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Clearing the Air: Expression of Nitrous Oxide Reductase from *Pseudomonas stutzeri*
in Transgenic Tobacco

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CLEARING THE AIR: Expression of Nitrous Oxide Reductase from *Pseudomonas stutzeri* in Transgenic Tobacco

Julianne Marie Staebler

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the Master of Science degree in Biochemistry

Department of Biochemistry, Microbiology and Immunology
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ABSTRACT

As atmospheric concentrations of N_2O, a potent greenhouse gas and destructor of ozone, continue to increase, it is important to explore a range of solutions for this issue. The bacterial enzyme responsible for catalyzing the final step of the denitrification pathway, conversion of N_2O to N_2, is nitrous oxide reductase (N_2OR). It is the aim of this study to produce model tobacco plants expressing the nitrous oxide reductase gene (*nosZ*) with the ultimate goal of supplying additional N_2OR to the plant-microbial-soil environment for more efficient reduction of N_2O and mitigation of agricultural N_2O emissions. Two nitrous oxide reductase expression cassettes under the control of 1) the d35S constitutive promoter and, 2) the rolD root-specific promoter were constructed and used to transform *Nicotiana tabacum* tissue. Integration and expression of the *nosZ* transgene in T_0 and T_1 generation plants was demonstrated by PCR and RT-PCR. Production of recombinant N_2OR in T_0 plants was shown by western immunoblot.

ACKNOWLEDGEMENTS

I wish to express sincere thanks to my supervisor, Dr. Illimar Altosaar, for his patience and encouragement throughout my degree. I am grateful for his assistance with my research as well as the professional opportunities he has provided to present my work to the scientific community. I also owe thanks to the members of the Altosaar lab for providing a fun and supportive work environment. I am especially grateful to David Blais, Evelin Loit and Melissa McNulty for their generous technical help and friendship, to Mohsin Zaidi for technical advice and to Yaseen Mottiar for his assistance in maintaining the greenhouse. I would like to express my gratitude for the financial support provided by the University of Ottawa and the Department of Biochemistry, Immunology and Microbiology. I must also express my appreciation to Dr. David Tepfer for providing the pLJ1 plasmid and Dr. Walter Zumft for his generous gift of anti-N_2OR serum. Finally, I would like to offer sincere gratitude to all my family and friends who have offered their constant support, especially my husband, Michael Staebler.

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LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
BA	6-benzyladenine
bp	Base pair
CaMV	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
cds	Coding sequence
d35S	Double 35S promoter
DEPC	Diethylpyrocarbonate
EC	Environment Canada
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic reticulum
ex	Extensin signal sequence
GHG	Greenhouse gas
IPCC	Intergovernmental Panel on Climate Change
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
Mb	Mega base
MW	Molecular weight
mRNA	Messenger ribonucleic acid
MS	Murashige-Skoog medium
Mt CO_2e	Megatonnes carbon dioxide equivalent
N_2OR	Nitrous oxide reductase
NAA	Alpha-naphthalene acetic acid
NOSter	Nopaline synthase terminator
nosZ	Gene encoding nitrous oxide reductase
Npt II	Neomycin phosphotransferase II gene
nt	Nucleotide
NT	Non-transgenic tobacco plant
PCR	Polymerase chain reaction
rolD	RolD promoter
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T_0	Primary transformants
T_1	Progeny from T_0 transformants
T-DNA	Transfer DNA
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	Tris (hydroxymethyl)aminomethane
WHO	World Health Organization
YEP	Yeast Extract Peptone medium

Chapter 1

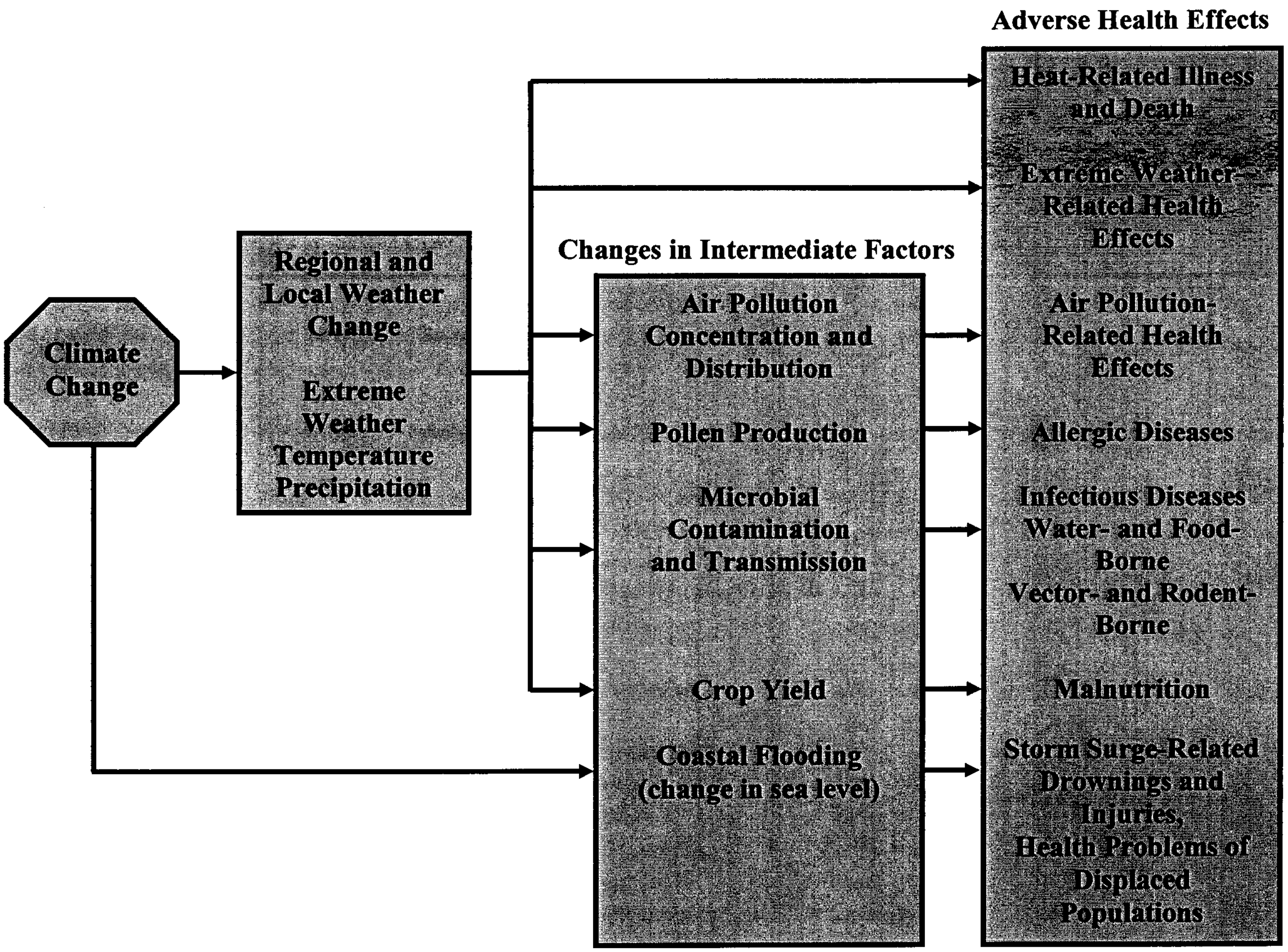
INTRODUCTION

1.1 Global climate change

Mounting evidence of rising global temperatures and a direct link to anthropogenic activities and increasing atmospheric greenhouse gas (GHG) concentrations has led to a lively debate within the scientific community on the subject of climate change (Khandekar et al., 2005). Despite the existence of some studies that dispute reported warming trends and their causes (McIntyre and McKitrick, 2003, 2005; Michaels et al., 2000), the majority of the scientific community accept human induced climate change as a reality. Extensive reports assessing current scientific knowledge in this field are prepared by the Intergovernmental Panel on Climate Change (IPCC). Recent IPCC data indicates that global average surface temperature has increased approximately 0.6°C since the 19th century and average surface temperature is projected to increase 1.4 to 5.8°C by 2100 (IPCC, 2001). Increases of this magnitude are expected to cause climatic events including rising sea levels, changing precipitation patterns causing flooding and drought, and increased incidence of extreme weather events such as hurricanes (Watson et al., 2005). These changes will ultimately result in ecological impacts such as: species distribution shifts (Walther et al., 2005; Walther et al., 2002; Root et al., 2003), loss of biodiversity (Thuiller et al., 2005) and modified agricultural productivity (Rotter and Van de Geijn, 1999). Human populations will not be exempt from these effects and changing patterns of human disease and mortality are expected (Patz et al., 2000) (Figure 1.1). In fact, the effects of climate change may already be evidenced by such weather events and their related deaths as the European heat wave of 2003 and the increasing frequency of Atlantic hurricanes in 2004-2005. Although these events cannot be attributed

Figure 1.1 Schematic of potential climate change impacts on health outcomes.

Predicted scenarios in which ecological consequences of global climate change may impact human health (adapted from Haines and Patz, 2004)

Adverse Health Effects
Climate Change
Regional and Local Weather Change
Extreme Weather
Temperature
Precipitation
Changes in Intermediate Factors
Air Pollution Concentration and Distribution
Pollen Production
Microbial Contamination and Transmission
Crop Yield
Coastal Flooding (change in sea level)
Heat-Related Illness and Death
Extreme Weather-Related Health Effects
Air Pollution-Related Health Effects
Allergic Diseases
Infectious Diseases
Water- and Food-Borne
Vector- and Rodent-Borne
Malnutrition
Storm Surge-Related Drownings and Injuries,
Health Problems of Displaced Populations

directly to global climate change, a recent study suggests that human influence on climate has more than doubled the risk of similar heat waves (Stott et al., 2004). Deaths resulting from environmental conditions are estimated to encompass 20% of worldwide deaths from all causes compared to 12% for cancer (WHO, 2004). Clearly the potential impacts on human health from climate change are of significant concern. In response to mounting world concerns about the consequences of anthropogenic GHGs and climate change, the Kyoto Protocol to the United Nations Framework Convention on Climate Change was negotiated and came into force in 2005. In response to the Kyoto Protocol commitment, the Climate Change Plan for Canada (2002) was developed and aims to reduce current GHG emmisions by 240 Mt CO_2e/yr (megatonnes of CO_2 equivalent) by 2012. All industrial and domestic sectors will have to participate in emission reduction and commit to research and innovation to accomplish this goal.

1.2 Nitrous oxide emissions from agricultural soils

The top three GHGs are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Although very little N_2O is released to the atmosphere, it is the most potent GHG and accounts for approximately 7% of yearly GHG potentials. It has 310 times the warming potential of CO_2 per molecule and an atmospheric half-life of 120 years. In addition to its heat absorbing capacity, N_2O participates in depletion of stratospheric ozone. Atmospheric concentrations of N_2O have grown by about 17% since the 18th century and at present, atmospheric concentrations of N_2O are increasing at a rate of about 0.3% per year (IPCC, 2001).

In 2003, Canada emitted 740 Mt CO_2e from all sectors of the economy and 62 Mt CO_2e or 8.4 % of all GHG emissions in Canada were from the agricultural sector (EC, 2005).

While agriculture contributes a relatively small proportion of GHGs it is responsible for up to 80% of N_2O emissions nationally, primarily through use of nitrogen fertilizers (Mosier, 2002). Although total sector emissions decreased 0.8% between 1990 and 2002, nitrous oxide emissions from soils rose 10% over the same period (EC, 2004). Evidently, the issue of N_2O emissions is of specific concern to the agricultural sector and presents an obvious area to address in order for Canada to meet GHG reduction goals. Unfortunately, mitigation of N_2O involves manipulation of the complex soil system and current knowledge on the dynamics of N_2O soil flux is still insufficient if not lacking entirely. Most N_2O is produced through the process of bacterial denitrification as part of the nitrogen (N) cycle and a lesser amount is lost as a by-product of bacterial nitrification (Figure 1.2). Dissimilative denitrification is the reduction of nitrate (NO_3^-) to dinitrogen gas (N_2) through the anaerobic respiration of N. The process occurs at the cytoplasmic membrane through the following steps;

$$\text{nitrate} \xrightarrow[\text{NAR}]{} \text{nitrite} \xrightarrow[\text{NIR}]{} \text{nitric oxide} \xrightarrow[\text{NOR}]{} \text{nitrous oxide} \xrightarrow[\text{N}_2\text{OR}]{} \text{dinitrogen gas}$$

Each step is catalyzed by a dedicated metalloenzyme: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR). Although denitrification completes the N cycle and returns N to the atmosphere as N_2 the process does result in some losses of N as N_2O. The magnitude of N_2O loss is determined by complex interactions of biological and physical soil processes that impact denitrification efficiency (Figure 1.3). These include: 1) Oxygen availability, which is determined by soil water content, land management practices that affect soil aeration and soil type (Elmi et al., 2003; Henault et al., 1998), 2) Soil nitrogen availability since substrate concentration and type will

Figure 1.2 The Nitrogen Cycle.

Depiction of the N cycle showing the biological processes of atmospheric N_2 fixation (and the artificial addition of N through fertilizer/manure application), uptake of N by plants and assimilation/dissimilation of N through the bacterial pathways of nitrification and denitrification. Completion of the cycle occurs when N is reduced to N_2 and returns to the atmosphere. Deleterious amounts of N_2O are also lost to the atmosphere at several points in the cycle.

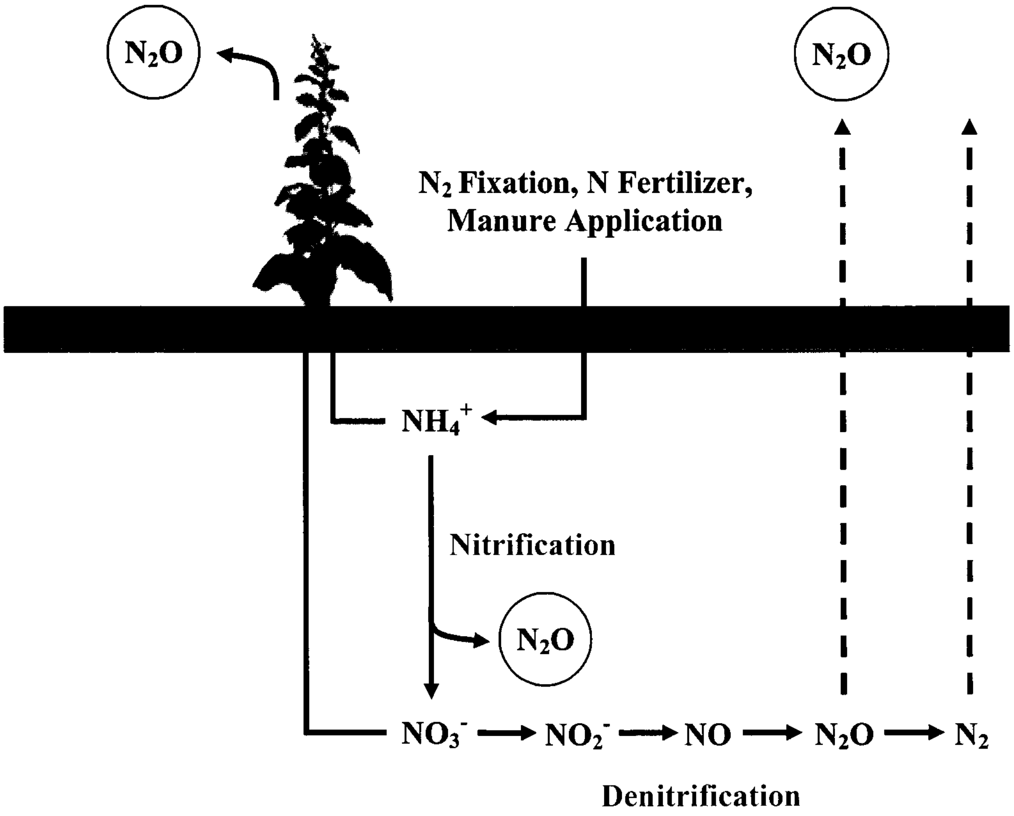
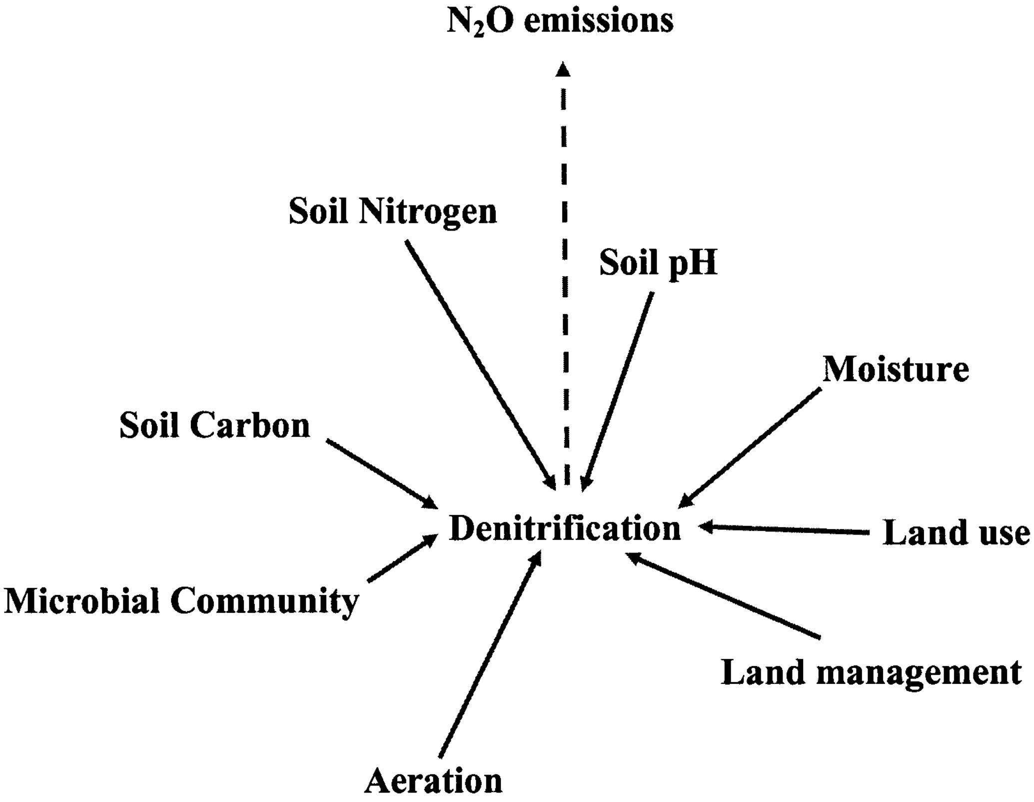

N₂O
N₂O
N₂ Fixation, N Fertilizer,
Manure Application
NH₄⁺
Nitrification
N₂O
NO₃⁻ → NO₂⁻ → NO → N₂O → N₂
Denitrification

Figure 1.3 Complexity of the soil environment and its impact on emissions.

Many physical and biological soil factors affect the rate of denitrification, which in turn results in a net soil N_2O flux. These factors also interact with each other in numerous and complex pathways, therefore, mitigation of emissions by manipulation of these factors is difficult. For example, no-till farming (a form of land management) can result in either increased or decreased N_2O emissions depending on the influence of other interacting factors, such as nitrogen source and soil carbon availability.


N₂O emissions
Soil Nitrogen
Soil pH
Moisture
Soil Carbon
Denitrification
Land use
Microbial Community
Land management
Aeration

determine reaction rates (Castaldi and Smith, 1998; Lemke et al., 1999), 3) Soil organic carbon availability, which impacts rates of microbial growth (Weier et al., 1993), 4) Structure of the microbial community due to variations in enzyme kinetic properties from different species (Cavigelli and Robertson, 2001), and 5) Soil properties including temperature, pH and salinity (Castaldi, 2000; Maljanen et al., 2002; Weier and Gilliam, 1986). The research data on the interactions and impacts of these factors is often inconclusive and contradictory. The current state of knowledge and limited control of environmental conditions restricts effectiveness of attempted land management strategies for mitigation of N_2O emissions. Significant effort is required to understand the complexity of soil N_2O flux and innovative solutions are needed.

1.3 The role of crop plants in the emission of nitrous oxide

Agricultural crops play a major role in the global carbon and nitrogen cycles and thus in the cycling of GHGs. As a result, Canada and the United States with over 60 million hectares and 150 million hectares of agricultural land, respectively, have a unique opportunity to manage atmospheric GHG concentrations through available biomass. This approach has been termed "Canada's Green Advantage" by the BIOCAP Canada Foundation. One significant contribution that agricultural crops and soils can make is the reduction of N_2O emissions. Mounting evidence indicates that plants play an important role in both transfer of N_2O to the atmosphere and production of N_2O. Studies have shown that N_2O is transferred from soil to the atmosphere through the transpiration stream in canola and barley (Chang et al., 1998), rye grass (Chen et al., 1999), rice (Yu et al., 1997), as well as corn and soybean (Chen et al., 2002). The amount of N_2O emitted directly from plant tissue is highly variable depending on the species, and likely result partially from differences in experimental design. Plant emitted N_2O ranges from 6-80% of total soil N_2O emissions (Chen et al., 2002; Yu et al., 1997). In addition to transpiration of soil N_2O, it has been demonstrated that plants themselves produce N_2O during N metabolism. Wheat leaves were shown to generate N_2O during NO_2^- reduction in chloroplasts (Smart and Bloom, 2001). These plant produced N_2O emissions are estimated to account for 5-6% of total N_2O emissions from agricultural plant–soil systems. N_2O production in transgenic tobacco expressing antisense nitrite reductase confirmed that N_2O is generated from NO_2^-, however, the mechanism through which N_2O is produced *in planta* is unknown (Goshima et al., 1999). In a more recent study, 17 plant taxa were analyzed for their ability to convert NO_3^- to N_2O. All but one taxa were shown to produce N_2O with a 58-fold variation in the quantity produced (Hakata et al., 2003). Although the mechanism of plant produced N_2O is not understood, it is clearly a common phenomenon.

In addition to direct production and transmission of N_2O, it is likely that plants exert an even greater influence on N_2O emission rates through plant-microbe interactions in the rhizosphere. It has long been known that root exudates deposit organic C in the soil which in turn supports the growth of soil microorganisms. Since organic C is also known to enhance rates of N_2O emissions through enhanced microbial activity (Baggs et al., 2003), it is logical to assume that root exudates play some role in the rate of denitrification occurring in the rhizosphere. In contrast, root exudates of the tropical grass *Bracharia humidicola* suppressed ammonium-oxidizing bacteria and thereby reduced N_2O production (Ishikawa et al., 2003). Despite gaps in knowledge of the fundamental mechanisms controlling N_2O production, it appears that plant systems and their interaction with the microbial-soil environment present an important approach for the management of GHGs.

1.4 Nitrous oxide reductase

In soils, the final step of bacterial denitrification, reduction of N_2O to N_2, is catalyzed by the enzyme nitrous oxide reductase (N_2OR) in the cellular periplasm (Figure 1.4). N_2OR is a dimeric copper enzyme comprised of two identical 66 kDa monomers encoded by the *nosZ* gene. This highly conserved enzyme has been identified and characterized in a wide array of bacteria including: Gram-positive *Bacillus azotoformans* (Suharti and DeVries, 2005), soybean microsymbiont *Bradyrhizobium japonicum* (Bedmar et al., 2005), non-denitrifying *Wolinella succinogenes* (Simon et al., 2004) and methylotrophic *Hyphomicrobium denitrificans* (Yamaguchi et al., 2003). The most thoroughly studied sources of N_2OR are the Gram-negative denitrifiers: *Pseudomonas aeruginosa*, *Pseudomonas nautica*, *Paracoccus denitrificans* and most extensively *Pseudomonas stutzeri*.

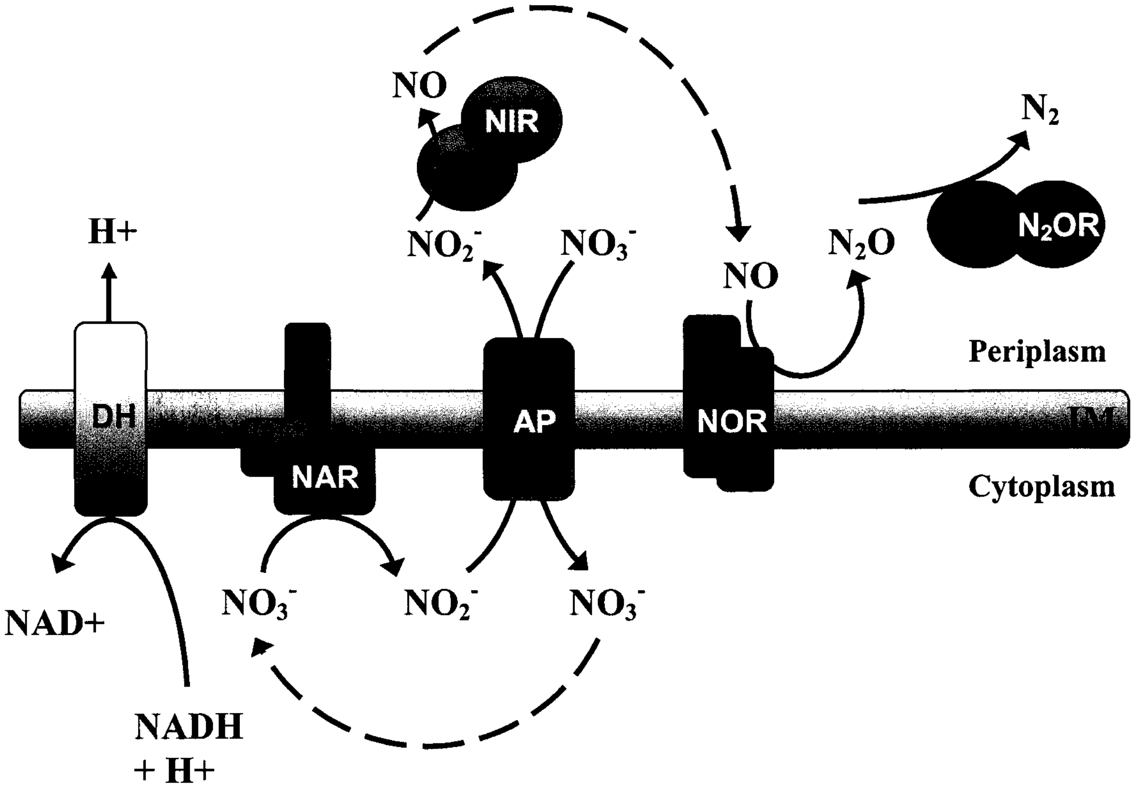
The genetic basis for reduction of nitrous oxide has been studied extensively since it was first identified by Viebrock and Zumft (1988). The *nosZ* gene is located within the 8 kb *nos* cluster on the 4.3 Mb chromosome of *P. stutzeri* and is closely linked with other denitrification genes (Jungst et al., 1991). Expression of *nosZ* is under complex transcriptional control from six different promoters (Cuypers et al., 1995) and unlike other denitrification enzymes, N_2OR is synthesized at a low constitutive level under aerobic conditions (Korner and Zumft, 1989). This low level expression is modulated by multiple environmental stimuli including: oxygen and nitrate (Baumann et al., 1996; Philippot et al., 2001), nitric oxide via the FNR-type transcription factor DnrD and to a lesser extent nitrous oxide (Vollack and Zumft, 2001).

The protein product of *nosZ* has an unusually long signal sequence of 50 aa that shares a conserved twin-arginine motif with other periplasmic enzymes. This motif is necessary for translocation of N_2OR to the periplasm via the Tat transport system (Dreusch

Figure 1.4 Denitrification at the bacterial membrane.

Organization of the anaerobic nitrate electron transport system, which involves the 8 electron reduction of nitrate catalyzed by the denitrification enzymes. Legend: DH, NADH dehydrogenase complex; NAR, nitrate reductase; AP, postulated nitrate/nitrite antiporter; NIR, nitrite reductase; NOR, nitric oxide reductase; N_2OR, nitrous oxide reductase; IM, inner membrane (adapted from Zumft, 1997).

$$N0_3^- \longrightarrow N0_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$$



et al., 1997; Heikkila et al., 2001). Maturation of the enzyme's copper centres occurs in the periplasm and functional enzyme is not produced in the absence of the signal peptide motif (Dreusch et al., 1997).

The crystallographic structure of N_2OR has been solved in two model organisms: *Pseudomonas nautica* (Brown et al., 2000a) and *Parococcus denitrificans* (Haltia et al., 2003). The structure of the copper centres described in these studies has been confirmed in *P. stutzeri* (Alvarez et al., 2001; Charnock et al., 2000; Rasmussen et al., 2000). Each monomer of N_2OR has two domains, one having a dinuclear Cu_A centre that acts as the electron transfer site, and the second being a β-propeller domain with a tetranuclear Cu-S centre, Cu_Z, which acts as the catalytic site (Figure 1.5A). Cu_Z is arranged in a tetrahedron, which is bridged by an inorganic sulfur atom (Brown et al., 2000b). The homodimer is arranged head-to-tail and electrons are transferred from Cu_A of one subunit to the N_2O binding site, Cu_Z, of the second subunit (Figure 1.5B).

In preliminary experiments, the proper assembly of the Cu_Z centre of N_2OR was shown to require a complex coded for by the *nos* cluster of genes (Figure 1.6A). A model for metal insertion has been proposed (Figure 1.6B) although components of the complex have not been fully characterized. Spontaneous incorporation of Cu into the Cu_A site has been described (Dreusch et al., 1997), however, attempts to rescue Cu_Z in vitro have not been successful. Functional assembly of Cu_Z is also lacking when *nosZ* is expressed in the non-denitrifiers *Escherichia coli* (Viebrock and Zumft, 1988) and *Pseudomonas putida* (Wunsch et al., 2003). Coexpression of *nosZ* with *nosD*, *nosF* and *nosY* is, however, sufficient for production of the catalytically active holoenzyme in *P. putida* (Wunsch et al., 2003). Together NosD, NosF and NosY form an ABC-type transporter that is thought to function in

Figure 1.5 Ribbon diagrams of the N_2OR monomer and dimer.

A, Crystal structure of the N_2OR monomer. Each monomer has two domains: the C-terminal Greek key motif domain (top) and the N-terminal β–propeller domain (bottom). The C-terminal domain contains the Cu_A centre having two copper ions (green spheres), which is the site of electron entry. The N-terminal domain contains the Cu_Z centre, which comprises four copper ions (green) arranged in a tetrahedron and bridged by a sulfur atom. The Cu_Z centre is the catalytic site where N_2O substrate binds. α-helices are shown in red and β-sheets in blue. **B**, Overall view of the N_2OR dimer; one monomer is shown in gray while the other monomer has the same color-code as defined above. In the dimer the C-terminal domain of one monomer faces the N-terminal domain of the other monomer. This brings the Cu_A and Cu_Z centres into close proximity to facilitate electron exchange. Figure was generated using Deep View/Swiss-PDBviewer v3.7. (Adapted from Brown et al., 2000a)

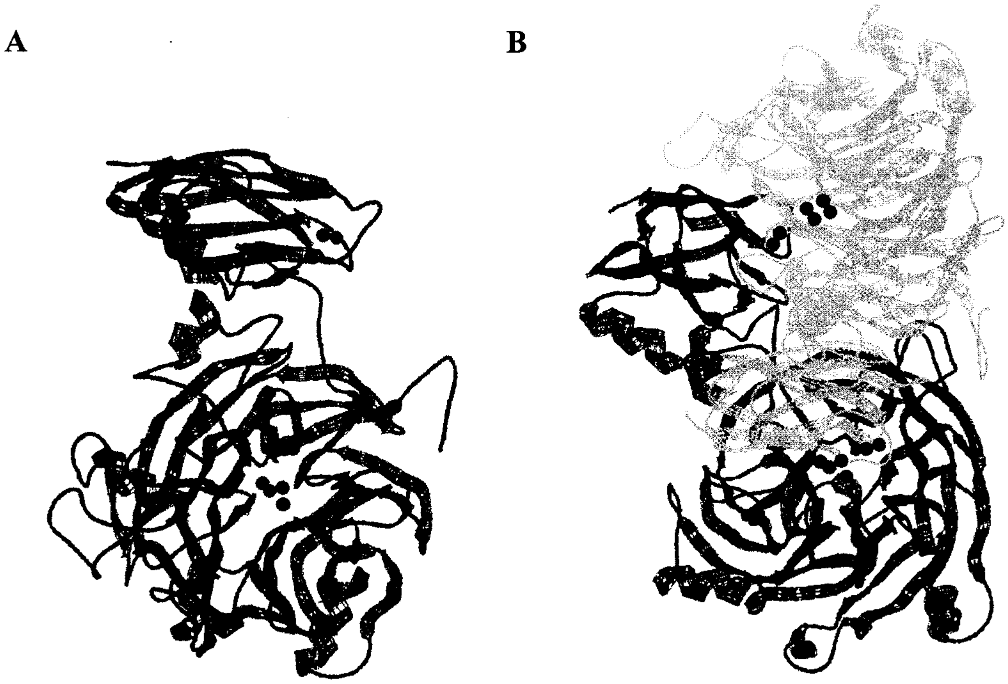
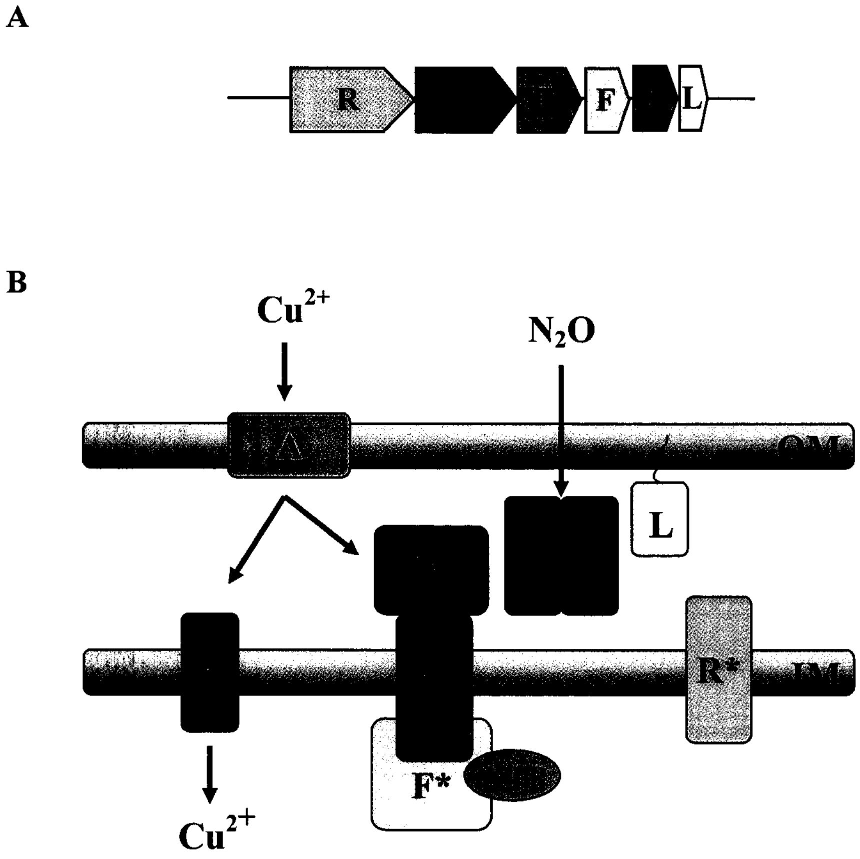

A
B

Figure 1.6 Proposed topology of the copper insertion apparatus for N_2OR.

A, Genetic organization of the *nosRZDFYL* genes. **B,** A, D, F, L, R, Y, and Z denote proteins NosA, NosD, NosF, NosL, NosR, NosY, and NosZ (or N_2OR), respectively. NosA is a putative pore forming protein through which copper is believed to enter the cell. NosD forms an ABC-type transporter together with NosY and the putative ATP-hydrolyzing protein NosF. The role of the transporter is thought to be delivery of either copper or sulfur to NosZ. NosL is a Cu-containing lipoprotein attributed with copper chaperone function. Membrane-bound NosR acts as a transcriptional regulator for *nosZ.* Proteins denoted with * are necessary for production of catalytically active N_2OR in a non-denitrifying background (Wunsch et al., 2003). The question mark symbolizes a presumed copper transporter. Legend: OM and IM, outer and inner membranes, respectively; NTP, nucleotide-binding site of NosF. (Adapted from Zumft, 1997)


A
R
F
L
B
Cu2+
N2O
Δ
OM
L
R*
IM
F*
Cu2+

copper or sulfur membrane transport for delivery to N_2OR (Honisch and Zumft, 2003). N_2OR draws electrons from an intermediate carrier, usually a c- or b-type cytochrome, and displays a low degree of recognition specificity to its electron donor (Zumft, 1997).

1.5 Rationale and objectives

Climate change is a national and global concern that is predicted to have severe environmental impacts and human health implications. The production of N_2O emissions by the agricultural sector represents an important contribution to anthropogenic GHGs responsible for the phenomenon of global warming and must be addressed. Crop plants are inextricably linked to N_2O flux from agricultural soils and present an interesting opportunity for N_2O mitigation. Although microorganisms capable of reducing N_2O are present and active in agricultural soils the loss of N_2O is inevitable since N_2O reductase is under complex regulatory control by environmental stimuli and is not expressed in all conditions. Fortunately, the genetic basis for the conversion of N_2O is well characterized and is available as a genetic engineering tool to improve the efficiency of N_2O reduction in soils. We conceptualized that plants engineered to express bacterial nitrous oxide reductase and secrete the enzyme in root exudates will produce a catalytic sink of N_2OR that will act to supplement endogenous soil N_2OR activity in the plant-microbe-soil environment. This soil remediant/compensatory system will have the potential to reduce N_2O from at least three sources: 1) N_2O produced in the rhizosphere and surrounding soil environment, 2) N_2O produced by the plant itself, as well as, 3) N_2O emitted through the plant transpiration stream. Such a green gene system should have a substantial impact on rates of N_2O emissions from agricultural soils on which the transgenic plants are grown.

Examples of remediation through rhizosecretion of recombinant enzymes have been reported (Uchida et al., 2005) and studies examining the potential of transgenic plants for environmental phytoremediation are well documented (Arthur et al., 2005). Several fungal and bacterial oxidoreductases have been functionally expressed in plants as phytoremediation strategies including, Mn-peroxidase (Iimura et al., 2002), pentaerythritol tetranitrate reductase (French et al., 1999), mercuric reductase (Rugh et al., 1998) and arsenate reductase (Dhankher et al., 2003). This type of plant decontamination strategy provides advantages, such as stable cultivation and control of the remediant organism and deep soil penetration, over traditional bioremediation.

While the N_2OR enzyme did not assemble correctly in the preliminary prokaryotic expression studies of Viebrock and Wunsch, it is yet unclear whether a more sophisticated proteome, which is found in a eukaryotic plant, will replace the function of the NosDFY complex. The copper chaperone CCH of the Arabidopsis plant transports copper to the RAN1 P-type ATPase (Mira et al., 2001), which then delivers copper to the secretory pathway (Hirayama et al., 1999). The transport of sulfur in plants is still being elucidated, however, 14 putative sulfate transporters have been identified in Arabidopsis and several others may exist (Leustek, 2002). As many as 129 ABC-type transporters may be expressed by Arabidopsis (Sanchez-Fernandez et al., 2001) and these could include a transporter with functions similar to NosDFY. This type of plant background may well prove to support the functional assembly of N_2OR and provide inadvertent complementation by host redox mechanisms. The first step in determining the potential for N_2OR activity in plants is expression of the *nosZ* structural gene in a model crop. If it becomes evident that the bacterial assembly complex is required by the plant, then engineering of additional *nos* operon components can be performed to address this issue.

The specific objectives of the current thesis were as follows:

1. Design and construction of expression cassettes for the production of recombinant N_2OR *in planta.*
2. Transformation of the model organism *Nicotiana tabacum* with N_2OR expression vectors and regeneration of mature plants.
3. Analysis of transformed plant lines for expression of the *nosZ* transgene and presence of the protein product.
4. Confirmation of stable inheritance and expression of the *nosZ* gene in T_1 progeny.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Pseudomonas stutzeri Zobell (ATCC 14405) was obtained from the American Type Culture Collection (Manassas, VA). David Tepfer (L'Institut National de la Recherche Agronomique, France) kindly provided the pLJ1 plasmid. Plasmids, pBI-525, pHBV-CO and pRD400, *Agrobacterium tumefaciens* LBA4404 and *Nicotiana tabacum* cv. Xanthi seeds were available in the lab from prior research. Competent *E. coli* DH5α was purchased from Invitrogen (Burlington, ON) and the pPCRScript cloning vector was obtained from Stratagene (LaJolla, CA). Anti-N_2OR serum was generously donated by Walter Zumft (Universität Karlsruhe, Germany). Oligonucleotides were synthesized by Alpha DNA (Montreal, QC). Unless otherwise noted, restriction and modifying enzymes were purchased from Invitrogen.

2.2 Methods

2.2.1 Construction of *nosZ* plant transformation vectors

2.2.1.1 PCR amplification of coding sequence and promoters

PCR reactions were carried out in a Mastercycler Personal (Eppendorf, Westbury, NY) with high fidelity *PfuTurbo* DNA polymerase (Stratagene) to ensure integrity of the PCR products. Detailed description of primers and PCR reaction conditions are listed in Table 2.1. A forward PCR primer was designed to anneal to the nitrous oxide reductase (*nosZ*) gene of *Pseudomonas stutzeri* Zobell (ATCC 14405) downstream of the 150 nt signal sequence identified by Dreusch et al. (1997). The primer annealing site corresponds to

Table 2.1 PCR reaction conditions for amplification of expression construct components.

Primers, reaction conditions and PCR cycling details used for amplification of the extensin signal sequence, *nosZ* cds, and rolD and d35S promoter sequences.

Product	Primers	Reaction components	Cycle conditions
Extensin signal sequence	exF 5’ GGTACCTATCTAGA**ATG**GGAAGAATTGCTAGAGG *Kpn*I *Xba*I exR 5’ GGAATTCATGCTAGCGGCTGTGGTTTCGGA *Eco*RI *Nhe*I	10 ng pHBV-CO DNA, 0.5 µM each primer, 1x *Pfu* buffer (includes 2 mM $MgCl_2$), 200 µM dNTPs, 1 unit *Pfu*Turbo.	95°C for 2m, 30c of 95°C for 30s, 50°C for 30s, 72°C for 30s and elongation at 72°C for 10m.
***nosZ* coding sequence**	nosZF 5’ GCGCTAGCCAGGCCGTCAAGGAGTCCAAG NheI nosZR 5’ CGAATTCTTGGATCC**TTA**GGCCGGCTCGACCATCA *Eco*RI *Bam*HI	90 ng *P. stutzeri* gDNA, 0.5 µM each primer, 1x *Pfu* buffer (includes 2 mM $MgCl_2$), 200 µM dNTPs, 10% DMSO*, 1 unit *Pfu*Turbo.	95°C for 5m, 30c of 95°C for 1m, 58°C for 1m, 72°C for 2m and elongation at 72°C for 10m.
rolD promoter sequence	RolDF 5’ GGGTACCAGTTGTATCCGAATACTCATA *Kpn*I RolDR 5’ GCTCTAGACTGCCTTGTAGGTGCGA *Xba*I	10 ng pLJ1 DNA, 0.5 µM each primer, 1x *Pfu* buffer (includes 2 mM $MgCl_2$), 200 µM dNTPs, 1 unit *Pfu*Turbo.	95°C for 2m, touchdown; 2c of 95°C for 45s, 58°C for 45s, 72°C for 45s, stepdown every 2c by 1°C until 49°C and 15c of 95°C for 45s, 49°C for 45s, 72°C for 45s and elongation at 72°C for 10m.
d35S promoter sequence	d35SF 5’ CGGTACCCAAGCTTGCATGCCTG *Kpn*I d35SR2 5’ GCTCTAGAATCCTCTCCAAATGAAATGA *Xba*I	10 ng pBI-525 DNA, 0.5 µM each primer, 1x *Pfu* buffer (includes 2 mM $MgCl_2$), 200 µM dNTPs, 1 unit *Pfu*Turbo.	95°C for 2m, touchdown; 2c of 95°C for 45s, 60°C for 45s, 72°C for 45s, stepdown every 2c by 1°C until 51°C and 15c of 95°C for 45s, 51°C for 45s, 72°C for 45s and elongation at 72°C for 10m.

Note: Start and stop codons are indicated in bold on primers exF and nosZR, respectively.
In cycle conditions: c = cycle, m = minute, s = second
* DMSO was added to the reaction to facilitate denaturation of the high GC template.

nucleotides 433-463 from the complete coding sequence (cds) reported by Viebrock and Zumft (1988) (NCBI accession # M22628). The reverse primer was designed to anneal at the *nosZ* stop codon.

To facilitate improved processing and extracellular secretion of the nitrous oxide reductase protein *in planta*, the leader sequence of the *Daucus carota* (carrot) *extensin* (ex) gene was chosen to replace the endogenous *P. stutzeri* signal sequence. The signal sequence was PCR amplified from the plasmid pHBV-CO.

Since one of the primary targets of recombinant N_2OR is the rhizosphere, the root-specific promoter of the *rolD* gene from *Agrobacterium rhizogenes* was chosen to examine the potential for root-specific expression. The promoter sequence 426D, described by Elmayan and Tepfer (1995), containing a 463 bp segment of the upstream untranscribed region of *rolD* was amplified from the plasmid pLJ1 (provided by D. Tepfer). Another promoter, the constitutive cauliflower mosaic virus d35S promoter (Kay et al., 1987), was selected to drive a second expression construct. The 648 bp promoter sequence was amplified from the plasmid pBI-525. Initial cloning of the d35S promoter sequence with primer d35SR (5' GCTCTAGATCCTCTCCAAATGAAATGA) introduced a methylation site at the *Xba*I recognition sequence preventing further cloning of the product. A second PCR was performed with primer d35SR2 (5' GCTCTAGAATCCTCTCCAAATGAAATGA) to eliminate the methylation site. All primers were designed following conventional guidelines using the Generunner software version 3.05.

2.2.1.2 Cloning of PCR products into the pPCR-Script Amp SK(+) cloning vector

PCR reactions were separated on 0.8% agarose gels stained with ethidium bromide. Products of appropriate size were cut from the agarose gels under UV light with a scalpel blade. DNA was isolated from the gel fragments using the QIAEX II Gel Extraction Kit (Qiagen, Mississauga, ON). The PCR-Script Amp cloning kit (Stratagene) was used to blunt-end ligate isolated PCR products to the pPCR-Script Amp SK (+) cloning vector. Cloning was performed following manufacturer's instructions. Positive clones were identified by blue and white selection and further confirmed by plasmid extraction and restriction digest analysis.

2.2.1.3 Sequencing of insert DNA from pPCR-Script Amp SK(+) clones

Once positive clones were identified by restriction digest analysis, plasmid DNA was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit with the ABI Prism 3100 Genetic Analyzer at the University of Ottawa Core DNA Sequencing and Synthesizing Facility. All clones were sequenced from both the T3 and T7 sequences on the pPCR-Script Amp vector. The *nosZ* coding region was further sequenced with the following internal primers: forward primers, nosS1C 5' CGCGTATCCGTCTGGACATC, nosS2C 5' AGGATGGCAAGGACAGCAAG, nosS3C 5' GGGCGAGAAGGTCAACTACATC, nosS4C 5' ACCGCAACGATCCGTTCTTC, and reverse primers, nosS1NC 5' ATCGTTCGGGTGCGGGATGATG, nosS2NC 5' GGCGATCATCGAGCAGGTTG, nosS3NC 5' TCTGATCACGGCGAGCCATG. Sequence alignments were performed with the online Clustal W sequence analysis tool from the European Bioinformatics Institute (EBI), a part of the European Molecular Biology Laboratory (EMBL), Hinxton, Cambridge, UK. (www.ebi.ac.uk/clustalw/)

2.2.1.4 Assembly of constructs into the binary expression vector pRD400

All plasmid extractions throughout the subcloning process were conducted with the QIAprep Spin Miniprep Kit (Qiagen). Restriction digestions were performed step wise as shown in Figures 2.1-2.2 to generate the expression cassettes d35S-*nosZ* and rolD-*nosZ* in the pPCR-Script cloning vector. Cassettes were then cloned into the binary expression vector pRD400 (Datla et al., 1992) to generate the expression vectors pd35S-*nosZ* and prolD-*nosZ* (Figure 2.3). Digestion products were separated on 0.8% agarose gels and products of expected size were cut from the agarose gel under UV light with a scalpel blade. DNA was extracted from agarose gel using the QIAEX II Gel Extraction Kit. Vector and insert products were ligated with T4 DNA ligase following manufacturer's instructions. Subcloning efficiency DH5α competent cells (Invitrogen) were transformed with ligation products following the standard heat-shock method described in the supplier's manual. Transformations were plated on X-gal/ampicillin media and positive clones were identified by blue and white selection following overnight incubation at 37°C. Positively selected clones were screened by colony PCR before proceeding to the next step of subcloning.

2.2.1.5 Verification of integrity of plant expression constructs by sequencing

Upon final assembly of the expression constructs the coding region was sequenced, as described previously, to ensure no mutation had occurred during cloning. Both constructs were sequenced with primers nosS1C, nosS2C, nosS3C, nosS1NC, nosS2NC, nosS3NC and nosS4NC.

Figure 2.1 Construction of the rolD-*nosZ* expression cassette.

The rolD promoter sequence (green) was subcloned from pPCR-Script-rolD into pPCR-Script-ex, containing the extensin signal sequence (orange), using the *Kpn*I and *Xba*I restriction enzymes. The bacterial *nosZ* cds lacking the bacterial signal sequence (blue) was then cloned into the resulting plasmid using the *Nhe*I and *Eco*RI restriction enzymes. Finally, the NOSter termination sequence (red) was added using the *Eco*RI and *Bam*HI restriction enzymes.

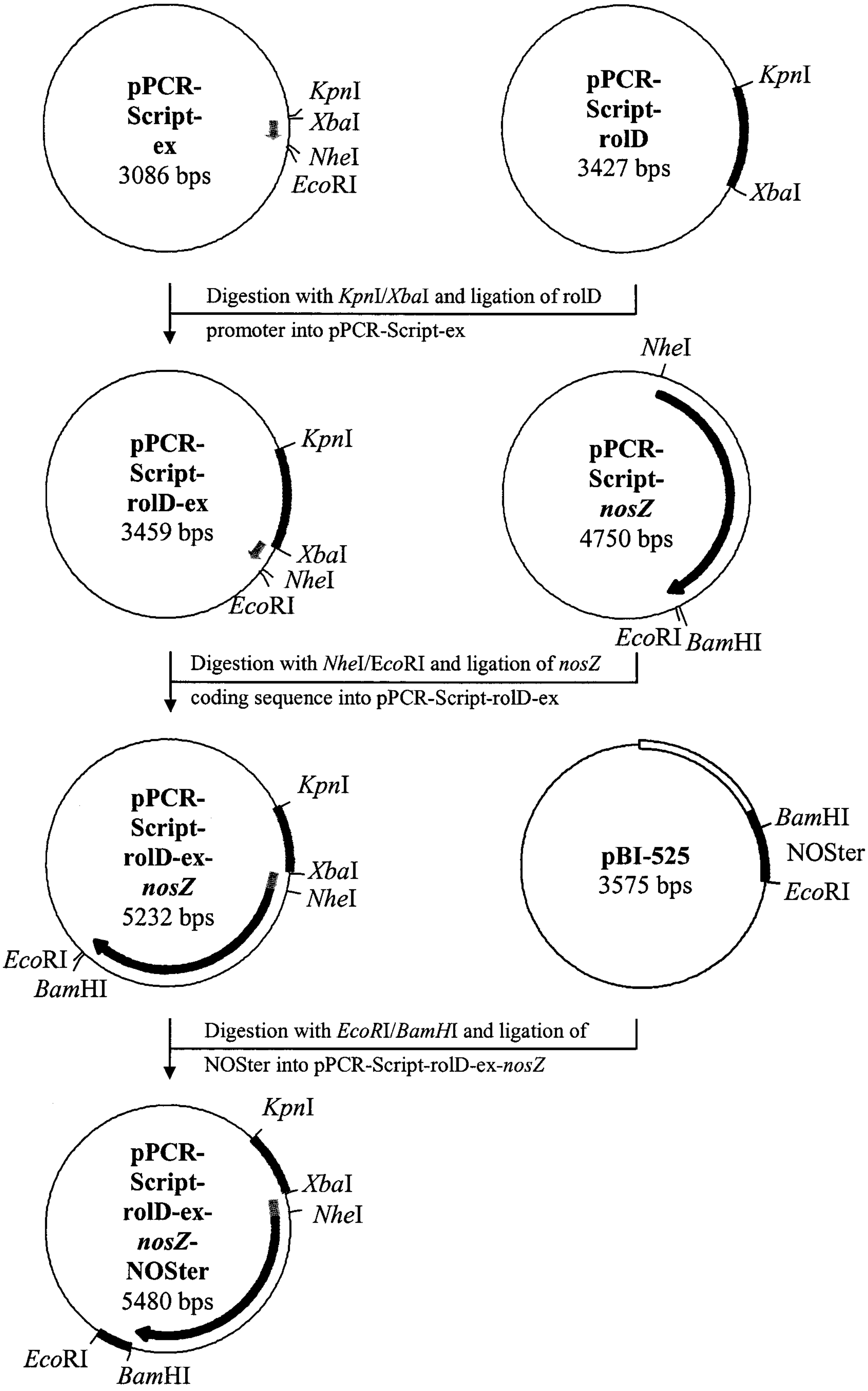

pPCR-Script-ex
3086 bps
KpnI
XbaI
NheI
EcoRI
pPCR-Script-rolD
3427 bps
KpnI
XbaI
Digestion with KpnI/XbaI and ligation of rolD promoter into pPCR-Script-ex
pPCR-Script-rolD-ex
3459 bps
KpnI
XbaI
NheI
EcoRI
NheI
pPCR-Script-nosZ
4750 bps
EcoRI
BamHI
Digestion with NheI/EcoRI and ligation of nosZ coding sequence into pPCR-Script-rolD-ex
pPCR-Script-rolD-ex-nosZ
5232 bps
KpnI
XbaI
NheI
EcoRI
BamHI
pBI-525
3575 bps
BamHI
NOSter
EcoRI
Digestion with EcoRI/BamHI and ligation of NOSter into pPCR-Script-rolD-ex-nosZ
pPCR-Script-rolD-ex-nosZ-NOSter
5480 bps
KpnI
XbaI
NheI
EcoRI
BamHI

Figure 2.2 Construction of the d35S-*nosZ* expression cassette.

The rolD promoter sequence (green) was replaced with the d35S promoter sequence (purple) in the pPCR-Script-rolD-ex-*nosZ*-NOSter vector using the restriction enzymes *Kpn*I and *Xba*I. Extensin signal sequence (orange), *nosZ* cds (blue), NOSter termination sequence (red) are also shown.

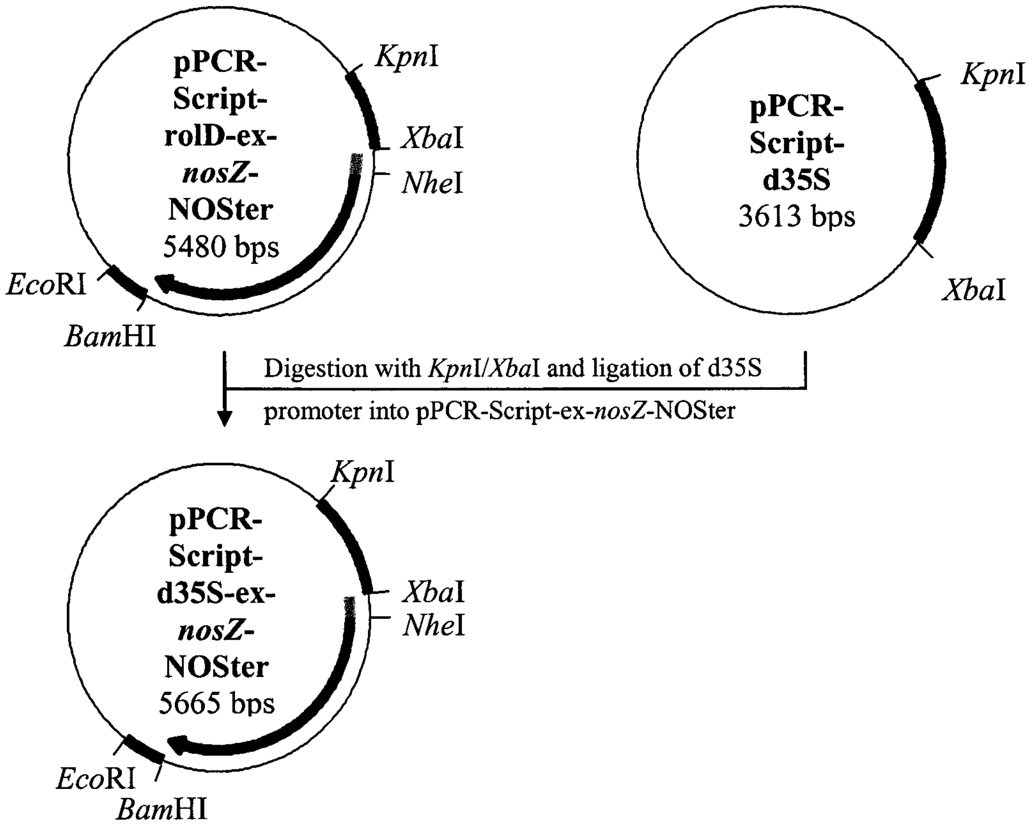
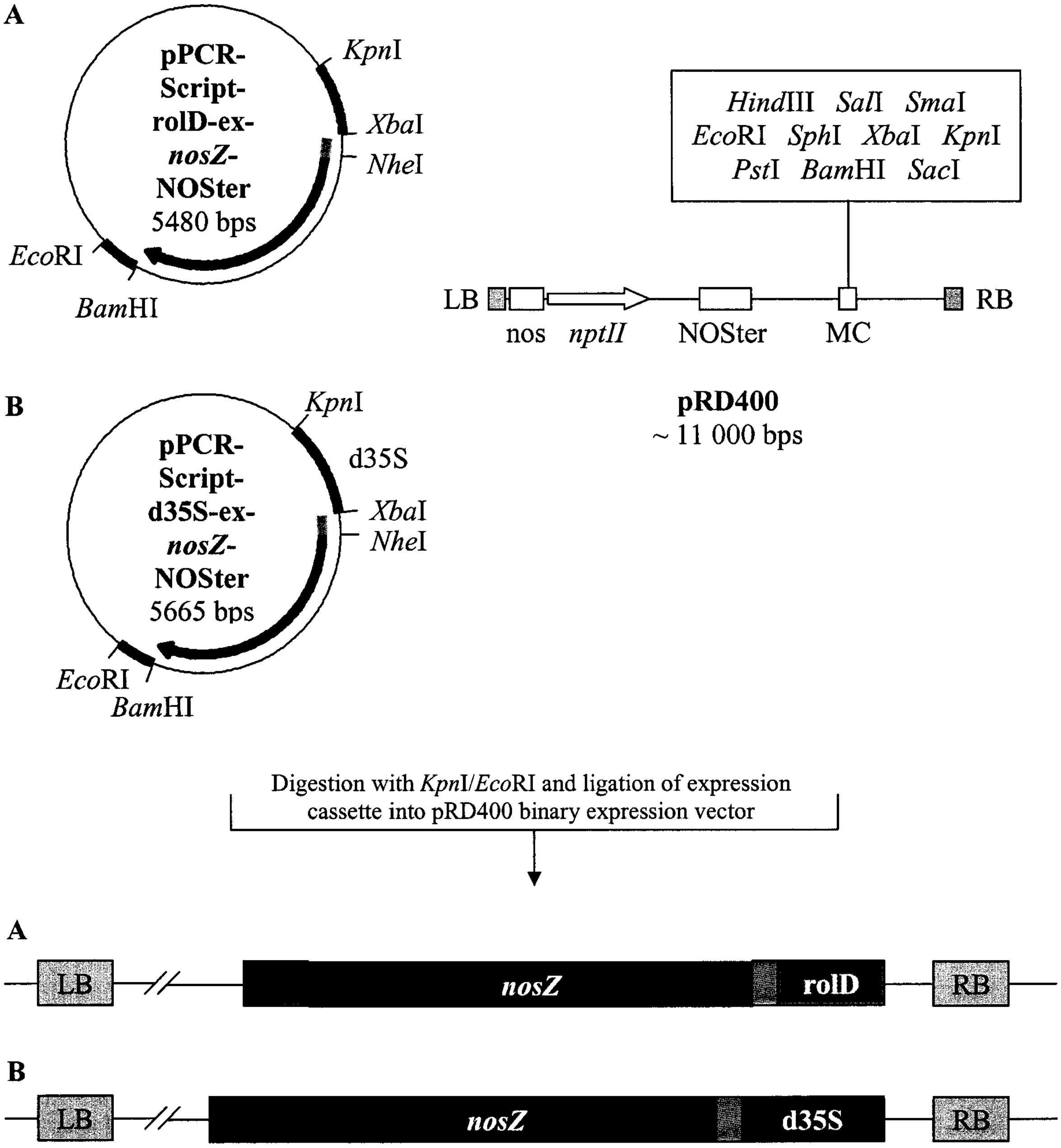

pPCR-Script-rolD-ex-nosZ-NOSter
5480 bps
KpnI
XbaI
NheI
EcoRI
BamHI
pPCR-Script-d35S
3613 bps
KpnI
XbaI
Digestion with KpnI/XbaI and ligation of d35S promoter into pPCR-Script-ex-nosZ-NOSter
pPCR-Script-d35S-ex-nosZ-NOSter
5665 bps
KpnI
XbaI
NheI
EcoRI
BamHI

Figure 2.3 Cloning of the d35S-*nosZ* and rolD-*nosZ* expression cassettes into the binary vector pRD400.

The rolD-*nosZ* (**A**) and d35S-*nosZ* (**B**) expression constructs were subcloned into the pRD400 transformation vector using the *Kpn*I and *Eco*RI restriction sites. Legend: Green, rolD promoter; orange, extensin signal sequence; blue, *nosZ* cds; red, NOSter (nopaline synthase gene polyadenylation signal); purple; d35S promoter sequence; LB, T-DNA left border; nos, promoter of the *nptII* gene; *nptII*, neomycin phosphotransferase II gene; MC, multiple cloning site; RB, T-DNA right border.


A
pPCR-Script-rolD-ex-nosZ-NOSter
5480 bps
KpnI
XbaI
NheI
EcoRI
BamHI
HindIII SalI SmaI
EcoRI SphI XbaI KpnI
PstI BamHI SacI
LB
nos
nptII
NOSter
MC
RB
pRD400
~ 11 000 bps
B
pPCR-Script-d35S-ex-nosZ-NOSter
5665 bps
KpnI
d35S
XbaI
NheI
EcoRI
BamHI
Digestion with KpnI/EcoRI and ligation of expression cassette into pRD400 binary expression vector
A
LB
nosZ
rolD
RB
B
LB
nosZ
d35S
RB

2.2.2 Transformation and regeneration of transgenic tobacco plants

2.2.2.1 Preparation of competent *Agrobacterium tumefaciens*

A. tumefaciens cells were made competent by chemical method (An, 1987). A single colony of *Agrobacterium* strain LBA4404 was grown overnight in 5 ml of liquid YEP (Yeast Extract Peptone) medium (10 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl) with shaking at 28°C. The overnight culture was used to inoculate 45 ml of liquid YEP medium and the culture was grown at 28°C with shaking to an OD of 0.5-1.0, approximately 6 hours. The culture was centrifuged at 3000*g* for 10 min at 4°C. The cells were resuspended in 1 ml of 20 mM sterile ice-cold $CaCl_2$ and glycerol was added to a final concentration of 15%. Aliquots of 0.1 ml were frozen on liquid nitrogen and stored at -70°C.

2.2.2.2 Transformation and verification of *A. tumefaciens*

Upon final verification of both the rolD and d35S constructs the binary vectors were introduced into competent *A. tumefaciens*. Approximately 1 μg of plasmid DNA was added to an aliquot of competent cells and the mixture was incubated on ice for 30 min. The cells were frozen in liquid nitrogen for 2 min and thawed at 37°C for 10 min. The cells were supplemented with 1 ml of YEP media and grown at 28°C with shaking for 6 h. The supernatant was discarded after 30 second centrifugation in a microcentrifuge and cells were resuspended in 100 μl YEP broth. The transformation mixture was then plated on YEP selection plates (50 μg/ml kanamycin) and incubated at 28°C for 2-3 days until colonies began to appear. Aliquots of 20 ml of YEP media were inoculated with single colonies from the selection plates and grown overnight at 28°C. Plasmid DNA was extracted from the overnight culture using the QIAprep Spin Miniprep Kit. Slight modifications were made to the standard manufacturer's protocol to improve DNA yield. Briefly, cells were centrifuged for 10 min at 3000*g* and resuspended in 1 ml P1 buffer. The cell suspensions were divided into aliquots of 250 μl and the protocol was followed as recommended. All aliquots were applied to the same spin column to concentrate the sample and the columns were washed with PB buffer to eliminate contaminating nucleases. Buffer EB was heated to 70°C to improve elution of the plasmid from the spin column. Plasmid DNA was digested with appropriate restriction endonucleases to positively confirm transformation of the cells and integrity of the plasmid.

2.2.2.3 *Agrobacterium*-mediated transformation of tobacco leaf tissue

The tobacco transformation procedure was modified slightly from the method of Horsch et al. (1985). Sterile tobacco tissue was prepared by germinating seeds of *Nicotiana tabacum* cv. Xanthi nc. in sterile Sigma culture pots on germination medium (2.15 g/L Murashige-Skoog (MS) medium, 3% sucrose, 0.8% agar, pH 5.8). Prior to plating, tobacco seeds were sterilized by vigorous vortexing in a solution of 20% bleach and 0.05% Tween-20 followed by repeated rinsing in sterile ddH$_2$O. Once seedlings reached an appropriate size, leaves were harvested under sterile conditions and were cut into 1 cm square sections with a blade under sterile ddH$_2$O. Small wounds were introduced to the sterile leaf sections with a scalpel blade to facilitate infection by *A. tumefaciens* and leaf sections were transferred to a 5 ml suspension of *A. tumefaciens* containing the appropriate expression vector. The suspension was prepared by inoculating 10 ml of YEP medium (10 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl, 1.5% agar, pH 7.0, 50 μg/ml kanamycin) with a colony of transformed *A. tumefaciens*. The culture was grown overnight at 28°C with gentle shaking (~ 300 rpm).

Cells were spun down and the supernatant discarded. The isolated cells were resuspended in 5 ml fresh YEP medium without kanamycin and the suspension was used as the final inoculant for the tobacco leaf sections. The tissue sections were incubated with the inoculant for several minutes before being transferred to co-cultivation plates (4.3 g/L MS medium, 3% sucrose, 1 mg/L 6-benzyladenine, 0.1 mg/L α-naphthalene acetic acid, 0.8% agar, pH 5.8). Infection of the tobacco tissue by *A. tumefaciens* and associated transfer of the T-DNA occurred on the co-cultivation media for 48 h at room temperature in darkness.

2.2.2.4 Regeneration of transformed tobacco

To inhibit growth of *A. tumefaciens* and allow regeneration of transformed tobacco cells, the tissue sections were transferred to regeneration medium (4.3 g/L MS medium, 3% sucrose, 1 mg/L 6-benzyladenine, 0.1 mg/L α-naphthalene acetic acid, 0.8% agar, pH 5.8, 500 mg/L timentin, 300 mg/L kanamycin). Non-transformed tobacco tissue sections that were not inoculated with *A. tumefaciens* were placed on regeneration medium as a negative control to ensure effectiveness of kanamycin selection.

Growth of calli from transformed tobacco cells was induced on the regeneration medium under photoconditions of 16 h light, followed by 8 h of dark at room temperature. This induction process occurred over a period of several weeks. Calli began to appear at approximately two weeks post-transformation and shoots were visible after four weeks. When shoots were approximately 1 cm in height they were excised from the callus under sterile conditions with a scalpel blade and transferred to rooting medium (4.3 g/L MS medium, 2% sucrose, 0.8% agar, pH 5.8, 500 mg/L timentin, 300 mg/L kanamycin). Only the first shoot from each callus was transferred to rooting medium to ensure the genetic variability of transformants, i.e. to obtain independent T_0 event lines. Induction of root growth occurred over several weeks under the same conditions as shoot regeneration. When roots were large enough, approximately 5 cm in length, the plantlets were transferred to potted soil. For the first 48 h after potting, plantlets were covered with transparent Sigma tissue pots to prevent desiccation. Pots were gradually removed over several days to allow plantlets to acclimate to reduced humidity in the greenhouse. Potted plants were maintained at the University of Ottawa CAREG research greenhouse under a photoperiod of 16 h at 26°C followed by a dark period of 8 h at 20°C. Plants were watered daily and fertilized with a water soluble fertilizer twice a week. After six weeks, plants began to flower and were self-pollinated as necessary. Seeds were collected once seed pods had matured and planted in soil to obtain first generation (T_1) progeny lines.

2.2.3 Analysis of mature transgenic tobacco plants

2.2.3.1 Polymerase chain reaction

Young leaf tissue was harvested from tobacco seedlings during transfer of the plants from rooting media to soil. Tissue was frozen and stored at -70°C until DNA extraction was performed at a later date. Frozen tissue was ground to a fine powder in liquid nitrogen prior to genomic DNA extraction with the DNeasy Plant Mini kit (Qiagen). The protocol for extraction of total DNA was followed as provided by the manufacturer. DNA concentration was measured at A_{260} with a Beckman DU600 spectrophotometer (Beckman Coulter, Mississauga, ON) prior to PCR. Purity of DNA samples was also monitored by the A_{260}/A_{280} ratio. Only samples with a ratio between 1.8 and 2.0 were used for subsequent PCR.

Integration of the expression cassette into the tobacco genome was verified by amplifying a segment of DNA starting within the respective promoter region (either d35S or rolD promoter) and extending into the *nosZ* coding region. DNA from plants transformed with the rolD construct was amplified with primers rolDF 5' GGGTACCAGTTGTATCCGAATACTCATA and nosS1NC 5' ATCGTTCGGGTGCGGGATGATG under the following conditions: 200 ng plant genomic DNA, 0.5 µM each primer, 1x *Taq* reaction buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 5% DMSO, 1 unit *Taq* polymerase (Invitrogen), with touchdown cycling conditions of 95°C for 5 min, 2 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 2 min, step-down every 2 cycles by 1°C until 50°C and 25 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 2 min and a final elongation step at 72°C for 10 min. Similarly DNA from plants transformed with the d35S construct was amplified with primers d35SF2 5' GCACAATCCCACTATCCTTCGC and nosS1NC 5' ATCGTTCGGGTGCGGGATGATG under the following conditions: 200 ng plant genomic DNA, 0.5 µM each primer, 1x *Taq* reaction buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 5% DMSO, 1 unit *Taq* polymerase (Invitrogen), with touchdown cycling conditions of 95°C for 5 min, 2 cycles of 95°C for 1 min, 63°C for 1 min, 72°C for 2 min, step-down every 2 cycles by 1°C until 55°C and 25 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final elongation step at 72°C for 10 min. Negative control reactions were performed under the same conditions with 200 ng of DNA from non-transformed tobacco plants. Positive control reactions were also performed under the same conditions with 1 ng of pPCR-Script-rolD-ex-*nosZ*-NOSter plasmid or pPCR-Script-d35S-ex-*nosZ*-NOSter plasmid where appropriate.

2.2.3.2 Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-PCR (RT-PCR) was performed on extracts from both leaf and root tissue. Young leaves were removed from mature plants frozen on liquid nitrogen and stored at -70°C until extraction of total RNA. A portion of the root-soil complex was cut away from the potted plant. The soil was rinsed away from the roots with running tap water and roots were immediately frozen in liquid nitrogen and stored at -70°C. Prior to grinding the frozen tissue, the mortar and pestle were prepared by baking at 180°C for 16 h. Additional materials to be used during extraction of total RNA were prepared by thorough washing with a solution of 0.1 M NaOH and 1 mM EDTA and rinsing with RNase-free DEPC-treated ddH_2O prior to autoclaving. Extraction of total RNA from both leaf and root tissue was performed with the RNeasy Plant Mini kit (Qiagen). The protocol recommended by the manufacturer was followed without changes. The optional treatment with DNase was included. Total RNA extracts were quantified at A_{260} and purity was assessed by the A_{260}/A_{280} ratio.

Reverse transcription was executed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The protocol for first-strand synthesis using random primers was followed without changes. A negative RT control, in which the SuperScript II reverse transcriptase was excluded from the reaction, was included for each sample. The reverse transcription incubations were carried out in a Mastercycler Personal (Eppendorf). The resultant cDNA samples were quantified at A_{260}.

The full length transcript was amplified using the primers exF 5' GGTACCTATCTAGAATGGGAAGAATTGCTAGAGG and nosZR 5' CGAATTCTTGGATCCTTAGGCCGGCTCGACCATCA. The PCR reaction used was: 50 ng cDNA template, 0.5 μM each primer, 1x *Taq* reaction buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 5% DMSO, 1 unit *Taq* polymerase. The cycling conditions used were a denaturing step at 95°C for 5 min, 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 2 min with a final elongation step at 72°C for 10 min. The positive control PCR reaction was the same as described except the cDNA template was replaced by 1 ng of plasmid DNA. A negative control of non-transformed tobacco cDNA was included as well as a no-RT enzyme control for each transgenic plant sample to ensure that no DNA contamination had occurred. Both leaf and root RNA extracts were handled in the same manner throughout the RT-PCR procedure.

2.2.3.3 Western hybridization

Leaf and root tissue powder stored at -70°C for RNA extracts was also used to prepare total protein extracts. Tissue powder was homogenized in a 1:1 ratio of 2x protein sample buffer (0.1 M Tris pH 6.8, 1 mM EDTA, 6% SDS and 20% glycerol). The mixture was incubated at 95°C for 10 min with regular inversion. Tissue debris was pelleted down for 10 min at 3000*g*, supernatant was transferred to a fresh tube and the centrifugation step was repeated. Crude total protein extract was quantified using the BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL). Extracts were used fresh whenever possible or were stored at 4°C for a maximum of 12 h. Immediately prior to electrophoresis of samples bromophenol blue was added to 0.1% and β-mercaptoethanol was added to 5%.

Crude cell lysate of *P. stutzeri* was used as a positive control in all western hybridization experiments. Lysate containing endogenous N_2OR was prepared as described by Charnock et al. (2000). Briefly, 50 ml of YEP media was inoculated with a 1 ml overnight culture of *P. stutzeri* and was grown at 30°C with shaking at 250 rpm for 6 h. Cells were induced to express *nosZ* with addition of 1% sterile $NaNO_3$ and shaking was reduced to 120 rpm (limiting oxygen conditions). Growth continued at 30°C for 16 h. The culture was centrifuged at 3000*g* for 10 min and the supernatant was discarded. The cell pellet was resuspended in 250 μl lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Triton-X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF) with vigorous vortexing. The slurry was incubated on ice for 30 min with vortexing every 10 min and centrifuged at 3000*g* for 10 min. The supernatant was transferred to a fresh tube and stored at -20°C.

Protein samples (175 μg leaf or root protein, 200 ng *P. stutzeri* protein) were heated at 95°C for 10 min in 2x protein sample buffer (0.1 M TrisCl pH 6.8, 1 mM EDTA, 6% SDS, 20% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol) and loaded onto an 10% SDS polyacrylamide gel with a 5% stacking gel. Gels were electrophoresed at maximum voltage (3000 V) and 16 mA in a Mini-PROTEAN 3 System (Bio-Rad, Hercules, CA) until the bromophenol blue had exited the gel.

Proteins were transferred onto nitrocellulose membrane (Bio-Rad) using the Trans-Blot SD semi-dry transfer cell (Bio-Rad). Filter papers, nitrocellulose membrane and resolving gel were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 1.3 mM SDS) for 15 min to allow equilibration. The transfer sandwich was arranged as follows: two sheets of filter paper were placed on the platinum anode of the apparatus, followed by the nitrocellulose membrane. The gel was centered on the membrane and finally covered with two sheets of filter paper. Transfer conditions were 10 V and maximum current (150 mA) for 1 h. Following transfer the nitrocellulose membrane was placed in blocking solution (5% skim milk in TBS-Tween [1.5 M NaCl, 0.2 M Tris pH 7.6, 0.5%

Tween-20]) and incubated with gentle rocking for 1 hr. The membrane was incubated with anti-N_2OR serum (provided by W. Zumft) in fresh blocking solution at a concentration of 1:5000 for an additional hour followed by 4 x 5 min washes with TBS-Tween buffer. A 1 h incubation with the secondary antibody, biotin-SP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), at a concentration of 1:25000 in TBS-Tween was also followed by 4 x 5 min washes with TBS-Tween buffer. The final antibody incubation with the tertiary antibody, anti-goat biotin horseradish peroxidase linked antibody (Cell Signaling Technology Inc., Beverley, MA), at a concentration of 1:1000 in TBS-Tween was carried out overnight at 4°C with shaking. To remove excess antibody, the membrane was washed with TBS-Tween for 4 x 5 min. Detection of proteins was carried out using the ECL western blotting chemiluminescent detection reagents according to the manufacturer's protocol (Amersham Biosciences, Baie D'Urfé, QC).

Chapter 3

RESULTS

3.1 Generation of transgenic tobacco plants expressing the *nosZ* coding sequence

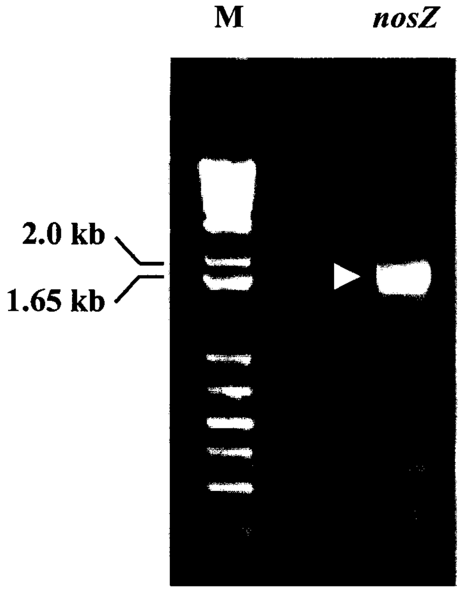
3.1.1 Cloning of two expression cassettes for expression of *nosZ* in tobacco

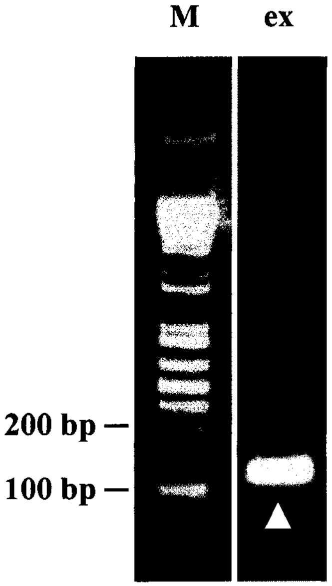
To engineer the expression of N_2OR in tobacco, a fusion of the plant extensin (ex) signal sequence from carrot with the bacterial *nosZ* cds was produced. Two vectors driving the expression of the *nosZ* fusion from two different promoters were constructed. The promoters used were the constitutive CaMV d35S promoter and the root-specific *A. rhizogenes* rolD promoter. These four components of the vector construct: d35S and rolD promoters, ex signal sequence and *nosZ* cds were amplified by PCR (Figure 3.1) and cloned into the pPCR-Script vector. Construction of the expression cassettes was performed in several steps as described in the methods section and included addition of the NOSter polyadenylation sequence from *A. tumefaciens* to achieve termination of transcription. The expression constructs were introduced into the binary vector pRD400 as a *Kpn*I-*Eco*RI fragment. The full length cds of both binary vectors, pd35S-*nosZ* and prolD-*nosZ*, were sequenced to confirm the ex::*nosZ* fusions were inserted in frame without mutations. Initial sequencing revealed a deletion mutation in the signal sequence at position 86 nt in both vectors. The mutation was corrected by replacement of the ex sequence with the original fragment from pPCR-Script-ex. A second round of sequencing revealed the cds to be free of mutations (Appendix I).

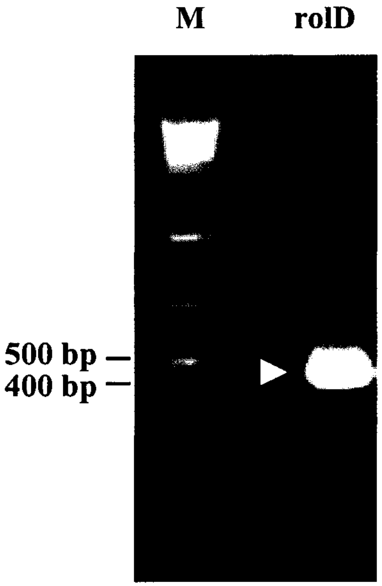
The two pRD400 expression vectors, pd35S-*nosZ* and prolD-*nosZ*, were introduced into the *A. tumefaciens* strain LBA4404 individually. The pRD400 vector carries the neomycin phosphotransferase II (*nptII*) gene for kanamycin resistance, therefore, positive

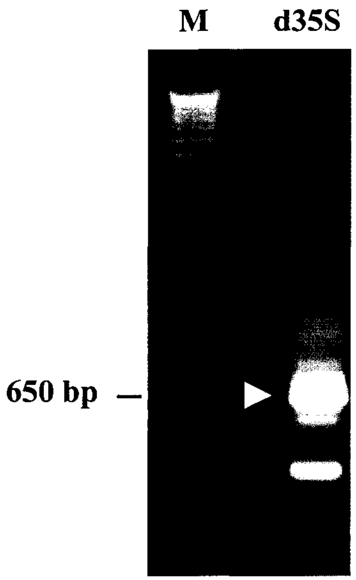
Figure 3.1 PCR cloning of the *nosZ* expression construct components.

To engineer the restriction enzyme recognition sites required for construction of expression cassettes, the extension signal sequence (ex), nitrous oxide reductase cds (*nosZ*) and promoters (rolD and d35S) were PCR amplified from appropriate template DNA. Each PCR reaction was separated on a 0.8% agarose gel. PCR products of expected size, 1790 bp (nosZ), 126 bp (ex), 463 bp (rolD) and 648 bp (d35S), were ligated into pPCR-Script and sequenced. PCR products positively identified by sequencing are indicated with white arrows. M: 1 kb plus DNA ladder (Invitrogen).


M
nosZ
2.0 kb
1.65 kb


M
ex
200 bp
100 bp


M
rolD
500 bp
400 bp


M
d35S
650 bp

transformants were isolated on kanamycin selection media. Plasmid DNA was isolated from positive colonies via minipreps and digested with *Hin*dIII to confirm transformation of the *A. tumefaciens* strain (Figure 3.2). The presence of digestion products of expected size, 1724 bp (prolD-*nosZ*) and 1857 bp (pd35S-*nosZ*), confirmed successful transformation of *A. tumefaciens* with the recombinant binary vectors.

3.1.2 Introduction of the *nosZ* gene into tobacco tissue

Following leaf disk transformation, approximately 30 kanamycin-resistant T_0 transgenic tobacco lines were regenerated from both *Agrobacterium* strains and designated d35S1-d35S32 and rolD1-rolD27. T_1 plant lines were grown from seed of the first generation and the overall horticultural traits, such as leaf broadness, flower size and colour, and pollen production, of both generations of transgenic plants were similar to those of non-transgenic (NT) plants. During regeneration of transformed tissue on kanamycin media a negative control of non-transformed tissue was incubated under the same conditions. Necrosis of the non-transformed sections confirmed the effectiveness of kanamycin selection.

3.2 Analysis of mature tobacco plants transformed with *nosZ* gene constructs

3.2.1 Confirmation of transgene integration into the tobacco genome

In addition to following kanamycin resistance as a measure of the presence of T-DNA containing the *nosZ* expression cassette, a segment of the recombinant DNA was amplified from plant genomic DNA by PCR. Genomic DNA was extracted from all regenerated tobacco plants and a segment of the d35S-*nosZ* or rolD-*nosZ* construct was amplified as shown in Figures 3.3 and 3.4, respectively. The expected PCR products of

Figure 3.2 Transformation of *A. tumefaciens* strain LBA4404 with binary expression vectors, prolD-*nosZ* and pd35S-*nosZ*.

A. Depiction of expected *Hin*dIII digestion products of rolD-*nosZ* and d35S-*nosZ* constructs. Extensin signal sequence shown in orange and NOSter termination sequence shown in red. Diagram is to scale.
B. Plasmid DNA from kanamycin resistant *A. tumefaciens* LBA4404 colonies transformed with prolD-*nosZ* or pd35S-*nosZ* was isolated and digested with *Hin*dIII. Digestion products were separated on a 0.7% agarose gel. rolD: digestion products of plasmid DNA isolated from *A. tumefaciens* transformed with prolD-*nosZ*; d35S: digestion products of plasmid DNA isolated from *A. tumefaciens* transformed with pd35S-*nosZ*; M: 1 kb plus DNA ladder (Invitrogen).

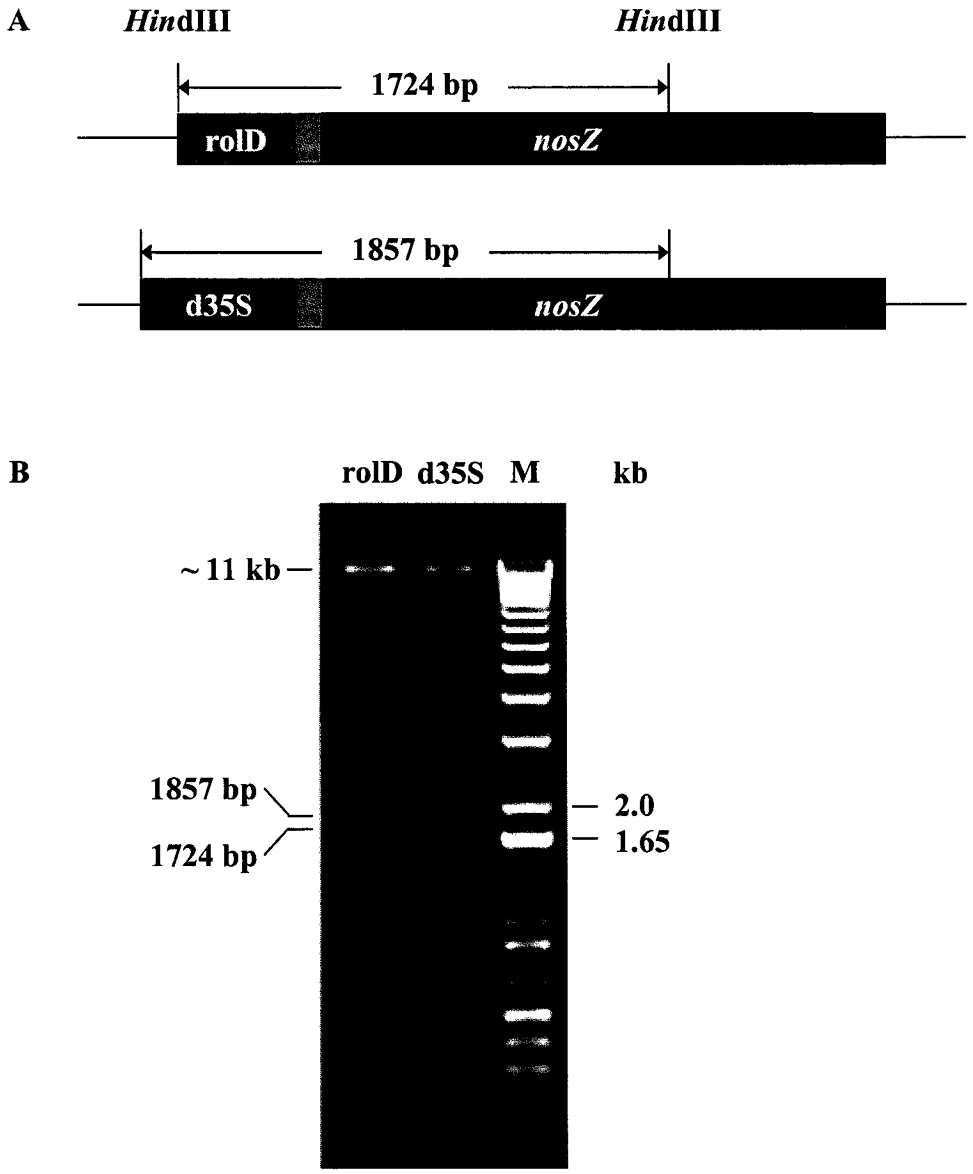

A
*Hin*dIII
*Hin*dIII
1724 bp
rolD
nosZ
1857 bp
d35S
nosZ
B
rolD
d35S
M
kb
~ 11 kb
1857 bp
1724 bp
2.0
1.65

Figure 3.3 Identification of d35S-*nosZ* T_0 transgenic tobacco by PCR screening.

A. Depiction of annealing position of primers used to amplify a fragment of the d35S-*nosZ* expression construct from plant genomic DNA. The expected size of the PCR product is 637 bp. **B.** Genomic DNA was extracted from 31 regenerated plants and a segment of the recombinant DNA was amplified as shown in A. M: 1 kb plus DNA ladder (Invitrogen); +: positive control, recombinant plasmid isolated from *E. coli*; NT: negative control, genomic DNA from a non-transformed plant. Transformed plants are identified by number. Plant 31 was lost.

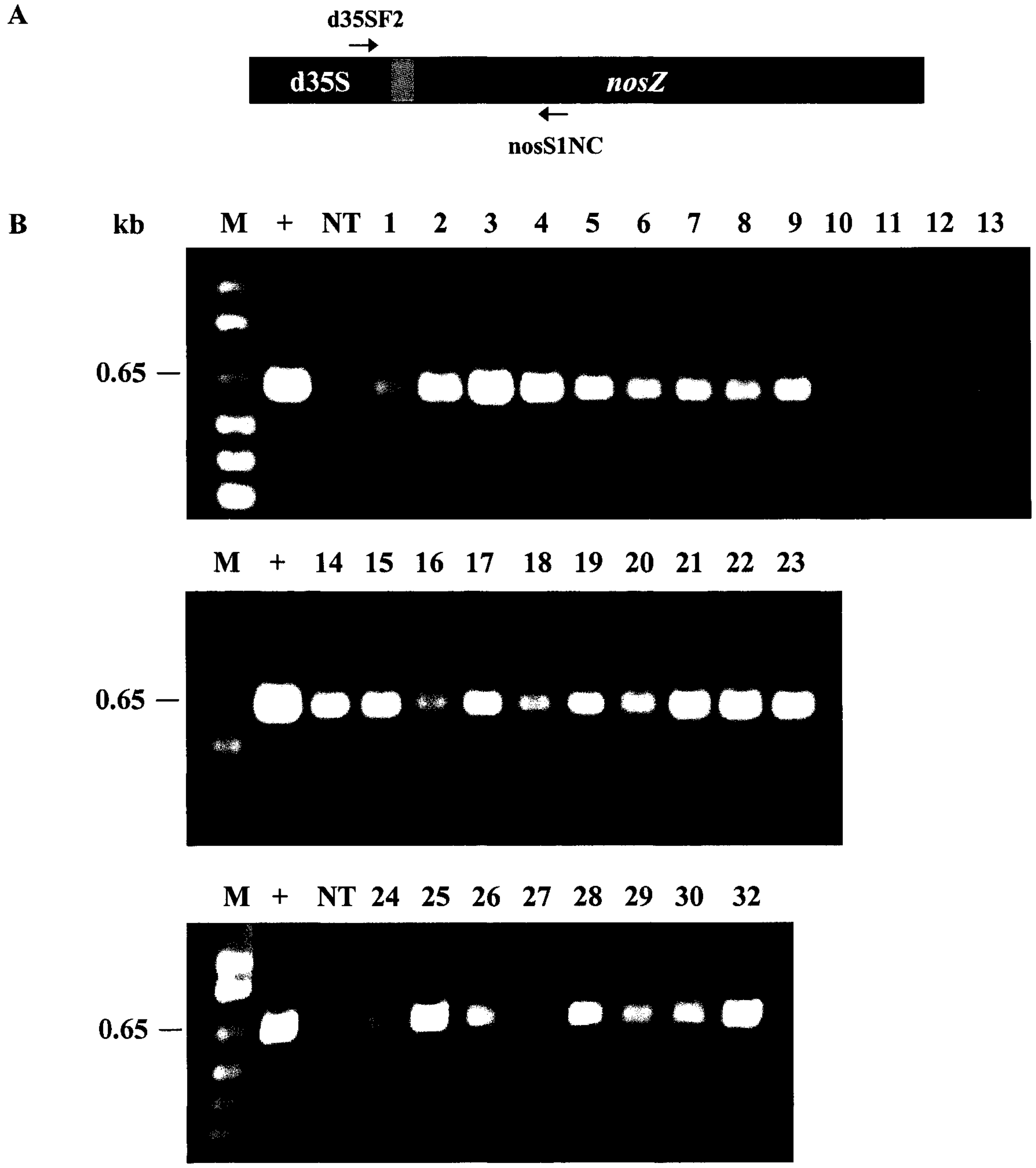
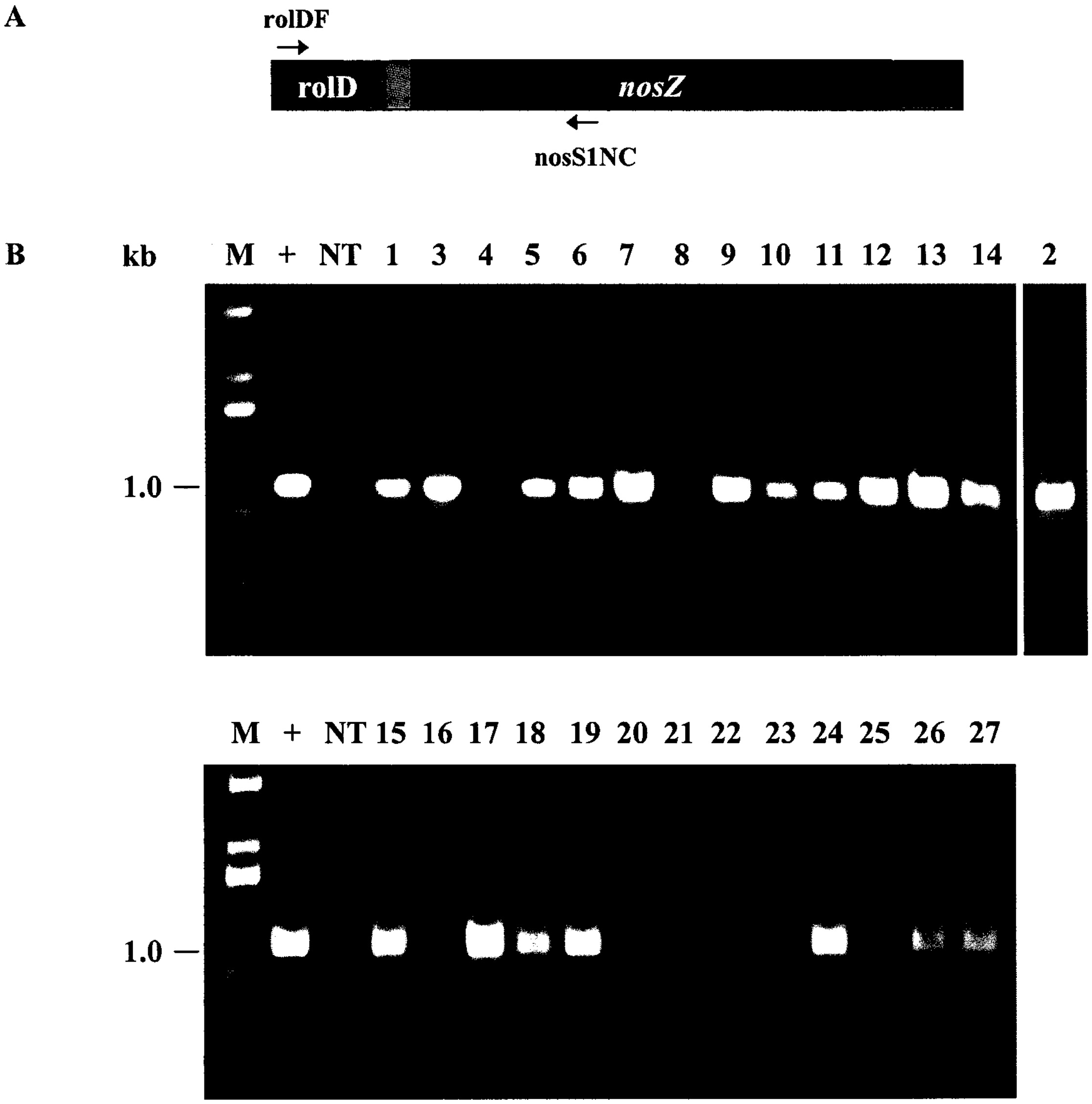

A
d35SF2
d35S
nosZ
nosS1NC
B
kb
M + NT 1 2 3 4 5 6 7 8 9 10 11 12 13
0.65
M + 14 15 16 17 18 19 20 21 22 23
0.65
M + NT 24 25 26 27 28 29 30 32
0.65

Figure 3.4 Identification of rolD-*nosZ* T_0 transgenic tobacco by PCR screening.

A. Depiction of annealing position of primers used to amplify a fragment of the rolD-*nosZ* expression construct from plant genomic DNA. The expected size of the PCR product is 1028 bp. **B.** Genomic DNA was extracted from 27 regenerated plants and a segment of the recombinant DNA was amplified as shown in A. M: 1 kb plus DNA ladder (Invitrogen); +: positive control, recombinant plasmid isolated from *E. coli*; NT: negative control, genomic DNA from a non-transformed plant. Transformed plants are identified by number.


A
rolDF
rolD
nosZ
nosS1NC
B
kb
M + NT 1 3 4 5 6 7 8 9 10 11 12 13 14 2
1.0 —
M + NT 15 16 17 18 19 20 21 22 23 24 25 26 27
1.0 —

637 bp and 1028 bp were detected from d35S and rolD plants, respectively. The incidence of PCR detection of the *nosZ* transgene was 93% (54 of 58 kanamycin resistant plants). A negative control was included and no amplification of recombinant DNA from a non-transformed plant sample was observed. PCR products of the same size were amplified from T_1 plant DNA (Figure 3.5).

3.2.2 *nosZ* expression at the plant RNA level

To assay for expression of recombinant *nosZ* at the RNA level, total RNA was isolated from mature leaf and root tissue of selected plants. Six plants transformed with each expression construct, which were shown by PCR to carry the *nosZ* gene, were selected. T_0 seedlings were transferred to soil on three potting dates and two plants from each date were chosen for further analysis. In addition these plants were selected to include the most phenotypic variation possible in the samples. Total RNA was reverse transcribed using random primers and the full length cDNA was PCR amplified with primers specific to the extensin signal sequence and *nosZ* (Figure 3.6). For each RT reaction a no-RT control, devoid of RT enzyme, was performed to ensure no DNA contamination of the RNA preparations had occurred (Appendix II). A product of approximately 1.8 kb, corresponding in size to the positive control and the expected 1869 bp full length cDNA, was detected in each of the leaf samples and all but three of the root samples (rolD24, d35S18, d35S29). No observable product was amplified from the cDNA of the non-transformed plant. Several T_1 lines, derived from T_0 lines that were confirmed to express recombinant N_2OR, were selected for RT-PCR of leaf RNA (Figure 3.7). The full length cDNA product was amplified from each of the T_1 plants tested.

Figure 3.5 Identification of T_1 transgenic tobacco by PCR screening.

A segment of the rolD-*nosZ* sequence or d35S-*nosZ* sequence was amplified as described for T_0 plants to confirm the transgenic nature of selected T_1 plants. M: 1 kb plus DNA ladder (Invitrogen); +: positive control, recombinant plasmids isolated from *E. coli*; NT: negative control, genomic DNA from a non-transformed plant. Transformed plants are identified by number and expression construct (rolD or d35S).

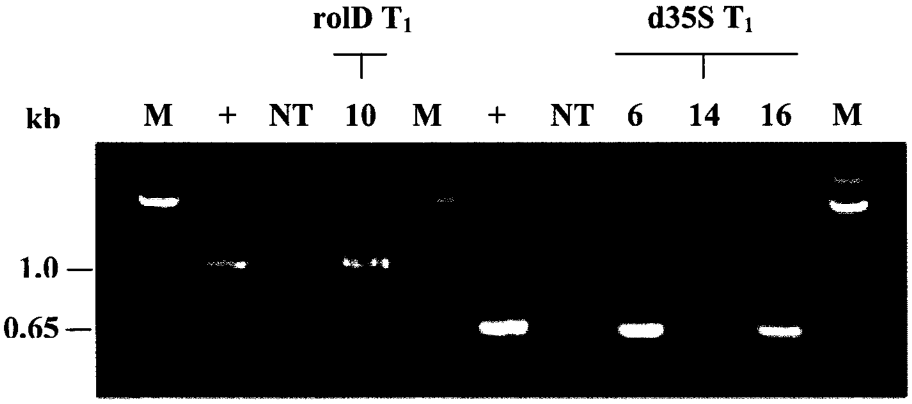

rolD T$_1$
d35S T$_1$
kb
M
+
NT
10
M
+
NT
6
14
16
M
1.0
0.65

Figure 3.6 Expression of *nosZ* in T_0 rolD-*nosZ* and d35S-*nosZ* transgenic tobacco.

RT-PCR detection of *nosZ* transcripts in leaf and root tissue of transformed tobacco plants. Total RNA was extracted from the leaves (upper panel) and roots (lower panel) of mature tobacco plants. RNA was reverse transcribed with the SuperScript First-Strand Synthesis System (Invitrogen) and the full length *ex::nosZ* cDNA was PCR amplified. The expected size of the full length cDNA is 1869 bp. M: 1 kb plus DNA ladder (Invitrogen); +: positive control, recombinant plasmid isolated from *E. coli*; NT: negative control, total RNA from non-transformed tobacco. Transformed lines are identified by promoter (rolD or d35S) and number. No-RT controls were also performed for each sample (Appendix II).

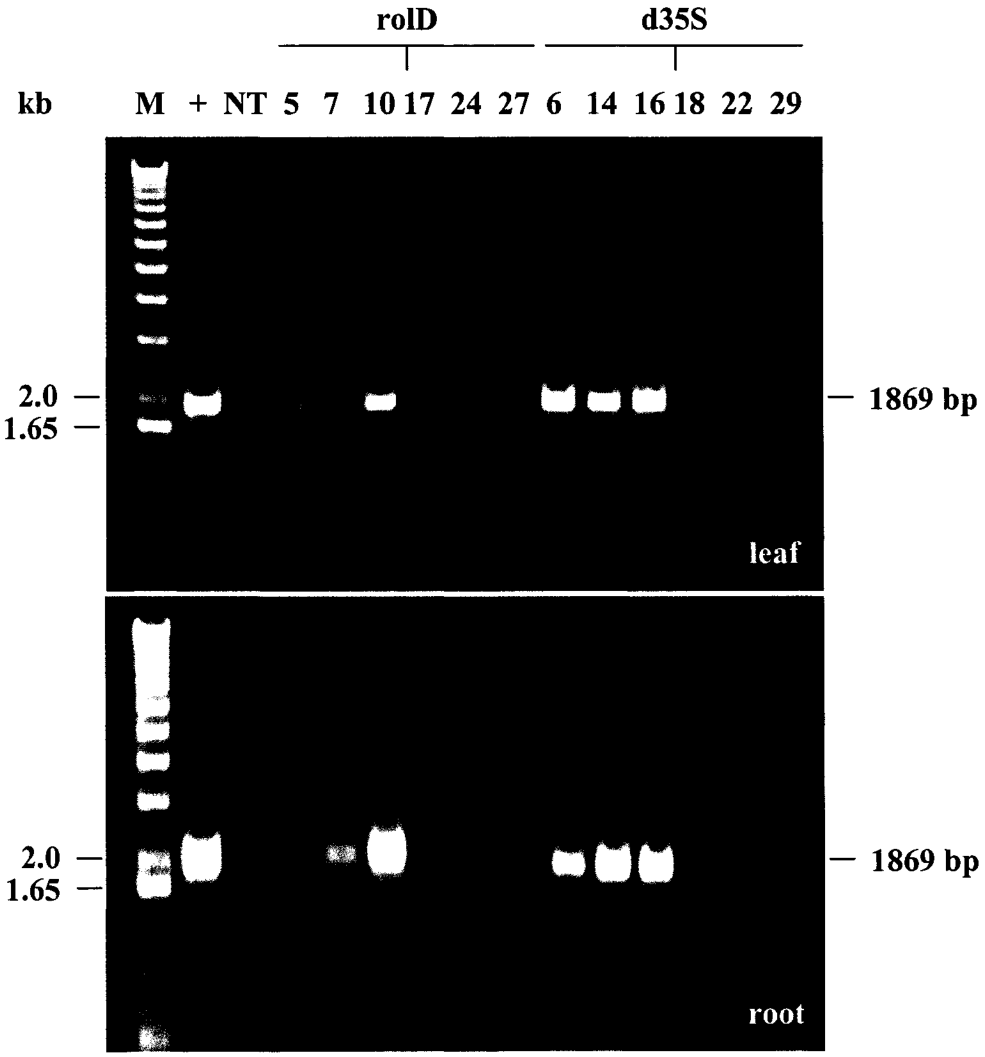
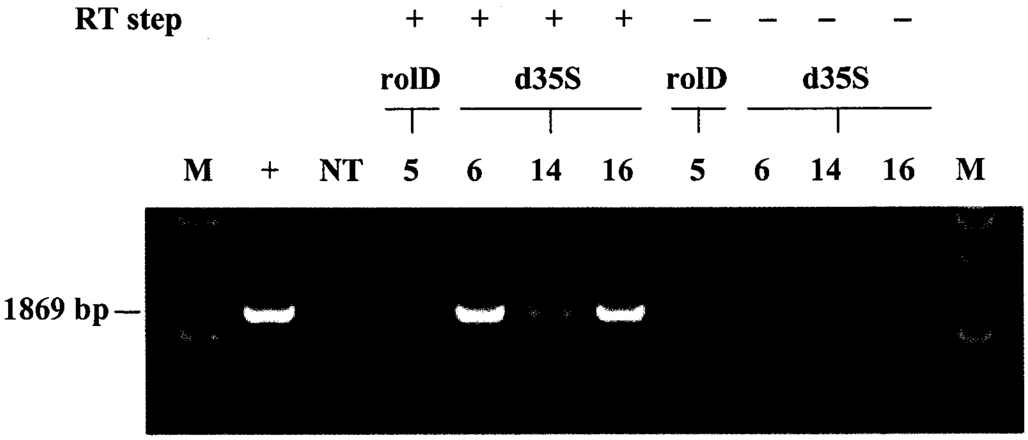

rolD
d35S
kb
M
+
NT
5
7
10
17
24
27
6
14
16
18
22
29
2.0
1.65
1869 bp
leaf
2.0
1.65
1869 bp
root

Figure 3.7 Expression of *nosZ* in T_1 transgenic tobacco.

The full length *ex::nosZ* transcript was amplified from leaves of selected T_1 plants by RT-PCR (as described for T_0 plants) to confirm expression of the transgene in T_1 progeny. M: 1 kb plus DNA ladder (Invitrogen); +: positive control, recombinant plasmid isolated from *E. coli*; NT: negative control, total RNA from non-transformed tobacco. Transformed lines are identified by promoter (rolD or d35S) and number. No-RT controls were also performed as shown on the right side of the gel image.

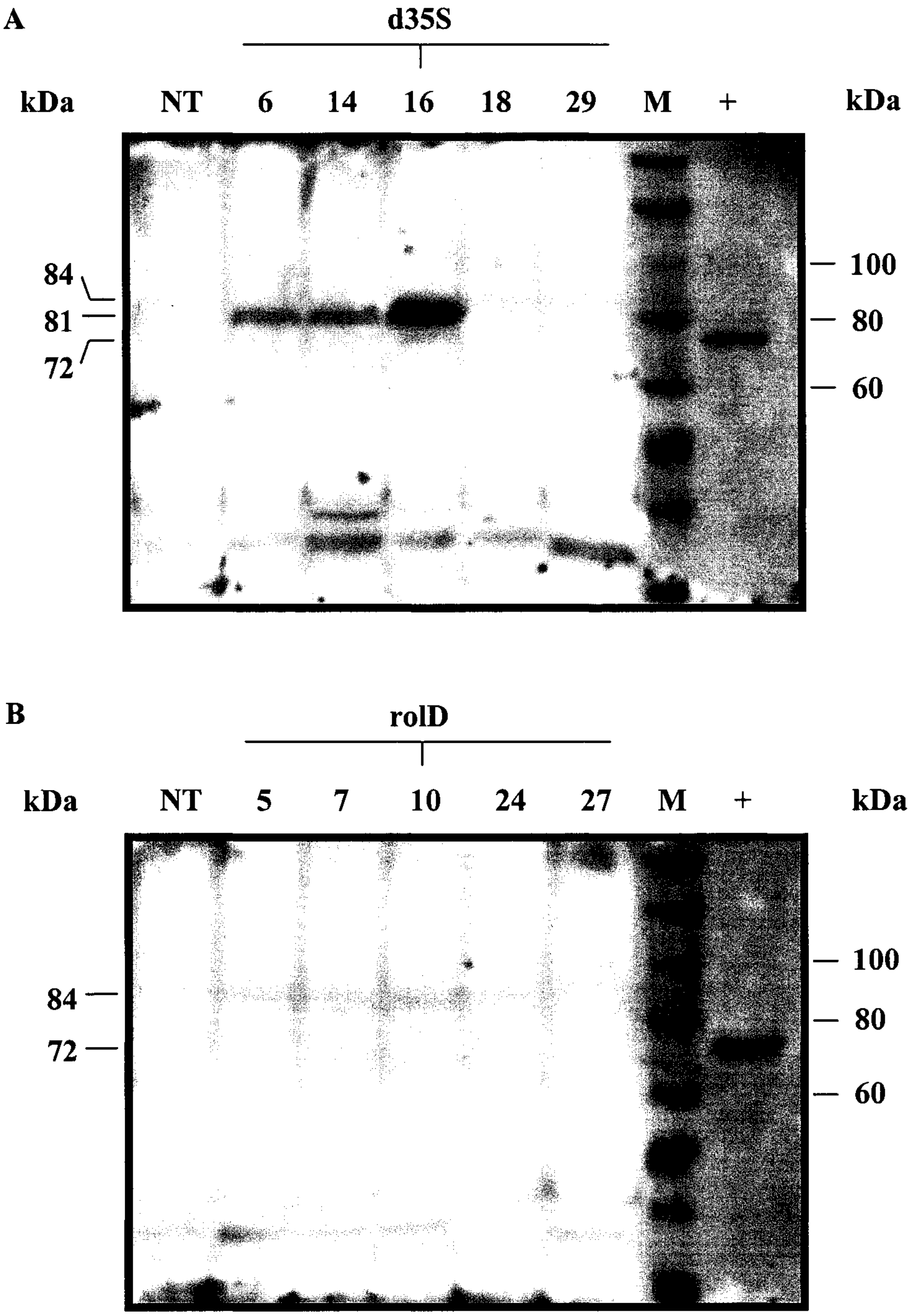

RT step
+
+
+
+
–
–
–
–
rolD
d35S
rolD
d35S
M
+
NT
5
6
14
16
5
6
14
16
M
1869 bp

3.2.3 Western immunoblot analysis of the N_2OR enzyme

Recombinant N_2OR protein produced by the transgenic tobacco lines was detected by separation of plant soluble proteins by SDS-PAGE followed by western immunoblot and chemiluminescent detection. Ten plant lines were selected for western immunoblot analysis. Protein extracts from a non-transformed plant were included as a negative control and cell extracts from *P. stutzeri* induced to express *nosZ* were included as a positive control. A specific protein band at 72 kDa was detected in the extract from *P. stutzeri.* The specificity of this band was confirmed by ruling out cross reactivity of the secondary antibody (Appendix III). Protein bands slightly larger in size, 81-84 kDa, were detected in the transgenic tobacco leaf extracts (Figure 3.8). Apparent molecular weight of the products was estimated by the standard curve of migration distance (Appendix IV). No similar protein was detected in the non-transformed plant extract. These results were consistently observed throughout optimization of the immunoblot procedure (Appendix V). Detection of the recombinant protein in root extracts was attempted by the same method (Appendix VI). No distinct bands could be identified in the transformed tobacco root extracts.

Figure 3.8 Production of recombinant N_2OR in the leaves of transgenic tobacco plants.

Western immunoblot analysis using anti-N_2OR serum to detect recombinant N_2OR in transgenic plants. Plant protein samples were extracted from leaf tissue and 175 µg of soluble plant proteins was separated by SDS-PAGE for each sample. The western blot was incubated with a 1:5000 dilution of anti-N_2OR serum and the secondary antibody biotin-SP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.). Proteins were visualized by chemiluminescent method. Panels **A** and **B** show protein extracts from plants transformed with the d35S-*nosZ* and the rolD-*nosZ* construct, respectively. Plants are identified by number. In both figures, NT: negative control, protein extract from a non-transformed plant; +: positive control, cell extract from *P. stutzeri* culture; M: biotinylated protein ladder (Cell Signaling Technology Inc.). The expected size of the N_2OR enzyme is 66 kDa.


A
d35S
kDa
NT
6
14
16
18
29
M
+
kDa
84
81
72
100
80
60
B
rolD
kDa
NT
5
7
10
24
27
M
+
kDa
84
72
100
80
60

Chapter 4

DISCUSSION

4.1 Overview

N_2OR is the enzyme that catalyzes the final step of nitrogen reduction, N_2O to N_2, during the bacterial process of anaerobic nitrogen respiration. The protein was first identified by Zumft and Matsubara (1982) and the *nosZ* gene of *P. stutzeri* was later cloned and characterized (Viebrock and Zumft, 1988). The protein has been extensively studied and dozens of members of the gene family encoding it have been identified in numerous soil and marine microorganisms. For the purpose of this study the main interest in N_2OR was the role it plays in the emission of N_2O from agricultural soils. N_2O is a potent greenhouse gas and accounts for up to 80% of agricultural GHGs. A significant portion of those emissions are a result of the incomplete reduction of N fertilizers by soil microorganisms.

The current study set out to generate transgenic tobacco expressing the *nosZ* gene of *P. stutzeri*. The plants produced by this work are being evaluated for their potential to catalyze the complete reduction of N_2O in agricultural soils, thus reducing the release of this GHG to the atmosphere. The *nosZ* gene has been expressed in non-denitrifying microorganisms, including *E. coli* (Viebrock and Zumft, 1988) and *P. putida* (Wunsch et al., 2003), however, this is the first description of its expression in a eukaryotic system.

4.2 Sequence and restriction analyses of the *nosZ* expression constructs

Two expression constructs, the first of their kind designed for expression of nitrous oxide reductase in a plant system, were created for this study. The first, designed to drive expression of the ex::*nosZ* fusion from the d35S constitutive promoter of CaMV, and the second, designed to drive the expression of the fusion from the rolD root-specific promoter of *A. rhizogenes*, were assembled as shown in Figures 2.1-2.2. Restriction analysis and sequencing confirmed that both constructs were free of mutations and were successfully assembled in frame. This particular expression system combining the d35S promoter and NOSter with pRD400 (Datla et al., 1992) has been used successfully in many GMO crops and has become a standard robust plasmid for deploying foreign proteins in plants (Marillia et al., 2003; Smykal et al., 2004; Wang et al., 2002)

4.3 Analysis of tobacco plants transformed with the pRD400-*nosZ* expression vectors

Transformed tobacco tissue was regenerated on kanamycin selection media. Control non-transformed tissue necrotized on this media confirming the effectiveness of selection (data not shown). A high efficiency of transformation was observed as calli, and later shoots, developed from all leaf discs. Not more than a single shoot was selected from each calli to ensure genetic variability of the transformants. This increased the chances of obtaining independent events and subsequent experimental transgenic lines for analysis in future soil scenarios. Shoots were transferred to rooting media and ultimately 58 putative transgenic plants, chosen for further study, were transferred to soil. Normal and uniform phenotypic traits were observed for all 58 plants. The mature tobacco plants also proved to be fertile and produced T_0 seed. These phenotypic observations suggest that *A. tumefaciens* mediated transformation of tobacco leaf sections combined with antibiotic resistance selection was successful in generating viable transgenic tobacco plants and that integration of the transgene did not disrupt any endogenous processes. Although alternative transformation procedures like biolistic gun bombardment (Altpeter et al., 2005) or protoplast electroporation (Li et al.,

2005) are used to generate transgenic plants, the *Agrobacterium* Ti-plasmid gene transfer method is the most widely used tool for introduction of foreign genes into plants and has many advantages (Gelvin, 2003).

4.4 Molecular characterization of transgenic tobacco plants

4.4.1 Molecular confirmation of transformation and integration of the *nosZ* transgene

Transgenic tobacco plants identified by kanamycin resistance were screened by PCR to confirm the presence of the transgene (Figure 3.3-3.4). To differentiate between the two constructs the recombinant DNA was amplified from a site within the promoter, d35S or rolD, to a site within the coding region of the *nosZ* gene. Amplification of the full length coding region proved inconsistent and a shorter segment was selected for amplification to avoid identification of false negatives. This fragment of the *nosZ* gene was positively identified in 54 of the 58 T_0 plants screened. Positive identification of the promoter-*nosZ* gene fragment from T_1 plant samples (Figure 3.5) confirmed that the *nosZ* transgene was stably inherited by the T_1 progeny. Despite the fact that PCR is prone to artifacts, it is still accepted as the state of the art test for selecting transformed plant lines (Novakova et al., 2005; Register, 1997; Zhang et al., 2005).

4.4.2 Confirmation of *nosZ* transgene transcription

To assess whether the transgene was being expressed and producing a full length transcript, several plants were selected for RT-PCR. An equal number of plants were selected from each planting date, two from each of three dates, and an effort was made to include as much phenotypic variability as possible. Total RNA was extracted from both leaves and roots of the d35S-*nosZ* and rolD-*nosZ* plants and the full length ex::*nosZ* fusion product was amplified from the cDNA. The expected 1869 bp product was observed for each of the leaf samples and most of the root samples of the T_0 lines (Figure 3.6) as well as the leaf samples of the selected T_1 lines (Figure 3.7). The absence of the transcript in some of the root extracts was more likely due to the failure of the RT-PCR reaction than a true absence of transcription in the tissue. Harvesting of the root tissue involved removal from soil and, while performed as quickly and thoroughly as possible, would inevitably have resulted in contamination of the sample with soil particles, which may interfere with the PCR reaction (Watson and Blackwell, 2000). In addition, the method of soil removal was somewhat time consuming and may have allowed partial degradation of RNA prior to freezing. Expression of *nosZ* from the d35S promoter appears to be higher overall than expression from the rolD promoter. Elmayan and Tepfer (1995) have reported 3-7 fold higher expression by the d35S promoter than the rolD promoter in all tobacco tissues. Thus, the findings with *nosZ* plants confirmed the performance of both 5' elements as reported in the literature. However, since PCR is prone to artifacts and this was a non-quantitative procedure, the extrapolation from gel band fluorescence intensity to promoter strength is simply an estimation. Techniques such as quantitative RT-PCR or northern hybridization might provide more insight into how strongly these two specific promoters are transcribed in this tobacco genetic background. Surprisingly, the rolD promoter did not target expression of n*osZ* specifically to root tissue. The Elmayan and Tepfer study reported a root/leaf expression ratio of up to 50 in mature tobacco plants (*N. tabacum* cv. PBD6), which is not reflected by these RT-PCR results. Plant age has been reported to have a significant impact on rolD driven expression in tobacco (Trovato, 1997), which may provide some insight into the unexpected results of this *nosZ* study. The *nosZ* tissues were harvested from mature plants that had already set seed thus, expression may have already begun to decline. Additionally, non-quantitative RT-PCR, while extremely sensitive, may not reveal relative small differences in expression and consequently may not reflect root-specific expression by the rolD promoter.

Variations in observed expression levels from the same promoter and the same tissue can be attributed to factors such as variability of T-DNA integration sites, gene silencing resulting from multiple transgene insertions.

4.4.3 Production of recombinant plant N_2OR

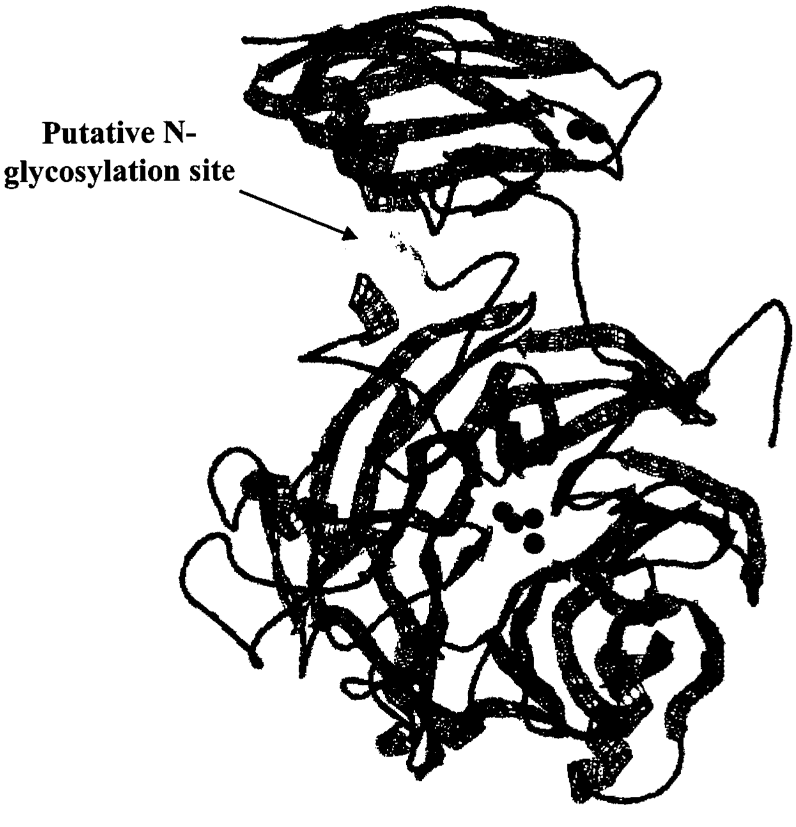
Protein extracted from ten transformants, five of each construct, was analyzed by western blot (Figure 3.8). A protein band of 72 kDa, as estimated by the standard curve of migration distance (Appendix IV), was detected in the positive control sample of *P. stutzeri* cell extract. The calculated molecular weight (MW) of N_2OR is 66 kDa, however, the N_2OR protein of *P. stutzeri* has been shown to separate at a higher MW in other SDS-PAGE experiments (Dreusch et al., 1997; Heikkila et al., 2001). Thus, it is not unexpected that endogenous N_2OR would have an apparent MW of 72 kDa in this experiment. For both rolD and d35S transgenic plants, a single high MW protein band was observed in each leaf extract. The apparent MW of this recombinant plant N_2OR varied from 81-84 kDa. No bands of comparable size were identified in the extract from a non-transformed plant. The shift in apparent MW between endogenous N_2OR and recombinant N_2OR may have several explanations. These could include experimental conditions such as the method of leaf protein extraction, incomplete SDS sample denaturation or acrylamide gel loading anomalies. A significant amount of plant extract was loaded on the gels to ensure presence of sufficient recombinant protein for detection. This may have resulted in slower movement of plant proteins through the matrix and resulted in a larger apparent MW.

Alternatively, post-translational modification of the protein in the plant host may explain the observed MW of 81-84 kDa. Two putative N-linked glycosylation sites are present within the sequence of the N_2OR protein, Asn-Glu-Ser-Arg at position 61 and Asn-Thr-Ser-Ser at position 279. The first three residues of the Asn-Glu-Ser-Arg site are highly conserved (based on the PDBsum EBI database entry 1QNI) and the potential of glycosylation is rated as 0.7 by the NetNGlyc 1.0 Server. The Asn-Thr-Ser-Ser sequence is far less conserved and the glycosylation potential is below threshold. Based on these observations it appears the N_2OR protein has a single probable N-glycosylation site. The position of this site is on an α-helix that physically separates the two domains of the N_2OR monomer (Figure 4.1). At this position glycosylation would not be expected to affect the copper centres, it may however affect the formation of the N_2OR dimer. Further biochemical analysis is necessary to identify whether glycosylation is occurring and if this modification has any impact on enzyme activity. Another possible modification is O-linked glycosylation, however, the initiation of this process is less conserved and far more difficult to predict than N-glycosylation (Hanisch, 2001).

The 81-84 kDa bands may also be a result of the extensin signal peptide not being cleaved. However, previous studies have demonstrated successful secretion of recombinant proteins mediated by the carrot extensin signal sequence from *Nicotiana tabacum* protoplasts (De Loose et al., 1991) and Arabidopsis root tissue (Richardson et al., 2001). Based on these findings, one can presume that the extensin signal peptide was successfully targeting the recombinant N_2OR protein to the endoplasmic reticulum and was being cleaved by tobacco signal peptidase. The extensin signal peptide is 34 aa residues long and would only add 3.5 kDa to the immunoblot-detected band thus it would not explain the full size shift observed.

Figure 4.1 Putative N-glycosylation site for recombinant N_2OR.

Analysis of the putative amino acid sequence of recombinant N_2OR reveals a possible N-glycosylation site on an α–helix that physically separates the two domains of the N_2OR monomer. This site, at position 61 of the peptide, is indicated in yellow. α–helices are shown in red and β–sheets are in blue. Figure was generated using Deep View/Swiss-PDBviewer v3.7.


Putative N-glycosylation site

When foreign proteins are expressed in transgenic tobacco, glycosylation is indeed a common cause of increased protein MW (Bardor et al., 1999; Lerouge et al.,1998) and thus, it seems the more plausible cause of the size shift.

The small size difference of the recombinant protein among plant lines (81 kDa to 84 kDa) is unexpected. This could be the result of differential patterns of glycosylation or signal peptide cleavage. Alternatively, the difference in apparent weight may simply be due to anomalies of the SDS-PAGE procedure. Since the size shift seems to correlate with a less intense signal it would seem the latter explanation is the most feasible. Based on chemiluminescent signal strength, it appears that the d35S plants 6, 14 and 16 are the highest expressers of N_2OR. Future work to establish the ability of these plants to reduce N_2O will focus on these lines.

Detection of the N_2OR enzyme in root extracts was attempted in this study (Appendix VI), however, the results were non-specific and could not help to positively identify the presence of recombinant N_2OR in the root tissue of transformants. It is probable that soil contaminants, including soil proteases, affected the quality of the root extracts or may have interfered with specific antibody interactions. Further study of the expression of N_2OR in root tissue may benefit from growth of plants hydroponically to eliminate the possibility of contamination from soil particles.

4.5 Future Considerations

Transgenic tobacco plants stably expressing the *nosZ* transgene and producing recombinant N_2OR have been produced and positively identified in this study. Further analysis of the tobacco plants will include examination of enzyme activity in tissue extracts and ability of the plants to reduce N_2O in a soil environment. If the recombinant N_2OR

produced by these plants is proven to be an apo-enzyme without functional copper centres, multigenic engineering of the NosDFY copper insertion complex may be necessary. There are several methods of multigene engineering available (François et al., 2002), such as co-transformation of multiple transgenes, which was the method used to produce golden rice (Ye et al., 2000). Additional molecular investigation may include N and C terminal sequencing, carbohydrate moiety analysis, quantitative expression studies and confirmation of stable integration in subsequent generations. In addition, localization studies to confirm functional secretion of the recombinant enzyme will be of interest. When the reduction of N_2O by the transgenic plant lines has been proven effective, studies to improve recombinant enzyme yield and activity will follow. These may include promoter studies, bioprospecting to identify *nosZ* genes with higher activity and enzyme engineering to improve stability and activity of the recombinant enzyme. Future studies will proceed in collaboration with the Green Crops Network funded by the NSERC Research Network Grant program (2005-2010).

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CONTRIBUTIONS OF COLLABORATORS

The following people have contributed to the experimental work in this thesis:

Yaseen Mottiar, Melissa McNulty and Evelin Loit of the Altosaar lab shared in the responsibilities of a rotating care schedule at the greenhouse facility.

David Blais of the Altosaar lab performed the PCR and RT-PCR experiments on T_1 progeny (using the protocols optimized by the author of the thesis).

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David Tepfter of L'Institut National de la Recherche Agronomique in France provided the pLJ1 plasmid from which the rolD promoter was obtained.

Walter Zumft of Universität Karlsruhe in Germany provided the anti-N_2OR serum used for western immunoblot detection of recombinant N_2OR.

APPENDICES

Appendix I: Nucleotide sequences of rolD-*nosZ* and d35S-*nosZ* expression constructs. The full sequences of both constructs, including the promoter and termination sequences, are provided. The putative amino acid sequence of the coding region is included. Start and stop codons are highlighted in bold. The linker between the extensin signal sequence and *nosZ* cds (*Nhe*I recognition site) is bolded and underlined. All sequences shown have been confirmed by dideoxy-chain termination sequencing.

rolD-*nosZ* construct:

ggtaccagttgtatccgaatactcatatatgtattgcaatacaataactggcaattggtt
***Kpn*I**
ttggagttggccgcccactacaatgaatttgttcgtgaactattagttgcgggccttggc

atccgactacctctgcggcaatattatattccctgggcccaccgtgaacccaatttcgcc

tatttattcattacccccattaacattgaagtagtcatgatgggcctgcagcacgttggt

gaggctggcacaactcatccatatactttctgaccggatcggcacattattgtagaaaac

gcggacccacagcgcactttccaaagcggtgccgcgtcagaatgcgctggcagaaaaaaa

ttaatccaaaagtaccctccaagcagcccatataaacgcgtttacaaatccgctaacctc

aacaatttgagcagagaaaattcgcacctacaaggcag**tctaga**
***Xba*I**
atgggaagaattgctagaggctcaaaaatgagttctctcattgtgtctttgcttgtagta
M G R I A R G S K M S S L I V S L L V V
ttggtgtcactcaatttggcttccgaaaccacagcc**gctagc**caggccgtcaaggagtcc
L V S L N L A S E T T A A S Q A V K E S
aagcagaagatccacgtcggccccggcgagctggatgactactacggcttctggtccggc
K Q K I H V G P G E L D D Y Y G F W S G
ggtcaccagggtgaagtccgcgtgctgggcgtgccgtcgatgcgcgagctgatgcgtatc
G H Q G E V R V L G V P S M R E L M R I
ccggtgttcaacgtcgactcggccaccggctgggggctgaccaacgaaagccgccacatc
P V F N V D S A T G W G L T N E S R H I
atgggcgacagcgccaagttcctcaacggtgactgccaccacccgcacatctccatgacc
M G D S A K F L N G D C H H P H I S M T
gacggcaagtacgatggcaagtacctgttcatcaacgacaaggccaacagccgcgttgcg
D G K Y D G K Y L F I N D K A N S R V A
cgtatccgtctggacatcatgaagtgcgacaagatgatcaccgtgccgaacgtgcaggcg
R I R L D I M K C D K M I T V P N V Q A
atccacggtctgcgtctgcagaaggtgccgcacaccaagtacgtattcgccaacgccgag
I H G L R L Q K V P H T K Y V F A N A E
ttcatcatcccgcacccgaacgatggcaaggtcttcgatctgcaggacgagaacagctac

F I I P H P N D G K V F D L Q D E N S Y
accatgtacaacgccatcgatgcggaaaccatggaaatggccttccaggtcatcgttgac
T M Y N A I D A E T M E M A F Q V I V D
ggcaacctcgacaacaccgacgccgactacactggccgtttcgctgctgctacctgctac
G N L D N T D A D Y T G R F A A A T C Y
aactcggagaaggccttcgatctgggcggcatgatgcgtaacgagcgcgactgggtggtg
N S E K A F D L G G M M R N E R D W V V
gtgttcgatatccacgccgtcgaagcagcggtcaaagctggcgatttcatcaccctgggc
V F D I H A V E A A V K A G D F I T L G
gactccaagacgcctgtgctcgatggtcgcaagaaggatggcaaggacagcaagttcacc
D S K T P V L D G R K K D G K D S K F T
cgttacgtgccagtgccgaaaaacccgcacggctgcaacacctcctccgatggcaaatac
R Y V P V P K N P H G C N T S S D G K Y
ttcatcgccgccggcaagctctcgccaacctgctcgatgatcgccatcgacaagctgccc
F I A A G K L S P T C S M I A I D K L P
gacctgttcgccggcaagctggccgatccgcgtgatgtgatcgtgggtgagcctgagctg
D L F A G K L A D P R D V I V G E P E L
ggtctcggcccgctgcacaccaccttcgacggccgcggtaacgcctacaccacgctgttc
G L G P L H T T F D G R G N A Y T T L F
atcgacagccaggtggtcaagtggaacatggaagaagctgttcgtgcctacaagggcgag
I D S Q V V K W N M E E A V R A Y K G E
aaggtcaactacatcaagcagaagcttgatgtgcactaccagccgggtcacctgcacgcg
K V N Y I K Q K L D V H Y Q P G H L H A
tcgctgtgtgaaaccaatgaagccgatggcaagtggctggtagcactgtccaagttctcc
S L C E T N E A D G K W L V A L S K F S
aaggaccgcttcctgccggttggcccgctgcatcccgagaacgaccaactgatcgacatc
K D R F L P V G P L H P E N D Q L I D I
tccggcgacgagatgaagctggtacatgacggcccgacctttgccgaaccgcatgactgc
S G D E M K L V H D G P T F A E P H D C
atcatggctcgccgtgatcagatcaagaccaagaagatctgggaccgcaacgatccgttc
I M A R R D Q I K T K K I W D R N D P F
ttcgccccgaccgtggaaatggcgaagaaggacggcatcaacctcgataccgacaacaag
F A P T V E M A K K D G I N L D T D N K
gtcattcgcgacggcaacaaggtgcgcgtgtacatgacctcgatggcgccggcgttcggc
V I R D G N K V R V Y M T S M A P A F G
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V Q E F T V K Q G D E V T V T I T N I D
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Q I E D V S H G F V V V N H G V S M E I
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S P Q Q T S S I T F V A D K P G L H W Y
tactgcagctggttctgccatgcgctgcacatggaaatggtcggccgcatgatggtcgag
Y C S W F C H A L H M E M V G R M M V E
ccggcc**taa**
P A -

ggatccccgatcgttcaaacatttggcaataaagtttcttaagattgaatcctgttgccg
***Bam*HI**
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tgtaatgcatgacgttatttatgagatgggtttttatgattagagtcccgcaattataca

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tgtcatctatgttactagatcgg**gaattc**
***Eco*RI**

d35S-*nosZ* construct:

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***Kpn*I**
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tgtgatatctccactgacgtaagggatgacgcacaatcccactatccttcgcaagaccct

tcctctatatnaggaagttcatttcatttggagagga**tctaga**
***Xba*I**
atgggaagaattgctagaggctcaaaaatgagttctctcattgtgtctttgcttgtagta
M G R I A R G S K M S S L I V S L L V V
ttggtgtcactcaatttggcttccgaaaccacagcc**gctagc**caggccgtcaaggagtcc
L V S L N L A S E T T A A S Q A V K E S
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K Q K I H V G P G E L D D Y Y G F W S G
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G H Q G E V R V L G V P S M R E L M R I
ccggtgttcaacgtcgactcggccaccggctggggctgaccaacgaaagccgccacatc
P V F N V D S A T G W G L T N E S R H I

```
atgggcgacagcgccaagttcctcaacggtgactgccaccacccgcacatctccatgacc
 M  G  D  S  A  K  F  L  N  G  D  C  H  H  P  H  I  S  M  T
gacggcaagtacgatggcaagtacctgttcatcaacgacaaggccaacagccgcgttgcg
 D  G  K  Y  D  G  K  Y  L  F  I  N  D  K  A  N  S  R  V  A
cgtatccgtctggacatcatgaagtgcgacaagatgatcaccgtgccgaacgtgcaggcg
 R  I  R  L  D  I  M  K  C  D  K  M  I  T  V  P  N  V  Q  A
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 I  H  G  L  R  L  Q  K  V  P  H  T  K  Y  V  F  A  N  A  E
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 G  L  G  P  L  H  T  T  F  D  G  R  G  N  A  Y  T  T  L  F
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 I  D  S  Q  V  V  K  W  N  M  E  E  A  V  R  A  Y  K  G  E
aaggtcaactacatcaagcagaagcttgatgtgcactaccagccgggtcacctgcacgcg
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 V  Q  E  F  T  V  K  Q  G  D  E  V  T  V  T  I  T  N  I  D
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S P Q Q T S S I T F V A D K P G L H W Y
tactgcagctggttctgccatgcgctgcacatggaaatggtcggccgcatgatggtcgag
Y C S W F C H A L H M E M V G R M M V E
ccggcc**taa**
P A -
ggatccccgatcgttcaaacatttggcaataaagtttcttaagattgaatcctgttgccg
***Bam*HI**
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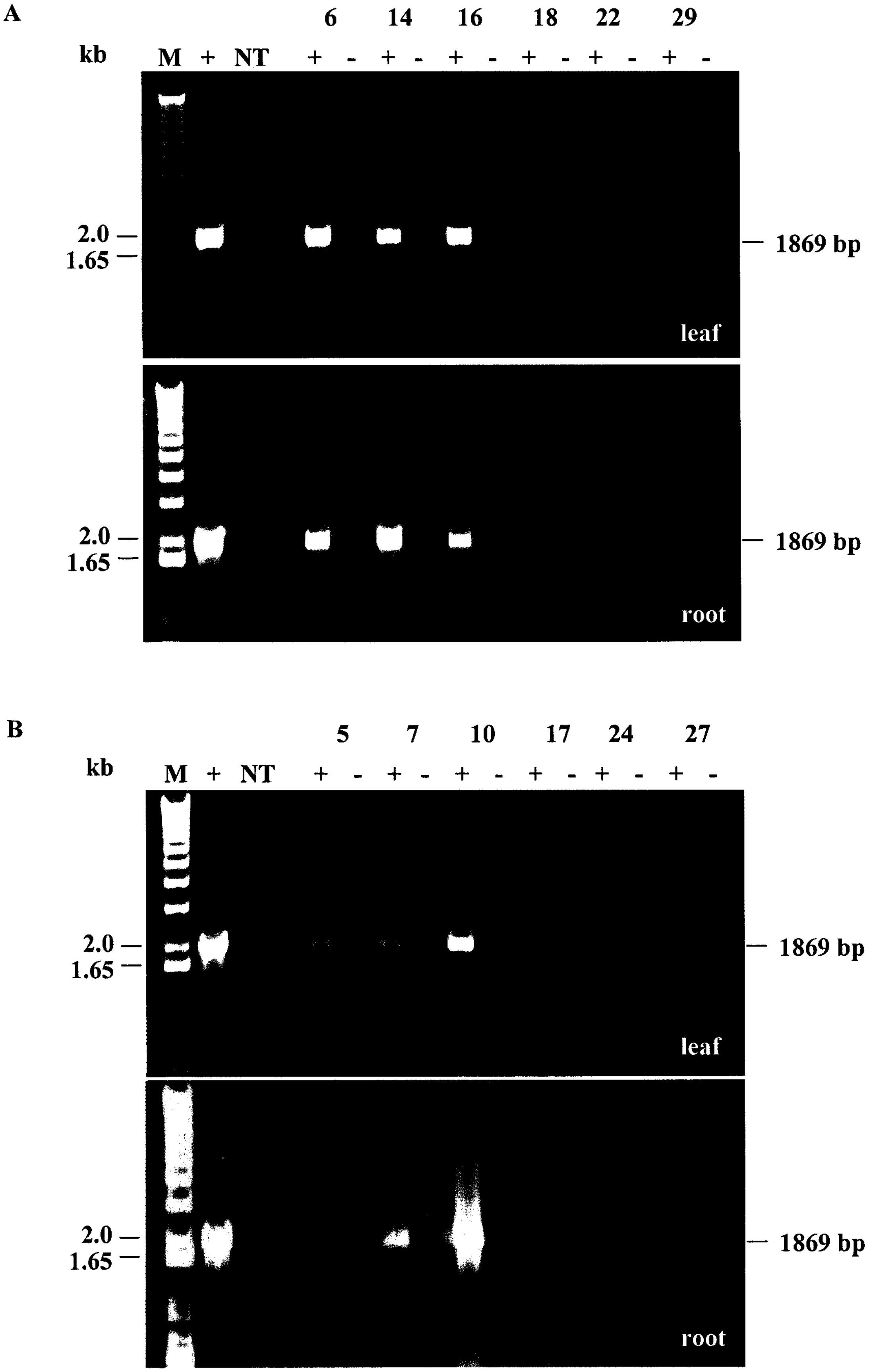
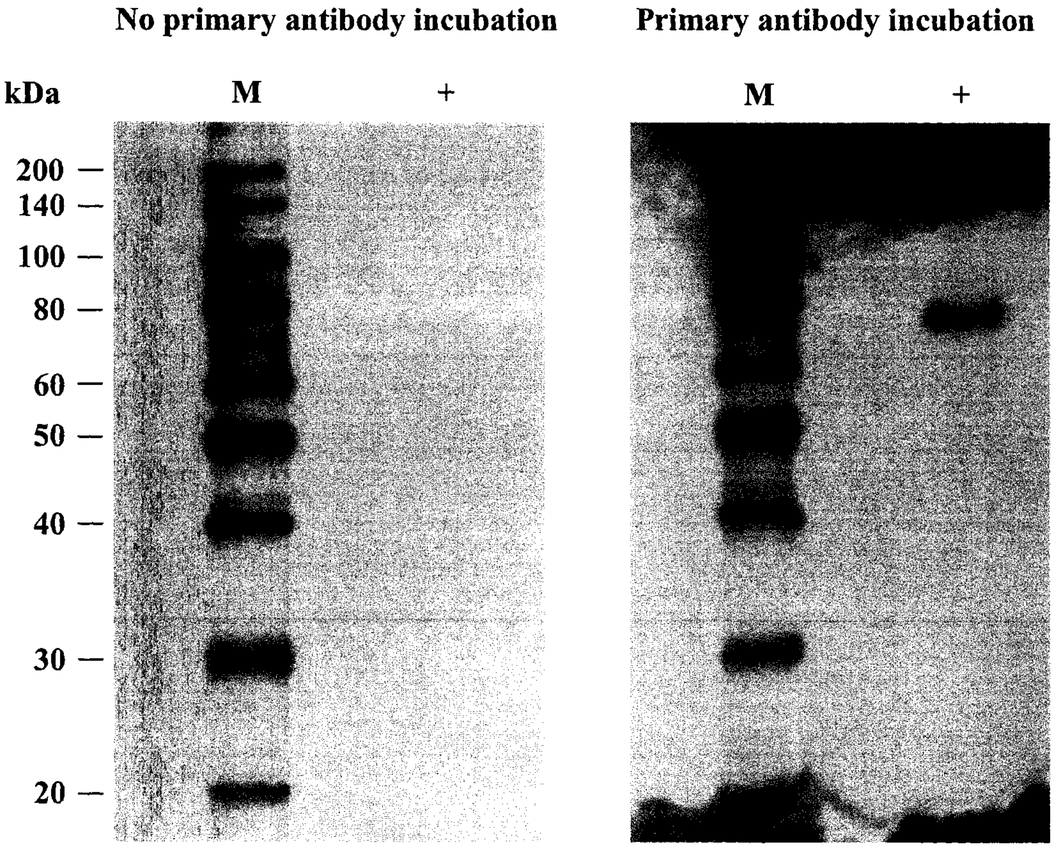
tttaatacgcgatagaaaacaaaatatagcgcgcaaactaggataaattatcgcgcgcgg

tgtcatctatgttactagatcgg**gaattc**
***Eco*RI**

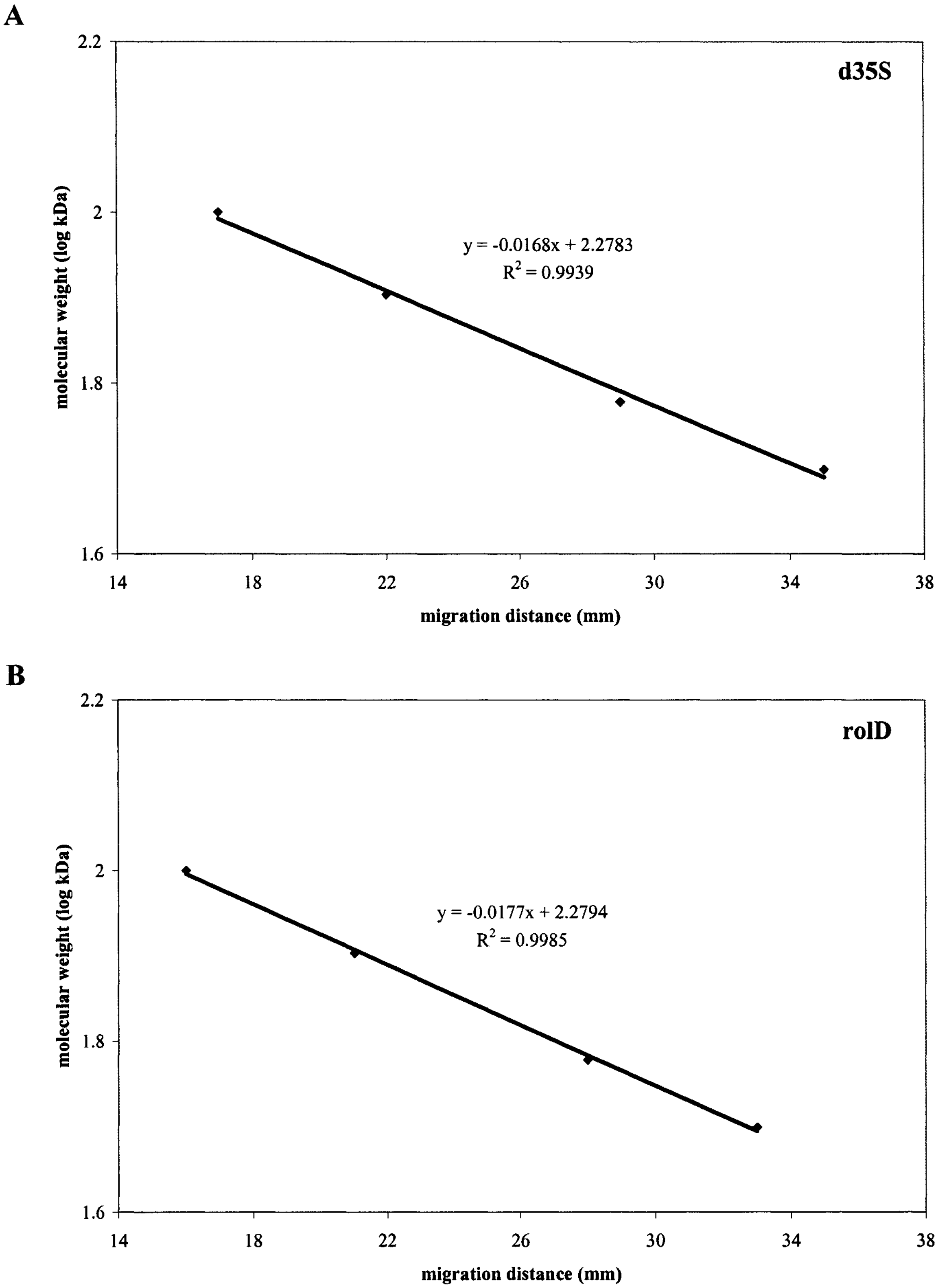
Appendix II: Expression of *nosZ* in T_0 rolD-*nosZ* and d35S-*nosZ* transgenic tobacco – no-RT controls. RT-PCR detection of *nosZ* transcripts in leaf and root tissue of transformed tobacco plants. Total RNA was extracted from the leaves (upper panel) and roots (lower panel) of mature tobacco plants. RNA was reverse transcribed with the SuperScript First-Strand Synthesis System (Invitrogen) and the full length *ex::nosZ* cDNA was PCR amplified. The expected size of the full length cDNA is 1869 bp. M: 1 kb plus DNA ladder (Invitrogen); +: positive control, recombinant plasmid isolated from *E. coli*; NT: negative control, total RNA from non-transformed tobacco. Transformed lines are identified by promoter (rolD or d35S) and number and for every plant sample a positive (+) and (-) reaction are included. Positive reactions included the reverse transcriptase enzyme while negative samples did not. No-RT reactions are included to confirm the absence of genomic DNA in the cDNA template. No DNA was found to contaminate the reactions. Panel **A**: Plants transformed with the d35S-*nosZ* construct. Panel **B**: Plants transformed with the rolD-*nosZ* construct.


A
6 14 16 18 22 29
kb
M + NT + - + - + - + - + - + -
2.0
1.65
— 1869 bp
leaf
2.0
1.65
— 1869 bp
root
B
5 7 10 17 24 27
kb
M + NT + - + - + - + - + - + -
2.0
1.65
— 1869 bp
leaf
2.0
1.65
— 1869 bp
root

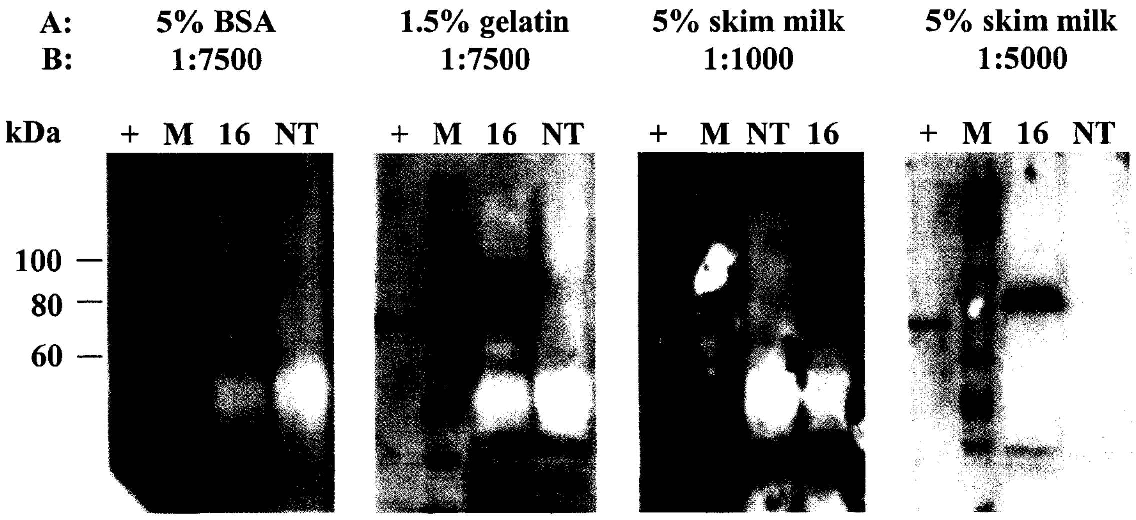
Appendix III: Cross-reactivity of the secondary antibody to protein extracts of *P. stutzeri*. Both western blots were handled simultaneously under the same conditions except that the blot shown on the left was not exposed to anti-N_2OR serum while the second, shown on the right, was incubated in a 1:5000 dilution of serum for 1 hr. M: biotinylated protein ladder (Cell Signaling Technology Inc.); +: protein extract of *P. stutzeri* culture.


No primary antibody incubation
Primary antibody incubation
kDa
M
+
M
+
200
140
100
80
60
50
40
30
20

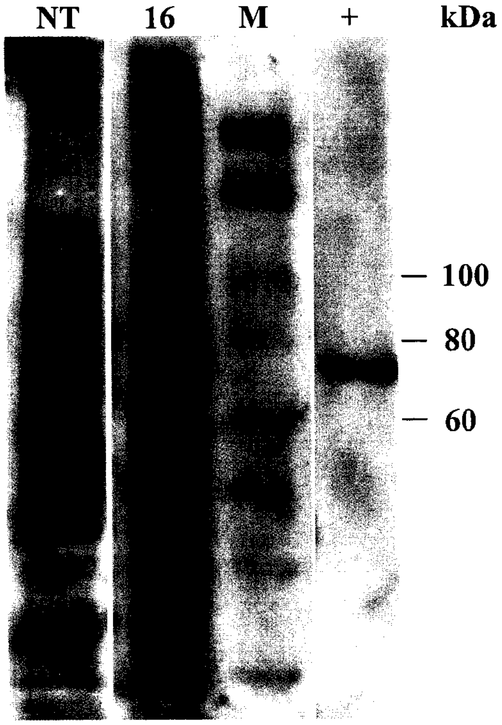
Appendix IV: Standard curves of log molecular weight vs. migration distance for western immunoblots of d35S and rolD leaf extracts. The log molecular weight (MW) values of the biotinylated protein ladder bands were plotted against the measured migration distances (mm). A line of best fit was calculated for each plot. The apparent MW of experimental proteins from *P. stutzeri* and transgenic tobacco were calculated using the resulting equations. Only data from the mid-sized protein bands (50-100 kDa) was plotted to avoid errors inherent in the extreme protein bands. A: Standard curve for the immunoblot of d35S plants, B: Standard curve for the immunoblot of rolD plants.


A
d35S
y = -0.0168x + 2.2783
R^2 = 0.9939
molecular weight (log kDa)
2.2
2
1.8
1.6
14
18
22
26
30
34
38
migration distance (mm)
B
rolD
y = -0.0177x + 2.2794
R^2 = 0.9985
molecular weight (log kDa)
2.2
2
1.8
1.6
14
18
22
26
30
34
38
migration distance (mm)

Appendix V: Optimization of western immunoblot detection of recombinant N_2OR in tobacco leaf extracts. Plant protein samples were extracted from leaf tissue and 175 µg of soluble plant proteins were separated by SDS-PAGE. Row **A:** Describes the blocking solutions tested, row **B:** Describes the dilution of the antiserum used. Following incubation with the secondary antibody biotin-SP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.) proteins were visualized by chemiluminescent method. +: positive control, cell extract of *P. stutzeri* culture; M: biotinylated protein ladder (Cell Signaling Technology Inc.); 16: protein extract from the transgenic plant d35S16; NT: negative control, protein extract from a non-transformed plant.


A:
B:
5% BSA
1:7500
1.5% gelatin
1:7500
5% skim milk
1:1000
5% skim milk
1:5000
kDa
+ M 16 NT
+ M 16 NT
+ M NT 16
+ M 16 NT
100
80
60

Appendix VI: Western immunoblot detection of recombinant N_2OR from root tissue extracts. 175 μg of soluble plant protein from root extracts was separated by SDS-PAGE. The western blot was incubated with a 1:5000 dilution of anti-N_2OR serum and the secondary antibody biotin-SP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.). Proteins were visualized by chemiluminescent method. NT: negative control, protein extract from roots of a non-transformed plant; 16: protein extract from roots of plant d35S16; +: positive control, protein extract of *P. stutzeri* culture; M: biotinylated protein ladder (Cell Signaling Technology Inc.). The expected size of the N_2OR enzyme is 66 kDa.


NT
16
M
+
kDa
100
80
60

CURRICULUM VITAE

JULIANNE STAEBLER

EDUCATION

Sept 2002 – present University of Ottawa Ottawa, ON
Master of Science, Biochemistry
In progress, A grade average.
Thesis: Clearing the Air: Expression of Nitrous Oxide Reductase from *Pseudomonas stutzeri* in Transgenic Tobacco.

Sept 1995 – Apr 1999 University of Guelph Guelph, ON
Bachelor of Science, Honours Molecular Biology and Genetics
Graduated with distinction.
Thesis: Cloning of an endopolygalacturonase from the plant pathogenic fungus *Colletotrichum gloeosporioides.*

EXPERIENCE

Jan 2004 – Dec 2004 National Research Council Ottawa, ON
Steering Committee Secretary
- Prepared meeting reports for the steering committee on Development of a Global Centre for Certified Reference Material for GM Crops

Sept 2003 – Apr 2004 University of Ottawa Ottawa, ON
Teaching Assistant
- Courses: Molecular Biology Laboratory and Biochemistry Laboratory

Jan 2002 – June 2002 McGill University Montreal, QC
Research Assistant
- Studied the application of FTIR spectroscopy as a bacterial detection technology for *Clostridium botulinum*

Apr 1999 – Dec 2001 University of Guelph Guelph, ON
Laboratory Technician
- Conducted and coordinated molecular mapping projects of maize and soybean populations using several molecular marker techniques including SSRs, RAPDs and AFLPs
- Implemented inventories and ordering systems for 3 research groups comprised of 20 staff, students and post-docs
- Provided training and support to a large group of graduate and undergraduate students
- Helped manage grant accounts and equipment inventories

EXPERIENCE CONT…

Apr 1998 – Aug 1998	University of Guelph	Guelph, ON

Research Assistant

- Learned molecular techniques and began cloning and characterization of a fungal virulence gene

CONFERENCE PRESENTATIONS

BIOCAP Canada Foundation 1st National Conference, Ottawa, Ontario, February 2-3, 2005
Oral Presentation: "Clearing the Air: *Pseudomonas* Nitrous Oxide Reductase in Transgenic Rhizospheres"

Eurosoil 2004, Freiburg, Germany, September 4-12, 2004
Oral Presentation: "Clearing the Air: Expression of Nitrous Oxide Reductase *in planta*"

10th Annual BioNorth Life Sciences International Conference and Exhibition, Ottawa, Ontario, November 17-19, 2003
Poster Presentation: "Clearing the Air: Expression of Nitrous Oxide Reductase *in planta*"

CONFERENCES ATTENDED

Biological GHG Sources and Sinks program workshop, Ottawa, Ontario, May 29-30, 2003

Green Crops Network meeting, Montreal, Quebec, May 4, 2003

58th Northeast Corn Improvement Conference, Ottawa, Ontario, February 13-14, 2003

ADDITIONAL ACTIVITIES

Attended Green Crop Network NSERC site visit and was instrumental in obtaining NSERC network grant for Altosaar lab (2003-2005)
Mentor to high school students in Aventis Biotech Challenge (2003-2004)
Volunteer for Let's Talk Science program (2003-2004)
Treasurer and liaison of Plant Agriculture Variety Club (2000)

AWARDS

Master's Admission Scholarship in Science and Engineering	$5500
Biochemistry Program Entrance Award	$4000