20µL chloroform and kept at 4°C until used. Assuming that 1 plug = 10⁶ pfu, 3 dilutions of the isolates were set up with λ buffer in a total volume of 100µL or less and adsorbed to 100µL of an overnight culture of Y1090 for 20-30 min at 37°C. This was added to 3mL of YT top agarose and poured onto fresh, small YT plates and incubated overnight at 37°C. Single plaque lifts were made as described above, of any plates that were not confluent. These were screened as above and the desired plaques picked with the small end of a pasteur pipette.

Purification of λ DNA

 λ DNA was purified by either the plate lysate method or by a liquid lysate method as detailed in Appendices 14 and 15. For the plate lysate method, large, fresh YT plates were used with fresh YT top agarose. The phage were adsorbed to Y1090 cells as described above at a concentration sufficient to give confluent lysis of the bacteria. If a single plaque did not give a high enough titre for DNA isolation, then up to 20 plaques would be picked from the same isolate and placed in 1mL of λ buffer (this was necessary in only one case).

<u>λ DNA Filter Hybridization</u>

Purified preparations of λ DNA were digested with either Eco RI or Not I. The digested DNA was electrophoresed on a 1% minigel and blotted onto nitrocellulose as described by Maniatis <u>et al</u>. (1982, see Appendix 6). The filters were probed with radiolabeled pCIB831 under the conditions described above for the screening of the λ plaques.

Subcloning of λ cDNA Inserts into pUC 18

a) Ligation of the Insert into pUC 18

Purified λ DNA was digested with Eco RI and the fragment isolated from a low melt agarose gel (Mainiatis <u>et al.</u>, 1982; see Appendix 12). The fragment was ligated into pUC 18 which had been digested with Eco RI and treated with alkaline phosphatase (see Appendix 16). The ligation volume was 10µL which contained a ratio of fragment to vector of

3:1. The control ligation contained vector DNA without any added fragment. The mixture was ligated overnight at 15° C or at room temperature for 1-4 hours. Finally, the ligase was heat denatured at 70° C for 10 min.

b) Transformation of Competent Cells with the LigationMixture

An aliquot of 3μ L of the ligation mixture was added on ice to 200 μ L of either MC1022 or JM101 competent cells (prepared as described in Appendix 17). The cells were kept on ice for 45 min, and the tubes occasionally mixed gently. This was followed by heat shocking the cells at 37° C for 2 min and replacing them on ice for 15 min. The cells were spread onto plates containing X-Gal, IPTG and ampicilin (Maniatis <u>et al</u>., 1982) and left overnight at 37° C. Six of the white colonies were picked and the plasmid DNA was isolated as described in Appendix 8.

Sequencing of cDNA Clone

The plasmids p1501, p1502, p1503 and p1503 (Sau 3A fragments) were sequenced using a Sequenase kit (United States Biochemical Corporation) and the protocols provided with the kit as described in Appendix 18. The sequencing reactions were labeled with ³⁵SdATP (Amersham). DNA was

prepared for sequencing using the large scale alkaline procedure (see Appendix 7).

Growth Conditions of Plants

Plants were grown hydroponically as described in detail in Chapter 1. Maize kernels were germinated on 1% agar for 2 days in a growth cabinet without a light supplement at 28°C. They were then transferred to hydroponic pots containing 1/10 Hoagland's solution and kept in a growth chamber at 28°C with a 16 h light/8 h dark regime. Nitrate was added and the plants were harvested as specified in the individual experiments.

RNA Extraction and RNA Blot Hybridization

RNA was extracted from plant organs by the method of Lahners <u>et al</u>. (1988, see Appendix 2). Equal amounts of RNA (a minimum of 10µg per lane) were denatured and electrophoresed on a 1.2% agarose gel which contained 2.2 M formaldehyde. This gel was used for RNA blot hybridization as described by Maniatis <u>et al</u>. (1982, see Appendices 4 and 5). The nitrocellulose filters were prehybridized for a minimum of 3 hours with RNA prehybridization buffer (recipe above in λ plaque lift protocol). DNA fragments were isolated from low melt agarose gels and labeled with ³²pdCTP (Amersham) by nick translation with the BRL Nick Translation System (see Appendices 12 and 13). The filters were hybridized with fresh RNA prehybridization buffer and the probe overnight. The next day the probe was removed and the filters washed for 20 min with 1X SSC and 0.1% SDS at room temperature (500mL per filter). This was followed by 3 washes (500mL per filter) at 68°C for 20 min each of 0.2X SSC and 0.1% SDS. The filters were air-dried and then exposed to film.

Extraction of Genomic DNA and DNA Blot Hybridization

Genomic DNA was extracted from 8 samples of 300mg of frozen maize leaf powder. Each 300mg was placed in an Eppendorf tube to which was added 700µL of Proteinase K buffer (50mM Tris-HCl pH 8.0, 100mM EDTA, 100mM NaCl and 1% SDS) and 35µL of Proteinase K (10mg/mL stock). The tubes were mixed and incubated overnight at 55°C. The following day 20µL of RNase A (10mg/mL) was added to each tube and they were incubated at 37°C for 1-2 hours. The mixture was extracted once with phenol, once with phenol:chloroform (1:1) and once with chloroform. An equal volume of RT isopropanol was added and the mixture spun for 1 min in the microfuge. The pellet was washed with 70% EtOH and then resupended in 320µL of water. To the suspension was added
80μ L of 10M NH₄OAc and 1mL of EtOH. The mixture was spun in the microfuge for 1 min and the pellet washed with 70% EtOH. The pellet was dried and resuspended in 75µL of TE.

The genomic DNA was digested with the appropriate restriction enzyme for 1-2 hours. At this time additional restriction enzyme was added and the digest incubated at 37°C overnight. The digested DNA was blotted onto nitrocellulose and the filters washed as described in Appendix 10.

The probes used for hybridization were prepared by the method of Heery et al. (1990). Plasmid DNA was digested with the appropriate restriction enzyme and the DNA fragments electrophoresed through agarose gels using 1X TAE as the running buffer. The region of the gel containing the DNA fragment to be used as a probe was isolated using a razor blade and placed in 0.5mL Eppendorf tubes which had been pierced in the bottom with a small, hot needle and contained approximately 2mm of packed siliconized glass wool The small Eppendorfs were placed in 1.5mL in the bottom. Eppendorf tubes and spun at 6000 rpm for 10 min at 4°C in a Biofuge A (Canlab). The liquid collected in the large Eppendorf tube contained the DNA and its volume was increased to 500µL with TE and 1/10 volume NaOAc was added. This solution was extracted once with phenol, and then with

chloroform. Ethanol (2.5 volumes) was added to the supernatant, the tube was mixed and then left on ice for 15-20 min. The DNA was pelleted by spinning in the microfuge at 4° C for 15 min at top speed. The pellet was dissolved in 15µL of TE initially. Probes used for screening genomic DNA blots were prepared by random priming (as described in Appendix 19).

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Results

Isolation of a cDNA Clone for Maize Root NR

A maize root λ gt11 cDNA library was used to isolate a root NR clone. The library was made from mRNA isolated from roots which had been induced with nitrate to enrich for mRNAs coding for NR and NiR. A partial cDNA clone of maize leaf NR, pCIB831, was used to screen the library.

When the library was screened, six plaques showed hybridization to the NR cDNA clone. These were rescreened, and three of the positive clones were shown to still hybridize to the NR sequence. These three phage were purified to homogeneity. The λ DNA from these three putative clones was purified and digested separately with Eco RI and Not I (the library had been constructed so that both an Eco RI and Not I site were located on either side of the cDNA inserts). The digested ENA was electrophoresed on an agarose gel and blotted onto nitrocellulose. Using pCIB831 as a probe, one of the isolates was found to contain an insert of approximately 1kb, while the other two inserts were only 200-300 bases long. The λ clone containing the 1kb fragment was digested with Eco RI and the fragment subcloned into pUC 18. The resultant plasmid was transformed into JM101 and named p1501 (Figure 20).

Sequencing of p1501

The ends of p1501 were sequenced and compared to the partial sequence obtained for maize leaf NR by Gowri and Campbell (1989). The portion of the clone which encodes the NR polypeptide was found to be approximately 75% homologous with the leaf clone, while the 3' untranslated region showed little homology. A restriction map was generated for p1501 and this was used to subclone smaller fragments for further sequence analysis (Figure 20). The complete DNA sequence was generated in this fashion as shown in Figure 21.

Comparison of p1501 to Other Known Sequences of NR

The sequence for p1501 is shown in Figure 21 and is compared to the maize leaf NR sequence (Gowri and Campbell, 1989) by using a computer package, DNASIS (Figure 22). In the coding region (bases 1-693 of the p1501 sequence) the nucleotide sequences are 72% homologous. At the amino acid level though (Figure 23), they are only 62% similar. In the non-coding regions of the root and leaf nucleotide sequences, very little homology is seen (Figure 22, bases 694-931 of the p1501 sequence). When the DNASIS program was Figure 20. Maps of p1501, p1502 and p1503. The plasmid p1501 was digested with the restriction enzyme SSt 1 to facilitate the sequencing of the root NR clone creating the plasmids p1502 and p1503. p1502 was small enough that it could be sequenced in both directions. As p1503 was still too large to be completely sequenced, it was digested with Sau 3A and the resultant fragments subcloned into pUC 18 and sequenced in both directions.

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Figure 21. Nucleotide and amino acid sequence of the maize root NR cDNA clone, p1501.

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1 CCG CAG AAG CTC GGC CTT CCC GTC GGC AGG CAC GTG TAC GTG TGC GCG 1 Pro Gln Lys Leu Gly Leu Pro Val Gly Arg His Val Tyr Val Cys Ala TCG ATA GGC GGC AAG CTC TGC ATG CGC GCG TAC ACG CCC ACG AGC CCC 49 Ser Ile Gly Gly Lys Leu Cys Met Arg Ala Tyr Thr Pro Thr Ser Pro 17 97 GTC GAC GAG GTC GGC CAC TTC GAT CTC CTC ATC AAG ATA TAC TTC AAG 33 Val Asp Glu Val Gly His Phe Asp Leu Leu Ile Lys Ile Tyr Phe Lys 145 GAC GAG GAC CCC AAG TAC CCC AAC GGC GGG CTC ATG TCG CAG TAC CTG 49 Asp Glu Asp Pro Lys Tyr Pro Asn Gly Gly Leu Met Ser Gln Tyr Leu GAC TCC CTG CCG CTG GGC GCG ACT ATT GAC ATC AAG GGT CCG CAT AGG 193 65 Asp Ser Leu Pro Leu Gly Ala Thr Ile Asp Ile Lys Gly Pro His Arg CAC ATC GAG TAC ACC GGC CGC CGC CGC TTC GTG GTG AAC GGC AAG CAG 241 His Ile Glu Tyr Thr Gly Arg Arg Arg Phe Val Val Asn Gly Lys Gla 51 CGT CAC GCG CGC AGG CTC GCC ATG ATC CAG GCC GGC AGA GGG ACC ACG 239 Arg His Ala Arg Arg Leu Ala Met Ile Gln Ala Gly Arg Gly Thr Thr 97 CCC GAC GAC GAC ACG GAG CAG GCC GTG CTG AGG GAC CAG CCC GAC GAC 337 113 Pro Asp Asp. Asp Thr Glu Gln Ala Val Leu Arg Asp Gln Pro Asp Asp GAC ACG GAG ATG CAC CTC GTG TAC GCG AAC CGA ACG GAC CAC GAC ATG 385 Asp Thr Glu Met His Leu Val Tyr Ala Asn Arg Thr Asp His Asp Met 129 CTC CTA AGG GAG GAG ATC GAC CGC GCT TGG CTG CCG CGC ACC CGG CGC 433 145 Leu Leu Arg Glu Glu Ile Asp Arg Ala Trp Leu Pro Arg Thr Arg Arg CTC AAG GTG TGG TAC GTG GTC AGC AAG GTC CCG GAG GAC GGG TGG GAG 481 161 Leu Lys Val Trp Tyr Val Val Ser Lys Val Pro Glu Asp Gly Trp Glu TAC GGC GTG GGG AGA GTG GAC GAG CAT GTC ATG AGG GAG CAC CTG CCT 529 Tyr Gly Val Gly Arg Val Asp Glu His Val Met Arg Glu His Leu Pro 177 CTG GGA GAC AGC GAG ACC ATT GCG CTC GTG TGC GGG CCG CCG GCG ATG 577 193 Leu Gly Asp Ser Glu Thr Ile Ala Leu Val Cys Gly Pro Pro Ala Met ATC GAG TGC ACA GTG CGC CCG GGC CTG GAG AAG ATG GGG TAC GAC CTC 625 Ile Glu Cys Thr Val Arg Pro Gly Leu Glu Lys Met Gly Tyr Asp Leu 209 GAC AAG GCT TGT CTC GTG TTC TGA GCT CTG AAT AGC GGC TAA CGG ATG 673 225 Asp Lys Ala Cys Leu Val Phe TCG TCA AGG TGC AAC TGT ACA TAG AAA TTC TGT GGT GCC TTG AAT CTT 721 GAA CCC TAG TAA CGT GTC GAT CTA GCT AGA ACT CTA CCG AGT TCT CTT 769 817 GTA ATA CTA GCG ATT TAA ACT GGC CAT GGA AGT TCA TAC TAG CTG GTG 865 CCA TGG CCG TCT TGT CAA GAT GAG ATG TAT TGG TCC TAC TAT ATA CAG 913 CGC AAT AAA AAC CCC AAG G

Figure 22. Comparison of maize root and shoot NR nucleotide sequences. The root sequence was derived from the sequencing of p1501. The shoot sequence is from Gowri and Campbell (1989).

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Figure 23. Comparison of maize root NR amino acid sequence to other known NR sequences from higher plants. In positions where at least two amino acids are the same, the area has been enclosed with lines. Amino acids in bold face represent positions where the amino acid is conserved in all of the sequences compared. Maize root NR sequence was derived from p1501. This was compared to tomato NR (Daniel-Vedele <u>et al.</u>, 1989), tobacco *nia*-2 NR (Vaucheret <u>et al.</u>, 1989), *Arabadopsis* NR2 (Crawford <u>et al.</u>, 1988), *Arabadopsis* NR1, barley NR (Cheng <u>et al.</u>, 1988), maize NADH:NR (Gowri and Campbell, 1989) and human cytochrome b5 reductase catalytic domain (Yubisui <u>et al.</u>, 1984). This figure was adapted from Daniel-Vedele <u>et al.</u> (1989).

1) 33 4 5 6 7 8	D Q V L G L P V G K H I F L C A TIVD D K L C H R A Y D Tobacco nia-2 D Q V L G L P V G K H I F L C A VI D D K L C H R A Y Arabidopsis 2 D M V L G L P V G K H I F L C A TIND K L C L R A Y Arabadopsis 1 D Q Q L G L P V G K H I F L C A TIND K L C L R A Y Arabadopsis 1 D Q Q L G L P V G K H V F V C A NI ND K L C L R A Y Maize root P 2 K L G L P V G K H V F V C A SII G G K L C M R A Y Maize leaf T E V L G L P I G K H I F V C A KIL E G K L C M R A Y Barley D Q V L G L P V G K H I F V C A TID G K L C M R A Y Cytochrome b5 Q H I L G L P V G Q H I Y L S A R I D G N L V V R P Y
1 2 3 4 5 6 7 8) T ST V D E V G F F E L V V K I Y P K G V H P K Y P N G G Q H S QHIL) T S T T D E V G Y F E L V V K I Y P K G T H P K Y P N G G Q M S Q Y L) S S T V D V V G Y F E L V V K I Y F G G V H P R Y P N G G L M S Q Y L) T S A T D A V G H I D L V V K V Y F K D V H P R Y P N G G L M S Q Y L) T S A T D A V G H F D L L T K I Y F K D E D P K Y P N G G L M S Q Y L) T S P V D E V G H F D L L T K V Y F K N E H P K Y P N G G L M T Q Y L) T S M V D E T G Q F E L L V K V Y F R D E H P K Y P N G G L M T Q Y L) T S M V D E T G Q F E L L V K V Y F R D E H P K Y P N G G L M T Q Y L) T S M V D E T G Q F E L L V K V Y F R D E H P K Y P N G G L M T Q Y L) T S M V D E T G Q F E L L V K V Y F R D E H P K Y P N G G Q M S Q Y L
1 2 3 4 5 6 7 8) D S I P I G A F - LD V K G P L G H I E Y Q G K G N F L V H G K Q K -) D S I P I G S F - LD V K G P L G H I E Y Q G K G N F L V H G K Q K -) D S L P I G S T - LEII K G P L G H V E Y L G K G S F T V H G K P K -) D S L P I G S H - I D I K G P L G H I E Y K G K G N F L V S G K P K -) D S L P I G S H - I D I K G P L G H I E Y K G K G N F L V S G K Q R -) D S L P U G G T - I D I K G P L G H V E Y T G R G S F V I N G K Q R -) D S L P V G G Y - I D V K G P L G H V E Y T G R G S F V I N G K Q R -) E S L V V G S S Y I D V K G P L G H V E Y T G R G N F V I N G K Q R -) E S L Q V G S S Y I D V K G P L G H V E Y T G R G N F V I N G K Q R -) E S M Q I G D T - I E F R G P S G L L V Y Q G K G K F A I R P D K K S
1 2 3 4 5 7 7 8) FAKKLAMIAGGTGITPVYQVMQSILKD-PE) FAKKLAMIAGGTGITPVYQVMQAILKD-PE) FADKLAMLAGGTGITPVYQIIQAILKD-PE) FAKKLAMLAGGTGITPIYQVQIIQAILKD-PE) HARRLAMIQAGRGTTPDDDDTEQAVLRDQPE) HARRLAMICQGSGITPMYQIIQAVVRDQPE) RARRLAMICGGSGITPMYQVIQAVVRDQPE) RARRLAMICGGSGITPMYQVIQAVLRDQPE) N PIIRTVKSVGMIAGGTGITPM/LQVIRAIMKD-PD
1 2 3 4 5 6 7 7 8) D D T E M Y V V Y A N R T K D D I L L K D K L D A W A E Q V P N R V K) D D T E M Y V V Y A N R T K D D I L L K E K L D S W A E K I P E R V K) D E T E M Y V I Y A N R T K D D I L L R E K L D G W A E Q Y P D R L K) D E T E M Y V V Y A N R T K D D I L L R E K L E G W A S K H K E R L K) D D T E M H L V Y A N R T D H D H L L R E E I D R A W L P R I R R R K) D E T E M H L V Y A N R T K D D I L L R D K L D R W A A E Y P Y R L K) D E T E M H L V Y A N R S K D D I L L R D K L D R W A A E Y P Y R L K) D E T E M H L V Y A N R S K D D I L L R D K L D R W A A E Y P O R L K) D T E C H L V Y A N R S K D D I L L R P K L E E L R N K H S A R F K
1 2 3 4 5 6 7 7 8) V W Y V V Q E S IIT Q - G H K Y STIG Y V T ES I L R E H I P EF) V W Y V V Q DIS I K EI - G W K Y SI G Y I T E A I L R E H I P E P) V W Y V V - E SAK EI - G WAY STIG Y I SIE A I HR E H I P DG) I W Y V V - EI A K EI - G WAY STIG Y I T E A VL R E H I P E G) V W Y V V - EI A K EI - G W E Y G V T T E A VL R E H I P E G) V W Y V V - S K V P - E D G W E Y G V V D E H V H R E H L P L G) V W Y V I - D Q V K E G W K Y S V G Y V T E A VL R E H V P E G) V W Y V I - D Q V K R P R D G W K F S V G Y V T E D I L R A H V P E G) V W Y Y T - D Q V K R P R D G W K F S V G Y V T E D I L R A H V P E G) L W Y T L - D R A P E - A W D Y G Q G Y V N E E M I R D H L P P P
1 3 4 5 6 7 7 8	1) - SHTTLALACGPPPHIQFAINPNLEKMGYDIKEEL 2) - SHTTLALACGPPPHIQFAVNPNLEKMGYDIKDSL 3) LDGISIALAMACGPPPHIQFAVOPNLEKMGYNIKEDE 4) LEGESLALACGPPPHIQFALOPNLEKMGYNVKEDL 5) - OSETIALVCGPPAHIECTVMPGLEKMGYDLDKAC 5) - GDDTLALACGPPPHIQFAISPNLEKMKYDMANSF 6) - GDDTLALACGPPPHIKIFAISPNLEKMKYDMANSF 6) - EEEFLVUMCGPPPHIOYACLPNLDHVGHP-TERC
	1) $L \vee F$ 2) $L \vee F$ 3) $L I F$ 5) $L \vee F$ 5) $V \vee F$

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used to adjust the sequences for maximum homology, only 41% of the bases were found to be homologous. When p1501 was compared to known amino acid sequences for NR from a variety of species (Figure 23), 75% of the amino acids in the root NR were the same as that found for other sequences. All of the NRs showed fairly high similarity to the human cytochrome *b*5 reductase catalytic domain.

Induction of Maize Root mRNA by Nitrate

Maize kernels were germinated as described in "Materials and Methods". They were grown hydroponically for one day and KNO_3 was added to a final concentration of 10mM two hours into the light period. Roots were harvested just before the addition of nitrate, and then 15, 30 and 60 min afterwards. The roots were cut into 2 segments, a 1cm root tip segment and a segment consisting of the rest of the root up to the kernel which was called the mature root.

Equal amounts of the RNA from these segments were electrophoresed through an agarose gel and blotted onto nitrocellulose. The filter was probed with radiolabeled p1501 DNA and autoradiogaphed (Figure 24). Prior to nitrate addition, little NR mRNA was detectable in the roots. Upon the addition of nitrate, the level of NR mRNA was observed to increase up to 7-fold compared to the no nitrate Figure 24. Induction of NR mRNA in maize root tips and mature roots upon the addition of nitrate to the medium. Seedlings were grown hydroponically as described in "Materials and Methods". The media was adjusted to 10mM KNO₃ and the roots harvested 0, 15, 30 or 60 min later. When harvested, the roots were divided into root tip and mature root segments. Total RNA was extracted from the tissues, transfer blotted and subsequently probed with p1501.



treatment in the tip and up to 2.5-fold in the mature root. Therefore, the p1501 clone hybridizes to a nitrate inducible mRNA of approximately 3kb, the expected size of the mRNA for NR. The appearance and decline of NR mRNA in the root tip was more rapid and reached up to twice the levels found in the mature root. During the period studied, a decline in the mature root mRNA was not seen.

Hybridization of Root and Leaf CDNA Clones to mRNA from Different Maize Organs

Maize seedlings were grown for one day in hydroponic pots, as described in "Materials and Methods", and then induced with 10mM KNO₃ nitrate for 2 hours. When harvested, roots were divided into a 1cm tip portion and a mature portion consisting of the rest of the root up to the kernel, before being frozen. Shoot material was obtained from plants which had been grown for an additional two days, then induced with 10mM KNO₃ for 2 hours. All of the leaf material above the coleoptile was harvested. Total RNA was extracted from the plant material, electrophoresed through a denaturing formaldehyde agarose gel and blotted onto nitrocellulose. Three separate DNA probes were used to analyse the NR mRNA present in the shoots and roots; the leaf cDNA clone (pCIB831), the protein coding region of the

root cDNA clone (p1503) and the 3' untranslated region of the root NR cDNA clone (p1502).

It can be seen in Figure 25, that each probe hybridizes strongly to the mRNA from the organ from which the probe was originally isolated i.e. the leaf probe hybridizes strongly to the leaf mRNA and the root probes hybridize to the mRNA of the two root segments. The root probes appear to be hybridizing to mRNA from both segments of the root approximately equally, suggesting that the NR mRNA is present throughout the root (this was also demonstrated in Figure 25).

It is interesting that there appears to be some hybridization of each probe to the mRNA from the other organ. This filter was washed under stringent conditions that should have tolerated very few mismatches. It was shown earlier (Figure 22) that the 3'untranslated region present in p1502 does not show any homology to the leaf cDNA clone. However, there is some hybridization of p1502 to the leaf mRNA suggesting that there may be some crosstranscription of the root message in the leaves. The leaf probe also appears to be weakly hybridizing to the root mRNA, again suggesting cross-transcription of the leaf NR mRNA in the root. Figure 25. Hybridization of root coding region, root noncoding region and leaf NR cDNA clones to mRNA from maize leaves and roots. Plants were grown hydroponically with 10mM KNO₃ as described in "Materials and Methods". Shoots were harvested above the coleoptile, and the roots were separated into root tip and mature root segments. Total RNA was extracted from these organs, transfer blotted and then probed with the appropriate cDNA clone.

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p831p15O3p15O2TMLTMLTML



Hybridization of Root and Leaf NRs to Separate Genes

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Genomic DNA was isolated from maize leaves, digested with 3 different restriction enzymes and blotted onto nitrocellulose as described in "Materials and Methods". The DNA of the different digests was probed on different lanes of the same filter with either the leaf NR cDNA clone or the root NR cDNA clone. The results are shown in Figure 26. It is clear that the two clones do not hybridize to the same bands and that they must therefore be encoded by separate genes. The low number of bands which hybridized with either clone suggest that there are very few copies of each gene in the genome. Figure 26. Southern blot of maize genomic DNA probed with leaf and root NR cDNA clones. Genomic DNA was extracted from maize leaves, digested with a)Hind III b)Eco RI and c)Bgl II and the digests transfer blotted as described in "Materials and Methods". The filters were probed with p1503 and p831.

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Discussion

The identification of a maize root NR partial cDNA clone was facilitated by the availability of a maize leaf NR partial cDNA clone. The largest cDNA clone that we identified, p1501, is approximately one-third the size of a full length cDNA clone. When the sequence of this clone was compared to the sequence of the maize leaf NR clone which had been identified by Gowri and Campbell (1989), we found no homology in the 3' non-coding region with the coding regions being 72% homologous (Figure 22). When this comparison was extended to other known amino acid sequences of NRs from other species (Figure 24), all of the NRs compared show a high degree of homology suggesting that NR has been highly conserved.

The amino acid sequences of a variety of clones have been compared to sequences of proteins of known function to try to determine the function of various parts of the NR coding sequence (Calza <u>et al.</u>, 1987; Crawford <u>et al.</u>, 1988; Daniel-Vedele <u>et al.</u>, 1989; Vaucheret <u>et al.</u>, 1989). It had been shown previously by Le and Lederer (1983) that the amino acid sequence of the heme-binding region of <u>Neurospora</u> <u>crassa</u> NR showed strong homology to members of the

cytochrome b5 superfamily. This appeared to be true of the higher plant NRs as well (Calza <u>et al.</u>, 1987). Homology between NR sequences and the molybdenum-pterin-binding domain of rat liver sulfite oxidase was found in the region of the NR sequence identified as the molybdenum-pterinbinding domain. A high degree of homology was also found between the FAD/NADH domain of NR and human erythrocyte cytochrome b5 reductase (Calza <u>et al.</u>, 1987; Crawford <u>et</u> <u>al.</u>, 1988; Daniel-Vedele <u>et al.</u>, 1989; Vaucheret <u>et al.</u>, 1989). Using the regions of homology as a guide, the division between the various NR domains has been determined at the sequence level (Daniel-Vedele <u>et al.</u>, 1989).

The maize root clone contains the sequence for the FAD-binding domain. When compared to the sequence for cytochrome b5 reductase (Figure 24), a high degree of homology can be seen between the two sequences. A catalytic thiol has been shown to have an important role in the binding of NADH to NR and other dehydrogenase activities (Barber and Solomonson, 1986). This is also thought to occur in cytochrome b5 reductase (Hackett <u>et al</u>., 1988). There appears to be only one conserved cysteine residue in the C-terminal domain of all of the NR sequences and the cytochrome b5 reductase sequence (Figure 24). This cysteine residue is found at the beginning of a long conserved

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sequence CGPP(P or A)MI (Daniel-Vedele <u>et al.</u>, 1989). Hackett <u>et al</u>. (1988) identified another amino acid residue (lysine-110) which appeared to be important in the binding of NADH to cytochrome b5 reductase. This residue is found to be conserved in the maize root NR sequence and in all of the other NR sequences which were compared.

The inducibility of NR mRNA levels by the addition of nitrate to seedlings has been demonstrated for each of the cloned NRs either by in vitro translation of mRNA and immunoprecipitation of the products with antibodies (Cheng et al., 1986; Calza et al., 1987) or by RNA blot hybridization of mRNA (Cheng et al., 1986; Crawford et al., 1986; Calza et al., 1987; Crawford et al., 1988; Gowri and Campbell, 1989; Melzer et al., 1989). When the maize root NR clone was used as a probe to look at the induction of NR mRNA in maize root segments, it was again apparent that nitrate had an effect at the mRNA level. The pattern of induction found for NR mRNA in root tips and mature roots is interesting not only because it shows a difference in the rates of expression between the two segments, but also this induction by nitrate is occuring much quicker than in any other systems yet studied. So far only Melzer et al. (1989) has looked at the expression of NR mRNA in roots as well as leaves. In their 7 day old barley roots, the NR mRNA peaked

at approximately 2 hours after induction while the leaves took 12 hours to reach maximal levels. The induction in the maize root tips appears to be very rapid, but these are younger plants, and there may be differences between genera and the concentrations of nitrate used. The differences between the tip and mature segments could be due to many factors such as uptake of nitrate, transport of nitrate between and within cells or differences in the action of regulatory proteins necessary for the production of mRNA. The rate of overall metabolism of the cells may also be different. Though the results clearly show that nitrate has an effect on NR at the mRNA level, it still has not been determined whether this increase is actually due to an increase in transcription or if it is due to a change in the stability of the transcripts.

Maize appears to contain at least two separate genes for NR as shown on a genomic blot (Figure 26) and by analysis of sequence data of cDNA clones (Figure 24). The two genes which have been identified appear to be predominantly expressed in either the roots or the shoots, but there does appear to be a small amount of crosstranscription (Figure 25). In barley, a single NADH:NR gene is expressed in both the roots and shoots of wild type plants. As has been found with maize, an NAD(P)H:NR is

expressed in the roots as well (Warner <u>et al</u>., 1987; Melzer <u>et al</u>., 1989). In <u>Nicotiana plumbagnifolia</u>, only one gene has been found which encodes NR (Gabard <u>et al</u>., 1987). Cheng <u>et al</u>. (1988) have identified clones for two different NR genes in the same species. They isolated two genomic clones in <u>Arabadopsis thaliana</u> which appeared to be closely related, but the localization of expression of these two genes was not determined.

Chapter 5: Localization of Nitrate Reductase in Maize Roots

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Introduction

The process of nitrogen assimilation has been investigated in many plant systems. The enzymes which reduce nitrate and assimilate ammonia have been identified and their regulatory properties examined (for review see Guerrero <u>et al.</u>, 1981; Beevers and Hageman, 1983; Oaks and Hirel, 1985). However, the localization of these enzymes at both the intra- and intercellular level has not been clarified completely.

Early attempts to localize NR within cells relied on the use of sucrose density gradients to separate organelles from the cytosol. Grant <u>et al</u>. (1970) and Dalling <u>et al</u>. (1972) found the majority of NRA in the supernatant of spinach, sunflower and tobacco leaves with only a minor component associated with the chloroplasts. In the roots of maize (Oaks and Gadal, 1979), rice, bean, pea and barley (Suzuki <u>et al</u>., 1981) NR was again found to be in the supernatant fraction suggesting that it was localized in the cytosol. Miflin <u>et al</u>. (1970), however, found that NRA in their barley root preparations migrated with NiRA in a particulate fraction which they could not identify. This result was subsequently shown to be an artifact of bacterial contamination by Blevins <u>et al.</u>, (1976). Recently Fischer and Klein (1988) were able to isolate intact chloroplasts from <u>Chlamydomonas reinhardtii</u>. Nitrate reductase was the only enzyme involved in nitrogen assimilation which did not appear to be localized in these chloroplasts.

The use of immunocytochemical techniques to try to confirm the results of the biochemical experiments has been marginally successful. Roldan et al. (1982) found NR to be localized in the cell wall-plasmalemma region and in tonoplast membranes of mycelial cells in <u>Neurospora crassa</u>. They suggested a role of NR in the absorption of nitrate in fungi. When Lopez-Ruiz et al. (1985a,b) tried to localize NR in the green algae Monoraphidium braunii, their antibody labeled the pyrenoid region of the chloroplast. The first study on higher plants of this type, used soybean cotyledons which had been super-induced for NR with norflurazon (Vaughn et al., 1984). Most of the label was found clustered in the cytoplasm with a small amount associated with the plastids. The effect of norflurazon on the types and distribution of NRs within cotyledons is not well understood. Nitrate reductase was subsequently localized in the stroma of chloroplasts in spinach leaves (Kamachi et al., 1987) and in the cytosol of mesophyll cells of maize leaf tissue (Vaughn and Campbell, 1988). This last result agrees with the

results of Harel <u>et al</u>. (1977) where both NR and NiR were found to be restricted to mesophyll cells in <u>Zea mays</u> by using a biochemical separation of the cell types. However, Gowri and Campbell (1989) subsequently found that their antibody was able to react with other proteins such as NADP⁺:glyceraldehyde-3-phosphate dehydrogenase. There are inherent problems in using antibodies to label proteins. Preparations may contain contaminants which confound results or, because of the nature of the protein to which the antibody was made, antibodies could be formed which will react with other proteins with regions or domains similar to the protein of interest. The conditions in which the antibodies are used can also alter the results (Solomonson and Barber, 1990).

In maize roots, the localization of NR within the different regions and cell types of the tissue has been a subject of interest and controversy. Early results showed that the highest levels of NRA were in the root tip with very little activity measurable in the mature regions of the root (Oaks <u>et al.</u>, 1972, 1980; Oaks, 1979; Polisetty and Hageman, 1983). Wallace (1975) found that by adding 3% casein to his extraction buffer, NRA could be measured in the mature regions as well as in the root tips in maize. He postulated that the casein was acting as a substrate for

proteolytic activity within the root extracts. After adding the protease inhibitor chymostatin to my extraction buffer, even higher levels of NRA have been recovered in mature portions of maize roots than in the tips (Long and Oaks, 1990; Chapter 1). Rufty <u>et al</u>. (1986) used a micro-surgical technique to separate the cells of the epidermis, cortex and stele and then measured each for the presence of NRA and NRP. When they used low concentrations of nitrate (0.2mM) to induce the plants, all of the NR was found in the epidermal cells. With a higher concentration of nitrate (20mM), NR was found in all of the cells. However, the majority of the enzyme activity and protein was still present in the epidermis.

In this chapter, attempts to localize NR in maize roots using immunocytochemical methods and a method for localizing the expression of NR mRNA in maize roots by the use of the tissue print hybridization technique originally developed by Cassab and Varner (1987) are described.

Materials and Methods

Growth Conditions of Plants

Maize kernels were germinated in large petri dishes (15cm diam.) which contained 1% agar and 1/10 Hoagland's solution with or without the addition of 10mM KNO₃. The plates were incubated in a growth cabinet at 28°C for 48 hours without supplemental light. At this time the roots were approximately 3cm long and could be prepared for electron microscopy. Samples of endosperm and scutellum were also prepared from these seedlings.

For tissue blotting, the two day old seedlings were transferred to hydroponic pots which contained 1/10Hoagland's solution. The plants were placed in the growth cabinet for an additional 48 hours at 28° C with a 16 h light/ 8 h dark cycle. On the second day in hydroponics, approximately 4 hours into the light period, KNO₃ was added to some of the pots to a final concentration of 10mM. Control plants had no nitrogen supplement. Plants were induced with KNO₃ for up to 1 hour before they were tissue blotted onto nitrocellulose.

Leaf material was prepared for electron microscopy from plants which had been grown hydroponically as described above with the exception that the plants were grown for a total of 4 days in hydroponics with 10 mM KNO_3 .

Nitrate Reductase Activity Assay

Plant material was extracted and assayed for nitrate reductase activity by the methods described in Chapter 1 with the exception that leupeptin, not chymostatin, was used in the extraction buffer as a protease inhibitor when leaf samples were extracted.

Tissue Preparation for Electron Microscopy

Maize roots were prepared for electron microscopy by a protocol previously developed as part of the requirements for the course Biology 764 modified from the method described by Greenwood and Chrispeels (1985). Root sections were prepared by removing and discarding the first 8mm of the root tip under a drop of fixative. The adjacent 4mm portion of the root was cut under fixative into 1mm pieces. These pieces of tissue were transferred to a larger volume of fixative (approximately 3-5 mL) for 2 h at 22°C, then for 14 h at 4°C. The fixative was made up of 3% paraformaldehyde, 0.3% glutaraldehyde and 25mM sucrose in 25mM KPO₄ (pH 7.2). After 4X 15 min washes with 25mM sucrose in 25mM KPO₄ (pH 7.2) at 4°C, the tissue was postfixed in 1% OSO_4 and 25% sucrose in 100mM KPO₄ (pH 7.2) at 4°C. This was followed by 4X 15 min washes with doubledistilled water. Dehydration was completed by 30 min steps of 10, 30, 50, 70, 80, 90, 95 and 100% EtOH. The tissue was infiltrated with gentle mixing by first placing the tissue into a mixture of 2 volumes of 100% EtOH and 1 volume of either Spurr's resin or L.R. White resin for 30 min (a total of approximately 5mL), then into a mixture of 1 volume of EtOH to 1 volume of resin for 30 min and then a third mixture of 1 volume of ethanol to 3 volumes of resin for 2 h. This was followed by eight changes of 100% resin over the next 48 h. The tissue was polymerized for 24 h at 60°C in gelatin capsules.

Immunocytochemical Labeling

a) Antibodies and Labels

A polyclonal antisera which had been made against purified maize leaf nitrate reductase in rabbits (Poulle and Oaks, unpublished results) was used in the labeling experiments. The antibody was passed through a column of CNBr-activated Sepharose 4B to which BSA had been bound by standard protocols (Pharmacia). It was hoped that the column would remove any epitopes in the antibody preparation which might bind to BSA or related proteins as it had been

shown previously that the antibody did react with BSA on Western blots (unpublished results). The maize NR antibody was preadsorbed with an equal volume of pure NR or 1% soluble starch for 1 h at 4°C and then used as a control in place of the primary antibody. Barley NR-monospecific antibody was obtained from the labs of R. Warner and A. Kleinhofs and spinach NR antibody was from the lab of H. Nakagawa. Colloidal gold conjugated to Goat anti-Rabbit IgG was provided by Dr. J.Greenwood.

b) Post-embedding Treatment

The method used for the post-embedding treatment was derived from the method of Greenwood and Chrispeels (1985). Sections (70-100nm in thickness) were collected on Formvar coated nickel grids. The grids were floated, section side down, on drops of the following solutions: 10 min on saturated aqueous NaIO₄, 5X 1 min washes on double-distilled water, 10 min on 0.1N HCl, 6X 1 min washes on PBST (10mM KPO₄ pH 7.2, 500mM NaCl, 0.2% Tween 20), 10 min on PBST and 2% gelatin, 6X 1 min washes on PBST, 10 min on 1° antibody diluted with PBST and 2% gelatin, 7X 1 min washes with PBST, 30 min on Protein A-gold (diluted with PBST and 2% gelatin), 10X 1 min washes on PBST, 6X 1 min washes with doubledistilled water, 1 min on 1% glutaraldehyde in 0.1M KPO₄ (pH 7.2), 6X 1 min washes with double-distilled water, stain 10
min with saturated aqueous uranyl acetate and rinse with double-distilled water. Preimmune serum was used in place of the 1° antibody as a control in each trial.

Preparation of Tissue Prints

Tissue prints were prepared by the method of McClure and Guilfoyle (1989) with a few minor modifications. Maize roots were cut longitudinally through the center of the root under a dissecting microscope using a thin, sharp razor blade (double edged-razor blades were broken in half) and a piece of wax as the cutting surface. The cut root was rinsed in double-distilled water and blotted gently on kimwipe tissue to remove any excess water. The root was then pressed, cut side down, on a piece of dry nitrocellulose (MSI) which was mounted on a piece of Whatman 3MM paper for 2.5 min. A small glass plate was placed on top of the root so that the root would be subjected to even force while blotting. Firm pressure was applied manually, without squashing the tissue. The root was removed carefully from the nitrocellulose with a pair of forceps and discarded. The tissue print was allowed to air dry. Once dry, the prints were placed between 2 sheets of Whatman 3MM filter paper and baked for 1 hour at 80°C.

Prints could be kept at room temperature for at least two weeks before staining and hybridization with a probe.

Staining of Tissue Prints with India Ink

Tissue prints were stained as described by McClure and Guilfoyle (1989). However, the staining was done before the hybridization procedure, rather than afterwards as done by McClure and Guilfoyle (1989) because the India Ink reacted with components of the hybridization solution. The tissue prints were rinsed repeatedly in ice water until the nitrocellulose membrane was uniformly wet. This could require soaking for up to 3 or 4 min, particularily if the prints had been kept at room temperature for a few weeks before staining. The prints were then immersed in ice cold India ink for 1 to 2 min. This was followed by destaining in ice cold water (usually 250mL of water was sufficient to rinse 2 blots but then it was necessary to change the water) and then rinsing in 2 changes of 0.2X SSC and 1% SDS (500mL The blots were placed immediately in the prewash each). solution, as described in the following section, without drying. This appeared to reduce the background from both the India Ink and the probe.

Hybridization Procedure

Freshly stained tissue prints were prewashed with 2 changes of 500mL of 0.2X SSC and 1% SDS for 4 to 8 hours at 65°C. The prints were prehybridized for 20 to 24 hours at 65°C in 10mL of RNA Prehybridization buffer (3X SSC, 5X Denhardt's solution, 20mM Tris-HCl pH 8.0, 0.1% SDS, 2mM EDTA) with the addition of 0.5mg/mL denatured, sheared salmon sperm DNA and 40µg/mL tRNA. A fresh 5mL of the same solution was used for hybridization of the blots with the probe. The maize root NR cDNA insert of the plasmid p1501 was labeled by random priming with ³⁵SdATP (Amersham) to a specific activity of 4-8 x 107 cpm/ug (see Appendix 19). The probe was boiled for 10 min and then cooled on ice for 10 min before addition to the hybridization buffer. The prints were hybridized overnight at 65°C. The next day, the tissue prints were washed 2X for 30 min with 500mL of 2X SSC and 1% SDS at room temperature followed by 2 more 30 min washes with 500mL of 0.2X SSC and 1% SDS at 65°C. The prints were air dried on Whatman 3MM paper and were then exposed directly to Kodak XAR-5 film at -70°C.

Results

Immunocytochemical Localization of Nitrate Reductase

The method used to prepare maize root sections involves the use of a mild fixation procedure designed to retain as much antigenicity of proteins as possible, while maintaining good ultrastructural preservation of the tissue. Once the tissue was fixed, it was postfixed with osmium tetroxide, dehydrated and then infiltrated with either Spurr's resin or L.R. White resin. Though L.R. White resin is often recommended for immunocytochemical localization studies (Craig and Miller, 1984) the Spurr's resin was found to infiltrate the tissue more successfully and to polymerize evenly whereas the L.R. White was often found to be chippy. Thin sections were cut and subjected to a post-embedding treatment. This procedure uses an oxidizing agent (NaIO4) to remove OsO_4 and reopen antigenic sites that may have been lost through crosslinking in the fixation procedure. The sections were then treated with various antibody combinations.

When an NR-specific antibody was used as a label with GAR IgG-tagged gold as its electron dense marker, the label was found in the amyloplasts associated with starch grains (Table 17, Figure 27). When preimmune serum (Figure 28) or GAR IgG-tagged gold alone (Table 17) was used in place of the NR-specific antibody no label was found on the sections. These controls suggested that the NR-specific antibody was reacting with a specific antigen on the sections. Because this was such an unexpected result, we decided to look at other organs in maize and to do more controls. As can be seen in Table 17, the amount of label on the plastids did not correlate with the amount of activity in the organ examined. In addition, the appearance of label in bundle sheath cells and not mesophyll cells, as expected by the results of Harel et al. (1977) could not be explained. The results became even more difficult to explain when the NR-antibody was preadsorbed with pure NR, because it would no longer bind to the sections as would be expected if the NR epitopes were now bound to the NR. Any epitopes which do not bind to pure NR should have reacted with the sections. When the Ab was preadsorbed to soluble starch, it labeled the sections in the same pattern as when it was not preadsorbed. These controls suggested that whatever antigen was being labeled on the sections could be found in a pure NR preparation, but not in soluble starch. Barley NR-specific Ab and spinach NR-specific Ab were also used but did not clarify the results as the barley Ab was

Table 17. Analysis of Nitrate Treated Maize Organs for their NRA and for the Amount of Label Observed on Plastids in Sections of the Organs.

	Maize organ				
	root (1	root uninduced)	endosperm	scutellum	leaf
NRA (µmols NO ₂ prod/h/GFW)	1.91	N.D. ^a	N.D.	2.23	6.41
primary_antibody		Amount	of label or	n plastids	
Maize NR-specific Ab	++++	+++	+++	+++++	+++
Maize NR-specific Ab preadsorbed with pure NR	N.D.	ĸ.D.	N.D.	N.D.	N.D.
Maize NR-specific Ab preadsorbed with starch	++++	+++	+++	+++++	+++
Preimmune serum	N.D.	N.D.	N.D.	N.D.	N.D.
GAR IgG-gold	N.D.	N.D.	N.D.	N.D.	N.D.
Barley NR- specific Ab	+++ (on	cell wall)		
Spinach NR- specific Ab	N.D.				

^a N.D. signifies that there was either no detectable NRA in the organ or no label on the sections.

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Figure 27. Electron micrograph of nitrate induced maize root cortical cells labeled with maize NR-specific Ab and GAR IgG-gold (X 42,500).

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Figure 28. Electron micrograph of maize root cortical cells labeled with preimmune serum and GAR IgG-gold (X 35,000).

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found on the cell walls. No label was found when the spinach Ab was used. The results of these experiments are difficult to explain in that the labeling on the starch grains appears to be specific. The localization of the label and the label appearing in tissues without NR suggests that our antibody is contaminated with epitopes to proteins constitutively expressed in our pure NR preparation. As discussed in Chapter 4, NR contains 3 large domains which show homology to other proteins. Antibodies made to these domains could cross-react with other proteins in the tissue sections with similar domains.

Tissue Printing of Maize Roots

The expression of NR mRNA in maize roots was localized by using the tissue printing technique. There were certain steps which appeared to be of particular importance in making this technique work. Nitrocellulose was found to be a better membrane for making prints than either Zetabind or Hybond nylon membranes. It gave a clearer imprint of the tissue, and the background from India Ink staining was much lower than with the nylon membranes. Staining of the prints with India Ink was changed to just before the prewash step. The buffer used for the prehybridization and hybridization steps interfered with the staining. Also, if the prints were kept wet after staining, and incubated for at least 4 hours in prewash buffer, background from the staining procedure was reduced, the prints appeared to hybridize better with the probe and the background was significantly reduced.

A variety of hybridization buffers were tried and the best results were found with a buffer which has been referred to in this thesis as RNA Prehybridization buffer (Chapter 4). The addition of a high concentration of salmon sperm DNA and tRNA were crucial to keeping the background from the hybridization reduced to a minimum. The most difficult obstacle to overcome with this technique was the high background levels i.e. non-specific binding of the probe to the tissue print. The use of a prewash, a long prehybridization step and the inclusion of high levels of blocking agents were necessary to overcome this problem.

Expression of NR mRNA in Maize Roots

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Tissue prints of roots grown with or without nitrate, were hybridized with a cDNA clone of maize root NR. The results are shown in Figure 29. After either 30 or 60 min of induction with nitrate, NR mRNA was found to be localized predominantly in a region begining 1-2cm above the root tip and continuing for another 1-2cm. Some mRNA was

Figure 29. Tissue prints of maize roots. Maize roots were grown hydroponically without a nitrogen supplement until the day of harvest. Roots were sectioned at 0 time, or after the addition of a final concentration of 10mM KNO₃ to the pots. Prints were probed with p1501. a) represents the India Ink stained image of b). b) is the autoradiographic image of a root exposed to nitrate for 30 min and c) is the image of a root exposed to nitrate for 60 min. d) is the autoradiogram of a root which did not receive a nitrogen supplement.

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also found in the mature regions of the root. The NR mRNA appeared to be expressed in cells throughout these regions with slightly higher levels apparent in the epidermal cells. If nitrate was not added to the roots, no expression was seen (Figure 29).

Discussion

The localization of NR in the starch grains of amyloplasts was an unexpected result in light of the biochemical and other immunocytochemical localization studies which have previously been reported (Grant <u>et</u> <u>al</u>., 1970; Dalling et al., 1972; Oaks and Gadal, 1979; Suzuki et al., 1981; Vaughn et al., 1984; Kamachi et al., 1987; Fischer and Klein, 1988; Vaughn and Campbell, 1988). The NR Ab appeared to label the starch grains in a specific manner as demonstrated with the controls i.e the use of preimmune serum or the preadsorption of the antibody with either pure NR or starch showed no label on the sections. The NR antibody also reacted with plant tissues that contained no detectable NRA but contained starch grains. Another unexpected result was found with maize leaves where label bound to the starch grains of bundle sheath cells, as nitrate reductase had been previously demonstrated to be localized in mesophyll cells of maize (Harel et al., 1977; Vaughn and Campbell, 1989). These results could be due to the particular conditions that were used for this experiment, the presence of a major contaminant in the pure NR preparation or the presence of antibodies which cross-

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react with proteins which contain similar domains to those found in NR. Nitrate reductase is a minor protein in plant tissues and this is likely to have been a factor in our results as well as the results of others who have tried to use immunocytochemical techniques to localize NR.

The development of the tissue print hybridization technique by Cassab and Varner (1987) has added a new, and what appears to be a very powerful technique for the rapid screening of a multitude of compounds in a tissue or organ sample which can bind to a membrane such as nitrocellulose. The technique can be applied to any tissue which is firm enough to be pressed onto a membrane without losing the definition of that tissue. It has been used to localize proteins with labeled antibodies, RNA with labeled nucleic acid probes, glycoproteins by flourescent lectins, soluble fluorescent compounds by direct observation and a variety of other compounds (Cassab and Varner, 1987; Taylor <u>et al</u>., 1989; Varner and Taylor, 1989).

The methods of McClure and Guilfoyle (1989), which had been originally developed to monitor the expression of auxin-induced RNAs in soybean seedlings were adapted to the maize root system. The localization of the expression of NR mRNA in the region beginning 1-2cm above the root tip and extending to a lesser extent throughout the mature root

agrees with my previous observation that NRA can be found throughout the root tissue including the mature segments. The absence of NR mRNA expression in the root tips either 30 min or 1 hour after induction was somewhat suprising. The induction of NR mRNA in the tip region was demonstrated in a previous chapter. However, it is quite possible that the plant material used for the extraction of RNA in this earlier experiment included some of the region which appeared to be highly inducible on the tissue blots. Another possibility is that a different gene is being expressed in the root tips and that under the stringent conditions used for hybridizing and washing of the tissue blots, the expression of this gene was not seen.

In the tissue prints, there appears to be higher expression of the NR mRNA in the epidermal cells than in the other cell types, though expression appeared to be present throughout the root. This observation agrees with the findings of Rufty <u>et al</u>. (1986). When this group used a micro-surgical technique to separate different cell types in maize roots after induction with nitrate, NRA and NRP were found predominantly in epidermal cells of roots induced with low concentrations of nitrate (0.2mM). When 20mM nitrate was used, NRA and NRP were found in each group of cells, but still at a higher concentration in the epidermal cells. The

results here, with $10 \text{mM} \text{ KNO}_{3}$, confirm at the level of gene expression the results obtained with 20 mM nitrate by Rufty <u>et al</u>. (1986).

GENERAL DISCUSSION

In maize roots, two isoforms of NR were identified in agreement with the results of Redinbaugh and Campbell These isoforms are an NADH monospecific form and an (1981). NAD(P)H bispecific form. The NAD(P)H:NR appears to be the dominant form throughout the root with the exception of the 1cm tip region. It has a low K_m for reductant, which is similar to the values found for other genera (Table 18). The NADH form, on the other hand, has an unusually high K_m The NAD(P)H:NR isoform may have a competetive for NADH. advantage because of its ability to use either reductant source. Emes and Bowsher (1991) suggest that NADPH is generated directly into the cytosol by the pentose phosphate pathway and it may therefore be readily available for use by NR. Each of the other biochemical characteristics examined for both the leaf and the root are very similar to the characteristics found in other genera (Table 18).

When NRA in maize roots was first measured, it was found that significant levels of NRA could be measured only in the root tip (Oaks <u>et al.</u>, 1972, 1980; Oaks, 1979; Polisetty and Hageman, 1983). The presence of NRA throughout the length of the root was first suggested by

Table 18. Survey of K_ms , Molecular Weights and pH Optima of Higher Plant NRs.

Species	pH optima	Native Molecular Weight	K _{m for-} NO ³	K _m for reductant
soybean leaf (NADH:NR) (NAD(P)H):NR)	6.5 6.5	300,000 220,000	110µM 4500µM	NADH 8.1µM NADH 1.5µM
(JOILY et al., 1975)	c 95		100-01	NNN 7 4-14
(NAD(P)H):NR) (Dean and Harper, 1988)	6./5		490µM	NADPH 7.2µM
wheat leaf (NADH:NR (Sherrard and Dalling, 1) 7.5 979)			NADH 33µM
barley leaf w.t.(NADH:NR nar1a (NADPH nar1a (NADH (Kuo <u>et al</u> ., 1982; Harke) 7.5) 7.7) 7.7 er <u>et al</u>	221,000 ., 1986)	130µМ 610µМ 620µМ	NADH 10אµM NADPH 10אµM NADH 68אµM
barley root (NADH:NR (Oji <u>et al</u> ., 1988)	.) 7.5	200,000	130µМ	NADH 2.6µM
spinach leaf (NADH:NR (Fido, 1987; Hewitt and) 7.5 Notton,	1980)	Mىر180	NADH 4.6µM
maize scutellum (NADH:NR (NAD(P)H:NR (Campbell, 1978)	() 7.5 () 7.5		200µM 600µM	
maize roots (NADH:NF (NAD(P)H:NF (Redinbaugh and Campbel)	l) 7.5 l) 7.5 ., 1981)	300,000 or 190,000 300,000 or 190,000	70אַע 300אַע	
maize roots (NADH:NR) (NAD(P)H:NR)	7.0 7.0	263,000 263,000	100µM 251յ։M	NADH 943µM NADH 15.5µM
(this investigation)				NADPH 14.5µM
<pre>maize leaf (NADH:NR) (this investigation)</pre>	I	263,000	109µM	NADH 13.3µM

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throughout the root when he added 3% casein to extraction buffer. This suggested that the casein was acting as a substrate to proteases which were released upon extraction of the tissue. The addition of chymostatin to extraction buffer has shown even higher levels of NRA in roots, particularily in the mature regions of the root. The use of the maize root NR cDNA clone to examine expression of NR mRNA in tissue blots of maize roots, supported these results as NR mRNA was found to be expressed throughout most of the length of the root. The elevated levels of NR mRNA in the epidermal cells agree with the findings of Rufty <u>et al</u>. (1986) who found the majority of NRA in the epidermis by micro-surgically separating cell types.

Maize root NRs are inducible by nitrate at the level of transcription, protein synthesis and appearance of activity. Nitrate reductase mRNA appears rapidly upon the addition of nitrate (10mM) to the system, but begins to decline again, in the tip, within an hour of induction. The levels of NRA, however, are slower to be induced, and continue to increase slowly for at least 10 hours until they reach steady-state. If nitrate is removed from the system, NAD(P)H:NRA is rapidly lost in the root tips as compared to the loss of NADH:NRA in this portion of the root. The loss in the tip is due at least in part to a dilution effect by

new cells produced after the removal of nitrate. In the mature part of the root, there is very little loss of NRA over a 6 hour period. These results differ from those of Oaks et al. (1972) who found high rates of turnover in the mature portion of the roots. This difference is accounted for by the improved extraction procedures developed in the course of this research. The role of MRP in vivo is still to be determined. However, in vitro it has been shown to inactivate NR rapidly (Wallace, 1973; Wallace and Oaks, 1985). It now appears that NR protein undergoes a slow rate of turnover in vivo, particularily in the mature portions of the root. Unlike NR in maize leaves, root NR does not appear to be regulated by a circadian rhythm or by light. The absence of light for an extended period, 2 days, is required for the complete disappearance of NR. By this time, carbon and the derivative reductant supplies have probably been depleted.

Whereas maize shoots contain an NADH:NR, the roots appear to have two isoforms of NR, an NADH:NR and an NAD(P)H:NR. In barley the NADH isozyme is encoded by the nar1 gene in both shoots and roots and the NAD(P)H isozyme is encoded by the nar7 gene in the roots of wild type plants. In maize, there appears to be some crosstranscription of the different genes in the roots and

shoots, however, the shoot NADH:NR cDNA clone does not hybridize strongly to root mRNA under stringent conditions as would be expected if the same gene was being expressed for the root NADH:NR. The NADH:NR isoforms in the roots and shoots also show distinct biochemical characteristics. At present, it is not known which isozyme the root cDNA clone encodes.

Nitrate reductase in maize roots is found, through the use of improved extraction procedures, to be at levels high enough to account for a significant level of reduction as had been previously suggested by Gojon <u>et al</u>. (1986) and Pate (1973). Using $({}^{15}N)NO_{3}^{-}$, Gojon <u>et al</u>. (1986) found that upon induction, 70% of the whole plant nitrate reduction took place in the roots of maize seedlings. After steadystate conditions were reached, both Gojon <u>et al</u>. (1986) and Pate (1973) found that roots accounted for approximately one-third of the whole plant reduction of nitrate. The results from the present study suggest that there is sufficient NR in maize roots to account for the reduction of nitrate in intact tissue.

The areas of research which are receiving the most attention with respect to nitrate reductase and the regulation of nitrate metabolism at present are centered around a) the attempt to identify the protein(s) involved in

the uptake of nitrate into plants, b) identification of the components involved in the transduction of the nitrate 'signal' to NR or NiR, c) identification of the cis-acting regions involved in the regulation of NR and NiR and d) the further elucidation of the structure of NR and the overall regulation of the enzymes of nitrogen assimilation and how they interact with the other metabolic pathways of plants is being examined. The genes and subsequently the proteins involved in nitrate uptake may be identified through functional complementation of mutants such as those identified by Oostindier-Braaksma and Feenstra (1972, 1973), Wallsgrove (1987) and Doddema and Telkamp (1979). Deletion analysis and the use of point mutations should elucidate the 5' upstream regions of NR and NiR which are necessary for the regulation of these enzymes. The identification of trans-acting factors may prove more difficult as intensive study has still not provided any mutants. At present, little is known about how NR is affected by factors other than nitrate and light or how carbon metabolism and reductant supply interact with nitrogen metabolism. These topics require much work at the molecular and biochemical level to try to elucidate pathways, limiting factors and the regulatory elements involved.

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In conclusion, the major findings of this thesis can be summarized as follows:

- 1) Maize roots contain two isozymes of NR.
 - a) An NADH:NR found predominantly in the root tip which has a very high K_m for NADH.
 - b) An NAD(P)H:NR which was found throughout the mature portions of the root which may have a competetive advantage due to its ability to use either NADH or NADPH as reductant.
- 2) Once NR was stabilized with chymostatin, it was found to be present throughout the root at levels which could account for significant reduction of nitrate in this organ.
- 3) Maize root NR is inducible by nitrate at the levels of transcription, protein synthesis and activity. Unlike leaf NR, it is not regulated by a diurnal rhythm or light.
- 4) At least one of the isozymes in roots is encoded by a gene separate from the gene which encodes leaf NR. The mRNA of this gene is expressed throughout the majority of the root.

LIST OF APPENDICES

- 1. Commonly used solutions and media.
- 2. Large scale preparation of RNA.
- 3. Miniprep of RNA.
- 4. Formaldehyde Agarose RNA gel.
- 5. Transfer of Formaldehyde-denatured RNA to nitrocellulose membrane.
- 6. Transfer of DNA from agarose gel to nitrocellulose membrane.
- 7. Large scale alkaline plasmid prep.
- 8. Alkaline miniprep of plasmid DNA.
- 9. Isolation of Genomic DNA.
- 10. DNA transfer and hybridization protocol of genomic DNA on Zetabind membranes.
- 11. Plaque lifts of λ DNA.
- 12. Isolation of DNA fragments from low melt gels.
- 13. BRL rcommended procedure for labeling DNA by nick translation.
- 14. Preparation of λ DNA from plate lysates.
- 15. Purification of λ DNA by differential centrifugation.
- 16. Alkaline phosphatase treatment of vector DNA.

- 17. Preparation of competent cells.
- 18. Protocol for sequencing using the Sequenase kit.
- 19. Labeling of DNA probes by random priming.

1. Commonly Used Solutions and Media

50X Denhardt's Solution	for 500mL
Bovine serum albumin	5g
Ficoll	5g
PVP (type 360)	5g

filter sterilize solution through a 0.45 μ m fliter

10X Dye Buffer

10X TBE
20% Ficoll
0.1% Bromophenol blue
0.1% Xylene cyanol

λ Buffer	for 1L	
NaCl	5.8g	
MgSO ₄ .7H ₂ O	2.0g	
1M Tris-HCl pH 7.5	50mL	
2% gelatin	5mL	

L Broth (LB)	for 1L
Tryptone	10g
Yeast extract	5g
NaCl	5g

.

 20X SSPE
 for 300mL

 3.6M NaCl
 63.1g

 0.2M NaPO₄ pH 7.7
 120mL of 0.5M

 20mM EDTA pH 8.0
 12mL of 0.5M

 Note: make up 0.5M Na₂HPO₄ and 0.5M NaH₂PO₄. Add NaH₂PO₄ to

 Na₂HPO₄ until pH 7.7.

20X SSC

3M NaCl

0.3M NaCitrate

Bring solution to pH 7.0 with HCl.

STET Buffer

50mM Tris-HCl pH 8.0

50mM EDTA

8% sucrose

5% Triton X-100

50X TAEfor 1LTris242gGlacial acetic acid57.1mL0.5M EDTA pH 8.0100mL

10X TBE

1M Tris base 1M Boric acid 1mM Na₂EDTA pH 8.0

10X TE

100mM Tris-HCl pH 8.0 10mM EDTA pH 8.0

YT	Media	for	1L
	Tryptone	8g	
	Yeast extract	5g	
	NaCl	5g	

Adjust to pH 7.2-7.4 with NaOH.

For top agarose add 7g agarose/L and autoclave. For plates add 15g agar/L and autoclave.

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2. Large Scale Preparation of RNA (Lahners et al., 1988)

1. Add 3 volumes of grinding buffer to flask: Grinding buffer: 50mM Tris-HCl, pH 8.0 4% Na p-aminosalicylic acid Add equal volume of Tris saturated phenol to grinding buffer.

- 2. Crush liquid N_2 frozen tissue in silver foil with pestle.
- 3. Pour into flask (or Starstedt tube) and grind with the polytron immediately for 2 min at high speed.
- Shake for 10 min at room temperature at 300 rpm in an Orbit Shaker (Labline).
- Centrifuge at 5,900 x g for 10 min in a Beckman J2-21 centrifuge with a JA 20 rotor.
- 6. Do a second phenol/CHCl₃ extraction on the aqueous layer and shake for 10 min at RT at 300 rpm.
- 7. Centrifuge at 5,900 x g for 10 min.
- Remove upper layer, add equal volume of CHCl₃:isoamyl
 (24:1) and shake for 10 min at 300 rpm.
- 9. Centrifuge for 20 min at 5,900 x g.
- 10. Remove aqueous phase into a baked graduated cylinder.
- 11. Adjust the aqueous phase to 2M LiCl (use 8M) and 1mM EDTA (use 500mM).

- 12. Pour into approximately 40mL Ultraclear tubes and precipitate overnight at 4°C. Tubes should be kept dark and covered with parafilm to eliminate evaporation.
- 13. Centrifuge at 25,000 rpm in a Beckman L8-70M Ultracentrifuge with a SW28 rotor at 4°C for 2 hours. Pour off supernatant, tip tubes upside down and carefully wipe the inside of the tubes with Kimwipes. Keep a sample of the supernatant to compare with the total RNA later on.

14.	Resus	pend pellet in 3mL of:	for 100mL
	400mM	Tris-HCl, pH 7.5	4mL of 1M
	20mM	NaOAc	0.67mL of 3M
	5mM	EDTA	2mL of 250mM
	1%	SDS	1g

93.33mL of water

- 15. Add 300µL of 3M NaOAc and 9mL of EtOH, precipitate on ice for 20-30 min and centrifuge for 10 min at 7000 rpm.
- 16. Resuspend in 0.9mL of water on ice by vortexing and pipetting the solution up and down. Transfer to an Eppendorf tube and spin for 5-10 min at top speed.
- 17. Transfer into 2 new Eppendorf tubes, EtOH precipitate again i.e. add 1/10 volume of NaOAc, 2.5 volumes of EtOH and leave on ice for 15-20 min. Centrifuge at 4°C for 15 min at top speed. Resuspend in 500µL water and

store at -70°C.

18. Read O.D., then take an aliquot and run 3-5µg on a 1.5% small agarose gel. Run the gel at 150mA and make sure that the gel box is clean before using it. 3. Miniprep of RNA.

- To a 15mL polypropylene tube add 1mL of grinding buffer:
 50mM Tris-HCl, pH 8.0
 4% Na⁺ p-aminosalicylic acid
- 2. Add 1mL of water (or Tris) saturated phenol.
- 3. Add 500mg of fresh or frozen tissue.
- 4. Grind with a polytron for 1 min at high speed.
- 5. Add 1mL of CHCl₃ (24:1 CHCl₃ : Isoamyl alcohol).
- 6. Vortex 1 min at high speed.
- 7. Centrifuge 10 min at 8,700 x g in a Beckman J_{2-21} centrifuge with a JA 20 rotor .
- Carefully remove the aqueous phase into a clean 15mL tube.
- 9. Add 2mL of $CHCl_3$ and vortex 1 min at high speed.
- 10. Centrifuge as before for 5 min.
- 11. Transfer aqueous phase to a sterile Eppendorf tube.
- 12. Make to 2M LiCl (use 8M) and 1mM EDTA (use 500mM).
- 13. Precipitate overnight at 4°C.
- 14. Centrifuge 30 min in a cold microfuge at top speed.
| 15. | Resuspend | pellet in 300µL of: | for 10mL | |
|-----|-----------|-----------------------|-----------|------|
| | | 40mM Tris-HCl, pH 7.5 | 0.4mL of | 1M |
| | | 29mM NaOAc | 67µL of 3 | BM |
| | | 5mM EDTA | of ئلر100 | 0.5M |
| | | 1% SDS | 500µL of | 20୫ |
| | | | | |

Incubate at 37°C for 5 min.

- 16. Pellet debris in microfuge for 5 min at 4°C at top speed.
- 17. Transfer supernatant to a clean tube and add 30µL of 3M NaOAc and 750µL of EtOH.
- 18. Allow to stand at -20°C for 30 min.
- 19. Spin at top speed for 15 min. Dry pellet. Resuspend RNA in 20µL of TE.
- 20. Store at -70° C.

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4. Formaldehyde Agarose RNA Gel (For Northern Blot)

Solutions:

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10X MOP's Buffer:	for 1L
0.2M MOPS	41.86g
0.05M sodium acetate	4.10g
0.01M EDTA	3.72g

Adjust buffer to pH 7.0 with NaOH. After autoclaving the solution turns yellow.

If using high quality formaldehyde or formamide, the following two treatments are not necessary.

Filtered formaldehyde: Filter a commercial solution (40% w/v formaldehyde) through Whatman N°1 paper. This removes any paraformaldehyde that may have precipitated out of solution.

Deionized formamide: Stir formamide with BDH 'Amberlite' monobed resin M8-3 till it reaches pH 7.0. Then vacuum filter through Whatman N°1 paper in a buchner funnel to remove resin (Do on pH meter, start filtering when pH approximately 8.0, as pH decreases very rapidly). Store in 500µL aliquots in Eppendorf tubes at -20° C. Note: all work with formaldehyde and formamide should be done in the fume hood.

1. For a 1.2% gel: 1.8g agarose

109mL water

15mL 10X MOPS

Boil to dissolve agarose then cool to approximately 60° C. Then add 3µL of EtBr

26mL formaldehyde

and pour into a meduium sized gel apparatus in the fume hood.

- 2. The gel is run submerged in 1X MOPS buffer.
- 3. Ideally RNA samples are suspended in sterile water to give approximately 10µg of total RNA in 5µL.
- 4. Denaturing solution: To a 500µL aliquot of formamide add 100µL of 10X MOPS and 150µL of formaldehyde. Add up to 5µL of RNA in water to 15µL of this solution in an Eppendorf tube.
- 5. Heat samples at 55°C for 15 min.
- Transfer the tubes to racks at room temperature and add
 2µL of loading buffer.
- Flush out wells with MOPS buffer to remove formaldeyde, it affects loading of the samples.
- 8. Load samples on gel immediately.

9. Run gel at 50mA until the samples are out of the wells, then increase the voltage to 100mA.

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5. Transfer of Formaldehyde-denatured RNA to Nitrocellulose (Northern blot)

1. Following electrophoresis, soak the gel for 5 min in several changes of water. (Note: gels containing formaldehyde are less rigid than non-denaturing agarose gels. Caution must be used in handling them.

2. Soak the gel for 1 hour in 3 changes of 20X SSC.

20X SSC: 0.3M Na citrate (352.9g/4L)

3.0M NaCl (701.3g/4L)

pH solution to 7.0

3. Photograph the gel with a transparent ruler lined up against the markers.

4. Place plexiglass sheet on a clean tray containing 20X SSC, wrap 2 pieces of filter paper around plexiglass and wet with 20X SSC. Roll filter paper a few times with a pipette to ensure that there are no air bubbles. Place 2 pieces of filter paper (larger than the gel) on top and wet with 20X SSC. Again ensure that there are no air bubbles and that the filter papers are saturated. Place 4 strips of parafilm around the edges of the filter paper (is easiest if a space approximately just smaller than the size of the gel is left open). 5. Invert the gel so that its original underside is now uppermost. Place the gel on the filter paper. Make sure that there are no air bubbles between the gel and the filter paper. Pull out the parafilm from under the gel so that only a few mm remain under it.

Using a fresh razor blade, cut a piece of nitrocellulose
 1-2mm larger than the gel in both dimensions. Handle the
 nirocellulose with gloved hands.

7. Float the nitrocellulose on the surface of a solution of 2X SSC until it becomes completely wet from beneath. Then immerse the filter in the 2X SSC for 2-3 min.

8. Cut off the top left hand corner of the nitrocellulose and carefully lay it onto the gel. Using a pipette remove any air bubbles between the membrane and the gel.

9. Wet 2 pieces of Whatman 3MM paper, cut to the same dimensions as the gel, in 2X SSC and place them on top of the nitrocellulose. Remove any air bubbles.

10. Place a stack (5-8cm) of paper towels evenly on top. Put a plexiglass sheet of top of the papertowel and place a 200mL bottle containing approximately 100mL of liquid centered on top.

11. Next day, remove the papertowels and the flip over the gel and nitrocellulose together. Mark the wells on the nitrocellulose with a soft pencil. Discard the gel. Soak

the filter in 3X SSC at room temperature for 5 min. Air dry the filter and then bake it between two sheets of 3MM paper at 80° C for 1 hour.

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6. Tansfer of DNA from Agarose gel to Nitrocellulose Membrane (Southern Blot)

1. Following electrophoresis, photograph the gel and measure the distance of the markers (can photograph the gel with a ruler on it).

2. Transfer the gel to a dish and trim away any unused areas of the gel with a razor blade.

3. Denature the DNA by soaking the gel in:

1.5M NaCl	150mL of 5M / 500mL
0.5M NaOH	83mL of 3M / 500mL

for 1 hour at room temperature with constant stirring or shaking.

4. Neutralize the gel by soaking in several volumes of a solution of:

 1.0M Tris-HCl (pH 8.0)
 121.1g/L

 1.5M NaCl
 300mL of 5M/L

for 1 hour at room temperature with constant shaking or stirring.

5. Set up the transfer apparatus and perform the transfer as described for the transfer of RNA up until step 11. At this step the membrane is washed in 6X SSC instead of 3X SSC.

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7. Large Scale Alkaline Plasmid Preparation

1. Grow 100mL of overnight culture with antibiotics. Spin down at 4,000 x g for 5 min in Oakridge tubes. (Used a Beckman J2-21 centrifuge with a JA 20 rotor).

 Resuspend pellet in 3.5mL of fresh Solution I with 5mg/mL lysozyme.

Solution I: 4.5mL 20% glucose

2.5mL 1M Tris-HCl, pH 8.0

2.0mL 500mM EDTA, pH 8.0

and add water up to a final volume of 100mL

3. Add 7mL of fresh Solution II, mix gently and put on ice. Solution II: 3.3mL 3M NaOH

2.5mL 20% SDS

bring up to 50mL with water.

4. Add 5.3mL cold 3M KOAc, mix very gently. Leave 10 min on ice, spin for 10 min at 18,000 x g at 4° C.

5. Transfer 13mL of the supernatant to a fresh Oakridge tube, add room temperature EtOH to the top, mix for 2 min and spin at 18,000 x g for 10 min at $4^{c}C$.

6. Drain EtOH and resuspend pellet in 2mL of TE. Transfer to a 15mL snap cap tube. Add 2mL cold LiCl, mix. Remove lids and spin for 10 min at 5,900 x g at 4° C. 7. Transfer supernatant to fresh 15mL tube. Add 2 volumes of room temperature EtOH. Spin at 5,900 x g for 10 min at 4° C. Wash pellet with 70% EtOH and dry.

8. Resuspend pellet in 400 μ L of water and transfer to an Eppendorf tube. Add 2 μ L of 10mg/mL RNase A and 1 μ L of T1 RNase and incubate at 37°C for 15-30 min.

9. Add equal volume of phenol, spin 5 min in a microfuge at top speed and remove aqueous phase to clean Eppendorf. Repeat using chloroform to wash the aqueous phase. Remove aqueous phase to fresh tube and add 1/10 volume of 3M NaOAc and 2 volumes of room temperature EtOH. Leave 5 min at room temperature then spin at top speed for 5 min at 4°C. Wash the pellet with 70% EtOH^a. Resuspend the pellet in 100-150µL of TE.

^a If pellet looks dirty (a bit yellow), resuspend in 200 μ L water. Add 200 μ L 4M NH₄OAc and 2 volumes of room temperature EtOH. After 5 min at room temperature, spin at top speed for 5 min at 4°C.

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- 8. Alkaline Miniprep of Plasmid DNA
- Spin down 1.5mL or 3.0mL of overnight culture in microfuge (2-5 min at top speed).
- Resuspend pellet in 100µL of GTE buffer and 4mg/mL lysozyme.
- 3. Incubate 5 min at room temperature.
- 4. Incubate 1 min on ice.
- 5. Add 200µL of 0.2M MeOH and 1% SDS (equal mix of 0.4M and 2%).
- 6. Invert tubes to mix.

- 7. Incubate 5 min on ice.
- 8. Add 150µL ice-cold 3M K⁺, 5M OAc⁻.
- Vortex gently (setting 3-5) in inverted position for 10 sec.
- 10. Incubate on ice 5 min (prepare phenol / chloroform(50:50)).
- 11. Spin 5 min at 4°C in microfuge at top speed.
- 12. Transfer supernatant (approx. 425µL) to new microfuge tube.
- 13. Add an equal volume of phenol / chloroform and vortex.
- 14. Spin 1-2 min in microfuge at top speed.
- 15. Transfer upper, aqueous phase (approx. 400µL) to new tube.

- 16. Add 2 volumes (800µL) of EtOH; vortex to mix.
- 17. Let stand 2 min at room temperature.
- 18. Spin 5 min at room temperature in microfuge at top speed.
- 19. Remove supernatant and wash pellet with (800µL) of 70% EtOH.
- 20. Spin 1-2 min in microfuge at top speed.
- 21. Carefully remove the supernatant.
- 22. Dry pellet briefly.

- 23. Resuspend in 48µL TE and 2µL 3mg/mL DNase-free RNase.
- 24. Add 6µL of 10X Proteinase K incubate at 37°C for 1 hour.
- 25. Add 1/10 volume 3M NaOAc and 2.5 volume EtOH at room temperature.
- 26. Spin 5 min at room temperature at top speed.
- 27. Do 2 X 1mL washes with 70% EtOH.
- 28. Dry pellet and resuspend in small volume of TE buffer.

<u>GTE buffer</u>	<u>per liter</u>	<u>per 200mL</u>	
50mM glucose	9.0g	1.8	
25mM Tris-HCl, pH 8.0	25mL of 1M	5.0	
10mM EDTA, pH 8.0	20mL of 0.5M	5.0	

3M K⁺, 5M OAc⁻

60mL 5M KOAc (122.68g/250mL) 11.5mL glacial acetic acid 28.5mL dH₂O

DNASE-free RNase

Prepare 3mg/mL Ribonuclease A in: 10mM Tris, 1mM EDTA, 15mM NaCl.

Heat to 100° C for 10-15 min; cool slowly to room temperatue. Store at -20° C.

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<u>10X Proteinase K</u>

0.5mL 1M Tris pH 7.8 (100µM)
100µL 0.5M EDTA (10mM)
1mL 10% Sarkosyl (2%)
2.5mg Proteinase K (500µg/mL)
3.4mL dH₂O
Store at -20°C.

9. Isolation of Genomic DNA

Note: all spins are done in a microfuge at top speed.

- 1. Grind up 200-300mg of leaves in Eppendorf with liquid N_2 (or 0.5-1.0g in mortar and pestle, then transfer to Eppendorf tube)
- 2. Add 700µL Proteinase K buffer

35µL Proteinase K (10mg/mL stock) Mix well

- 3. Incubate overnight at 55°C
- Add 20µL RNase A (10mg/mL)
 Incubate at 37°C for 1-2 hours
- 5. 1X phenol (Add 0.5mL phenol/tube) Spin 5 min
 1X phenol/chloroform/isoamyl alcohol Spin 3 min
 1X chloroform/isoamyl alcohol Spin 1 min
- 6. Add equal volume of room temperature isopropanol Mix well

Spin 1 min - pellet may be transparent

- 7. Add 0.5mL 70% EtOH (loosen pellet) Spin 1 min, remove EtOH
- Resuspend pellet in 320µL H₂O
 Add 80µL 10M NH₄OAc, 1mL EtOH, mix
 Spin 1 min, wash pellet with 70% EtOH
- 9. Dry pellet and resuspend in 70-100µL TE

<u>Proteinase K Buffer</u>	<u>100mL</u>
50mM Tris-HCl, pH 8.0	5mL of 1M
100mM EDTA	20mL of 0.5M
100mM NaCl	2mL of 5M
1ቄ SDS	10mL of 10% SDS

<u>Proteinase K</u> - 10mg/mL in sterile water, store at -20°C

<u>RNase A</u> - 10mg/mL made up as in Maniatis (1982) (heat treated to kill DNase)

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10. DNA Transfer and Hybridization Protocol of Genomic DNA on Zetabind Membrane

1. Run samples on a 0.7% agarose gel (high quality agarose) containing no more than $30\mu g$ of EtBr. Gel should be made up in 1X TAE buffer.

2. Photograph the gel with a transparent ruler lined up against the markers.

3. Gel should be denatured for 30 min in 500mL of:

0.4N NaOH

0.6M NaCl

4. Neutralize for 30 min in 500mL of:

0.5M Tris-HCl, pH 7.5

1.5M NaCl

5. Rinse the gel with water briefly and soak in 20X SSC before blotting.

note: by this time the blotting apparatus should have been set up as described for the RNA transfer hybridization procedure.

To treat the Zetabind membrane:

wet in water first

soak in 20X SSC for 20 min before placing on gel 6. Blot the gel overnight with a light weight on top (1/2 filled 200mL bottle). 7. The next day, mark the lanes on the membrane and then rinse the membrane 2X 15 min in 2X SSC at room temperature. Let the membrane dry completely as this step permits binding of the DNA to the membrane.

8. Before prehybridization of the membrane, wash the Zetabind membrane for 25 min at 60° C in a solution of:

0.1X SSC

0.5% SDS

in the water bath. Set the shaking speed such that the filters are gently moving around. All of the loading dye should be washed of *f*.

9. Prehybridize the filter at 42°C for at least for 3 hours in:

5X SSPE 10X Denhardt's Solution 0.5% SDS

Place the plastic bags containing the filters in a tupperware container filled with water to keep the temperature constant during the prehybridization, and later during the hybridization.

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10. Hybridize overnight in:

5X SSPE 0.5% SDS 50µg/mL salmon sperm DNA 10% dextran sulphate

50% formamide

Note: mix the ssDNA and the formamide first and heat to approximately 60°C until the DNA has dissolved (approximately 15 min). Next add the dextran sulphate and then the rest of the ingredients.

One full random primed reaction should be added to 16mL of hybridization solution. This is enough to probe a large filter. Adjust the amount of hybridization solution and probe accordingly.

11. Wash the filter as follows:

1X at RT for 15 minwith 2X SSC0.1% SDS1X at RT for 15 minwith 0.1X SSC0.1% SDS2X at 65°C for 30 min with 0.1X SSC

0.1% SDS

If the background still seems high on the filters after the two 30 min washes, then do 2 more 30 min washes. Keep the filters wet, by wrapping them in saran wrap until the filters are exposed so that they may be rewashed if necessary.

12. Expose the filters to Kodak OMAT-AR film overnight and develop.

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1. The plates containg the plaques were cooled for a minimum of 1 hour at 4° C.

2. A nitrocellulose filter was placed on the surface of a plate and marked with ink and a 22 guage needle by poking three holes around the edges of the plate in an asymetric pattern. The first filter was kept on the plate for 1 min, the second for 2 min, the third for 4 min and the fourth for 8 min (usually only 1 or 2 lifts were done per plate, dependant on the number of probes to be tried). Each of these filters were marked with the same holes as the first so that they could be compared at a later time.
3. Three glass dishes containing Whatman 3MM filter paper saturated with either denaturing, neutralizing or SSC solutions were set up. Each nitrocellulose filter (plaque side up) was denatured for 1 min on filter paper containing;

500mM NaOH

1.5M NaCl.

4. This was followed by neutralization for 2 min on; 500mM Tris-HCl, pH 8.0

1.5M NaCl.

5. The last step was a soak for 1 min in 2X SSC.

6. The filters were air-dried on Whatman 3MM paper, and then placed between two sheets of Whatman filter paper and baked for 1 hour at 80°C.

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12. Isolation of DNA Fragments from Low Melt Gels

Note: unless otherwise specified, all spins are done at top speed in a microfuge.

 Run digested DNA on a low melt agarose gel of the appropriate percentage made up with 1X TAE buffer.
 Note: may want to check 1µL of the digest on a quick minigel before running the entire digest on the low melt gel to ensure that the digestion is complete.

2. Cut out desired bands from low melt gel, minimizing the amount of excess agarose, and place them in Eppendorf tubes. 3. Heat the Eppendorfs at 70° C for 10-15 min. Bring the volume up to 500µL with TE.

4. Add 1/10 volume of 3M NaOAc made up in TE.

5. Add equal volume of phenol (Tris-saturated) and return to 70° C for an additional 5 min.

6. Vortex the tubes for 1 min and centrifuge for 5 min. A white band of agarose should be visible at the interface. Remove the upper layer to a fresh Eppendorf.

Add an equal volume of phenol. Vortex 1 min.
 Centrifuge 1 min. Remove the upper layer to a fresh tube.
 Add an equal volume of chloroform. Vortex 1 min.
 Centrifuge 1 min. Remove the upper layer to a fresh tube.

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Add 2.5 volumes of ethanol. Leave on ice for 15-20 min.
 Spin at 4°C for 15-20 min. Because of agarose left in the mixture should get a very obvious white pellet.

11. Without disturbing the pellet, pour off the EtOH.

12. Wash the pellet with 70% EtOH by adding 0.5mL 70% EtOH and inverting the tube a 2-3 times to take up any liquid left at the bottom. Take care not to dislodge the pellet. Centrifuge for 5 min at 4° C.

13. Pipette out supernatant so as not to lose the pellet. Dessicate the pellet till dry.

14. Resuspend the pellet in TE (usually 10 or 15µL to start).

15. Run out a small aliquot of the fragment on a minigel to check that the fragment preparation is free from other contaminating DNA and to estimate the amount present. 13. BRL Recommended Procedure for Labeling DNA by Nick Translation

The following procedure and solutions are found in the BRL Nick Translation System.

1. Add the following reagents to a 1.5mL microcentrifuge tube placed on ice, then mix briefly:

5µL solution A_2

which contains:0.2mM each of dATP, dGTP and dTTP

500mM Tris-HCl, pH 7.8

50mM MgCl₂

100mM 2-mercaptoethanol

100µg/mL nuclease-free BSA

XµL DNA fragment (10-100ng)

5µL (α^{32} P)-dCTP (Amersham)

YµL Solution E (H_2O) to bring the solution up to a volume of 45μ L

2. Add 5µL of Solution C (DNA Polymerase I/ DNase I:

0.4U/µL DNA Polymerase I, $40pg/\mu$ L DNase I, 50mM Tris-HCl (pH 7.5), 5mM Mg-acetate, 1mM 2-mercaptoethanol, 0.1mM PMSF, 50% (v/v) glycerol, 100µg/mL nuclease-free BSA). Mix gently but thoroughly.

,3. Incubate at 15°C for 60 min.

4. Add 5µL Solution D (Stop buffer: 300mM Na₂EDTA, pH 8.0)

5. Separate the unincorporated nucleotides from the labeled DNA on a 1mL syringe column, plugged with a glass fiber filter (Whatman GF/C glass microfibre filters) and filled with Sephadex G-50 equilibrated with TE.

6. The labelling mixture is loaded onto the column and the column is subsequently washed with 50µL aliquots of TE until a peak in radioactivity comes off. This should be followed by a larger peak. Collect the tubes of the first peak which contain the highest levels of radioactivity and use those as the probe.

14. Preparation of λ DNA from Plate Lysates

1. After incubation at $37^{\circ}C$ (as described in Maniatis <u>et</u> <u>al</u>., 1982) the plate is overlaid with 5mL (small plates) or 10mL (large plates) of 10mM Tris-HCl (pH 7.5) and 10mM EDTA. Leave overnight at $4^{\circ}C$.

2. Scrape off top agarose and buffer into a 30mL Corex tube. Spin at 5,900 x g for 10 min in a Beckman J2-21 centrifuge with a JA 20 rotor. Collect supernatant in a fresh tube.

3. Add RNase A to a final concentration of 1μ g/ml. Incubate at 37°C for 30 min.

4. To 4mL of supernatant, add 0.4mL 0.5M EDTA pH 8.5, 0.2 mL 2M Tris-HCl (pH 8.5) and 0.2mL of 10% SDS and mix. A white precipitate may form in the tube if placed on ice.
5. Add 10µL Diethylpyrocarbonate (DEP), heat for 30 min at 65°C in open tubes in the fume hood (precipitate dissolves immediately at 65°C).

6. Cool on ice, add 1mL 5M potassium acetate (not buffered, pH approximately 8-9). Leave for 1 hour on ice. A white precipitate will form.

7. Spin at 25,000 x g for 10 min. Nucleic acids remain in the supernation. Decant to a fresh tube and add 11mL EtOH. Mix thoroughly and leave at -20° C overnight or for 10-15 min in a dry ice/EtOH bath.

8. Spin the tubes at 20,000 x g for 30 min to pellet the nucleic acids, then decant the EtOH. Dry the tubes in a vacuum desiccator then redissolve the DNA in 0.4mL (or less) of 10mM Tris-HCl (pH 7.5) and 1mM EDTA. Then make the Tris up to 0.1M.

15. Purification of λ DNA by Differential Centrifugation (from Barbara Moffat)

1. Grow a 25mL lysate of lambda DNA in the following manner. A flask with 25mL of LB is innoculated with 0.25mL of a Y1090 overnight culture (i.e. a 3mL culture grown from fresh innoculum). When the cells reach an OD_{600} of 0.2-0.4 a single plaque is added. Shake the cells well until lysis occurs. Add 250µL of chloroform and shake for another 10 min.

2. Spin down in an Oak Ridge tube, at $4,000 \times g$ in a Beckman J2-21 centrifuge with a JA 20 rotor.

3. Pour off supernatant into a fresh Öak Ridge tube, trying not to disturb the pellet or the chloroform bubble.

4. Spin down 2.5 hours at 32,500 x g.

5. Pour off supernatant; allow phage in the pellet to resuspend overnight in 1mL of λ buffer at 4°C.

6. Transfer to storage tube the next day (It may be necessary to spin out the small amount of debris, as it may interfere with the isolation of the DNA. Alternatively one can extract the solution with phenol/chloroform (1:1) and the EtOH precipitate i.e. add 2.5 volumes EtOH and 0.1 volume NaOAc and precipitate on ice for 20 min. Spin for 15 min at top speed and dry the pellet. Resuspend in λ buffer).

- 16. Alkaline Phosphatase Treatment of Vector DNA
- 1. Digest the vector with the appropriate restriction enzyme.
- 2. Add 1µL of alkaline phosphatase.
- 3. Leave at 37°C for 30 min.
- 4. Add 1/10 volume of NaOAc.
- 5. Heat 45 min at 65°C.
- Bring the volume up to 500µL with TE and add the appropriate amount of NaOAc to adjust the concentration back to 1/10 volume.
- 7. Phenol extract twice i.e. add an equal volume of phenol, mix for 1 min, centrifuge for 5 min at top speed, remove the supernatant to a fresh tube.
- 8. Extract with chloroform (24:1 Chloroform: isoamyl alcohol).
- 9. Add 2.5 volumes of EtOH, leave on ice 15-20 min, centrifuge for 15 min at 4°C at top speed, then discard the EtOH.
- 10. Dry the pellet and resuspend in 10µL of TE.

17. Preparation of Competent Cells

Note: use ice cold CaCl₂ and glycerol

- Transfer 1mL of overnight culture to a flask containg 100mL of LB.
- Grow cells in shaking incubator to O.D.₆₀₀ 0.4-0.5 (approximately 2 hours).
- 3. Place on ice for at least 10 min.
- Centrifuge at 5,900 x g for 5 min in a Beckman J2-21 centrifuge with a JA 20 rotor.
- Resuspend the pellet in 1/2 volume (50mL) of 0.1M CaCl₂.
 Vortex if necessary.

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- 6. Put on ice for 30 min.
- 7. Centrifuge at 5,900 x g for 5 min.
- Resuspend in 1/10 volume (10mL) of 0.1M CaCl₂. Vortex gently if necessary.
- 9. Put on ice for at least 30 min.
- 10. Add 15% glycerol.
- 11. Aliquot cells into 200µL aliquots in Eppendorf tubes.
- 12. Freeze cells rapidly in either liquid N_2 or a dry ice/ EtOH bath.
- 13. Keep cells at -70°C.

18. Protocol for Sequencing Using the Sequenase Kit

DNA Denaturing

1. Pipet 4-5ug of supercoiled plasmid into a microfuge tube and bring the volume up to 20µL with water.

2. To this tube add $2\mu L$ of a solution of 2M NaOH and 2mM EDTA. Incubate at room temperature for 5 min.

3. Neutralize the reaction by the addition of 3μ L 3M sodium acetate (pH 5.0) and 7μ L of distilled water.

4. Vortex for 10 seconds. Add 75 μ L absolute ethanol. Let stand at -70°C for 5 min.

5. Centrifuge 5 min at top speed in the microfuge.

Decant the supernatant. Add 200µL cold 70% EtOH.
 Centrifuge 5 min.

7. Decant the supernatant and dry the pellet. Resuspend the pellet in 7µL distilled water.

Annealing Template and Primer

 For each set of 4 sequencing lanes, a single annealing (and subsequent labelling) reaction is used. In a centrifuge tube combine the following:

Primer		1	կրը
Sequenase	Reaction	buffer	Հու
DNA		•	7µĽ

The total volume should be 10µL.

2. Warm the tube to 65° C for 2 min, then allow the temperature of the tube to cool slowly (approximately 30 min) to room temperature in a beaker of 65° C water. Once the temperature is below 35° C, the annealing is complete.

Labeling Reaction

1. Dilute the Labeling Mix (dGTP) 5-fold with water (4 μ L Labeling Mix and 16 μ L water) and store at -20°C.

2. Dilute the Sequenase enzyme 1:8 in ice-cold TE buffer to make up 2µL per reaction. Store on ice for up to 1 hour.

3. To the annealed template-primer add the following:

Template-primer	10µL
DTT 0.1M	1µՆ
Diluted Labeling Mix	2µՆ
³⁵ SdATP (10µCi/µL)	0.5µL
Diluted Sequenase	2µL

Mix thoroughly and incubate 5-10 min at room temperature.

Termination Reactions

 Have on hand 4 tubes labeled G, A, T and C.
 Place 2.5µL of the ddGTP Termination Mix in the tube labeled G. Repeat for the other tubes using the corresponding ddNTP. 3. Pre-warm the tubes at 37°C at least 1 min.

4. When the labeling incubation is complete, remove 3.5μ L and transfer to each of the 4 labeled tubes. Mix and return to the 37°C water bath for another 5 minutes.

5. Add 4μ L Stop Solution to each of the reactions, mix and store at -20°C until ready to load on the gel (up to 1 week).

6. When the gel is ready to load, heat the samples to 75- 80° C for 2 min.

Preparing the Gel

 Clean the 2 glass plates with detergent followed by several rinses of distilled water. Then clean with ethanol.
 Place the larger plate down and place the spacers around the perimeter. Lay the smaller plate on top and adjust the spacers.

3. Place clamps along the bottom and half way up the sides. Place a P200 tip between the two plates at the top.

4. To make a 6% gel combine: 8.6g acrylamide

0.45g bis-acrylamide

72g urea

15mL 10X TBE buffer

60mL deionized water

5. Heat on a stir plate 3-5 minutes.

6. Filter through Whatman No.1 paper. Adjust the final volume to 150mL.

7. Add 750µL of a 10% solution of fresh ammonium persulfate and mix.

8. Add 75µL TEMED and mix quickly.

9. Using a 50mL syringe pour the gel at a constant flow, lifting the plates vertically to remove air bubbles. Once the gel is approximately 2/3 full, lay the plates flat and remove the pipet tip. Allow the extra fluid to drain into a bucket.

10. Place the rest of the clamps around the plates and insert the comb upside-down. Allow to polymerize for at least 1 hour.

Running the Gel

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1. Once the gel has polymerized, remove the clamps and then remove the bottom spacer and fill the air space created with 1X TBE. Remove the comb and place it back between the plates with the teeth down, just touching the gel surface. Fill the wells with 1X TBE.

 Carefully place the gel in the electrophoresis apparatus. The bottom chamber should already be filled with 1X TBE and the main seal should be in place. Ensure that there are no air bubbles caught in the bottom of the gel. Place the two sponge squares at the top corners of the plates and secure the gel in place with the clamps.
 Pour some butfer into the top chamber to check for leaks. Then fill the top chamber and pre-run the gel at constant power (60 W) for at least 1 hour. A few µL of running dye may be loaded while the gel is pre-running to see how the lanes are running.

5. Once the gel has warmed up, wash the wells with 1X TBE to remove any urea and load 2µL of each reaction in adjacent wells. Loadings may be staggered to allow for the reading of more sequence.

6. When the run is finished, remove the gel from the apparatus and lay on a flat surface. Remove the side spacers and place a flat spatula between the two glass plates at the bottom and twist very gently to separate the two plates.

7. Place the plate with the gel in a large dish containing 10% methanol and 10% glacial acetic acid. Allow to fix for 30-60 min.

8. Siphon off the fixing solution. Use a piece of Whatman 3MM paper cut a little larger than the gel to remove the gel from the glass plate. This can be done by placing the filter paper on the gel and smoothing it down with a gloved hand. The filter paper is then rolled back from 1 edge with

the gel sticking to it.

- 9. Place the gel face up and cover with Saran Wrap.
- 10. Dry the gel under vacuum for 2 hours at 80°C.
- 11. Remove the Saran Wrap and expose to X-ray film.

19. Labeling of DNA Probes by Random Priming (Feinberg and Vogelstein, 1983)

For 1 reaction: XµL DNA (100ng) YµL H₂O 1µL Bovine serum albumin (10mg/mL = 25X stock) 1.25µL Hexamer (0.1units/µL) 10µL Reaction buffer 5µL (³²P)-dCTP or ³⁵SdATP (Amersham) 0.5µL Klenow ------

25µL total volume

Add DNA, H₂O and hexamer to an Eppendorf tube. Boil for 2 min. Cool quickly on ice. Add all other components of the reaction mixture.

Leave the mixture at room temperature for 3.5 hours to overnight. Then run through a 1mL column made up of Sephadex G-50 equilibrated in TE in a 1mL syringe plugged with a filter (Whatman GF/C glass microfibre filters). The reaction mixture is loaded on the column. Aliquots of 50µL
of TE are loaded on the column until the labeled DNA comes off in a peak. Collect the fractions of this peak with the highest levels of radioactivity to be used as a probe.

notes:

Hexamers

-pdN₆ - nucleotides from Pharmacia already in solution -make stock solution of 1 O.D./ μ L in water then dilute this 1:10 for use (0.1units/ μ L = 20X stock)

2.5X Reaction Buffer for 1mL (Final concentration) 0.5M HEPES pH 6.6 500 μ L of 1M 12.5µL of 1M 12.5mM MgCl₂ 25mM B-Mercaptoethanol 2µL 125mM Tris-HCl pH 8.0 125µL of 1M 12.5µL of each 125µM of dATP, dTTP, dGTP if using (³²P)-dCTP as a label of 10mM stocks or dCTP, dTTP, dGTP if using ³⁵SdATP as a label

water

323µL

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