# PHYTOREMEDIATION OF NITROUS OXIDE:

# EXPRESSION OF NITROUS OXIDE REDUCTASE FROM *PSEUDOMONAS STUTZERI* IN TRANSGENIC PLANTS AND ACTIVITY THEREOF

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## Abstract

As the third most important greenhouse gas, nitrous oxide **(**$N_2O$) is a stable greenhouse gas and also plays a significant role in stratospheric ozone destruction. The primary anthropogenic source of $N_2O$ stems from the use of nitrogen in agriculture, with soils being the major contributors. Currently, the annual $N_2O$ emissions from this "soil–microbe-plant" system is more than 2.6 Tg (one Tg equals a million metric tons) of $N_2O$-N globally. My doctoral studies aimed to explore innovative strategies for $N_2O$ mitigation, in the context of environmental microbiology's potential contribution to alleviating global warming. The bacterial enzyme nitrous oxide reductase ($N_2OR$), naturally found in some soils, is the only known enzyme capable of catalyzing the final step of the denitrification pathway, conversion of $N_2O$ to $N_2$. Therefore, to "scrub" or reduce $N_2O$ emissions, bacterial $N_2OR$ was heterologously expressed inside the leaves and roots of transgenic plants. Others had previously shown that the functional assembly of the catalytic centres ($Cu_Z$) of $N_2OR$ is lacking when only *nosZ* is expressed in other bacterial hosts. There, coexpression of *nosZ* with *nosD*, *nosF* and *nosY* was found to be necessary for production of the catalytically active holoenzyme. I have generated transgenic tobacco plants expressing the *nosZ* gene, as well as tobacco plants in which the other four *nos* genes were coexpressed. More than 100 transgenic tobacco lines, expressing *nosZ* and *nosFLZDY* under the control of rolD promoter and d35S promoter, have been analyzed by PCR, RT-PCR and Western blot. The activity of $N_2OR$ expressed in transgenic plants, analyzed with the methyl viologen-linked enzyme assay, showed detectable $N_2O$ reducing activity. The $N_2O$-reducing patterns observed were similar to that of the positive control purified bacterial $N_2OR$. The data indicated that expressing bacterial $N_2OR$ heterologously in plants, without the expression of the accessory

Nos proteins, could convert $N_2O$ into inert $N_2$. This suggests that atmospheric phytoremediation of $N_2O$ by plants harbouring $N_2OR$ could be invaluable in efforts to reduce emissions from crop production fields.

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## List of Abbreviations

| | |
|---|---|
| Å | Ångström (1/10,000,000,000 of a metre) |
| ABC transporter | ATP-binding cassette transporter |
| As | Arsenic |
| bp | Base pair |
| Bt | *Bacillus thuringiensis* |
| Cd | Cadmium |
| cDNA | Complementary deoxyribonucleic acid |
| CFCs | Chlorofluorocarbons |
| $CH_4$ | Methane |
| Co | Cobalt |
| $CO_2$ | Carbon dioxide |
| Cr | Chromium |
| $^{137}Cs$ | Cesium-137 |
| Cu | Copper |
| $Cu_A$ | Copper centre A |
| $Cu_Z$ | Copper centre Z |
| DEAE | Diethylaminoethyl |
| DNases | Deoxyribonuclease |
| DnrD | Transcription factor (a member of the Crp-Fnr family) |
| d35S | Double 35S promoter |
| EDTA | Ethylene diamine tetraacetic acid |
| F | Fluorine |
| Fe | Iron |
| GHG | Greenhouse gas |
| GMO | Genetically modified organism |
| $GtCO_2eq$ | Billion tonnes of carbon dioxide equivalent |
| GWP | Global warming potential |
| HFCs | Hydrofluorocarbons |
| Hg | Mercury |
| IPCC | Intergovernmental Panel on Climate Change |
| kb | Kilo base |
| kDa | Kilo Dalton |
| LB | Luria broth |
| Mb | Mega base |
| Mn | Manganese |
| mRNA | Messenger ribonucleic acid |
| MS | Murashige and Skoog medium |
| $N_2$ | Nitrogen |
| $NH_3$ | Ammonia |
| $NH_4^+$ | Ammonium |
| Ni | Nickel |
| NO | Nitric oxide |
| $NO_3^-$ | Nitrate |

| | |
|---|---|
| $N_2O$ | Nitrous oxide |
| $N_2OR$ | Nitrous oxide reductase |
| NOSter | Nopaline synthase terminator (DNA sequence from *Agrobacterium*) |
| *nosZ* | Gene encoding nitrous oxide reductase |
| NOx | Nitrogen oxides |
| NT | Non-transformed/non-transgenic |
| ODP | Ozone-depleting potential |
| ORF | Open reading frame |
| PAH | Polycyclic aromatic hydrocarbon |
| Pb | Lead |
| PBS | Phosphate buffered saline |
| PCB | Polychlorinated biphenyl |
| PCR | Polymerase chain reaction |
| PFCs | Perfluorocarbons |
| ppb | Parts per billion |
| ppm | Parts per million |
| RNase | Ribonuclease |
| rolD | RolD promoter |
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| Se | Selenium |
| $SF_6$ | Sulfur hexafluoride |
| $T_0$ | Primary transformants |
| $T_1$ | Progeny from $T_0$ transformants |
| Tat | Twin-arginine translocation |
| TBST | Tris-buffered saline with Tween |
| TCE | Trichloroethylene |
| TgN | Teragrams of nitrogen |
| TNT | Trinitrotoluene |
| Tris | Tris(hydroxymethyl)aminomethane $(HOCH_2)_3CNH_2$ |
| Zn | Zinc |

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# Chapter 1

# General Introduction

## 1.1 Greenhouse gases and climate change

### *1.1.1 Global warming and climate change*

Earth is currently undergoing a global climate change phenomenon (Kerr, 2007). Earth's atmospheric temperature increased by approximately 0.74°C and sea level rose at an average rate of 6.8 inches (1.7 millimetre/year) during the past 100 years (Trenberth et al., 2007). During 1993-2003, the rise in sea level was at an average rate of 3.1 mm/year. Furthermore, global warming also has relevance in the increasing frequency of droughts, floods, heat waves and other severe weather-related events such as hurricanes and monsoons (Anthes et al., 2006; Hoerling and Kumar, 2003; Milly et al., 2002). The resulting climatic change has been found to affect the seasonal timings of activities, distributions, and community compositions of many organisms including animals and plants. Some have argued that the earth's atmosphere's composition of gases may serve as a type of canopy similar to that in a glasshouse or greenhouse (Callendar, 1938).

The greenhouse gases (GHGs) not only allow solar radiation to pass through the atmosphere, but also partly prevent heat from being radiated out into the space (Hegerl and Cubasch, 1996). The GHGs make a considerable contribution to keeping the earth warm enough for life processes to proceed. Otherwise, the mean global surface temperature would be about -18°C, instead of the current 15°C (Hunter, 2008). Global warming may be partly caused by the increase in greenhouse gas concentration in the atmosphere (Bera et al., 2009).

According to the well-accepted climate theory, known as the greenhouse effect, global climate change could be induced by the increase of greenhouse gases in Earth's atmosphere (Jones and Henderson-Sellers, 1990; Schneider, 1989). Global atmospheric concentrations of GHGs may have increased markedly as a result of human activities. GHGs could limit the amount of solar radiation which is reflected from the surface of the Earth, change the heat budget of the atmosphere, and lead to an increase in the average surface temperature of the earth (Callendar, 1938).

### *1.1.2 Greenhouse gases*

The common greenhouse gases include carbon dioxide ($CO_2$), methane ($CH_4$), nitrous oxide ($N_2O$), hydrofluorocarbons (HFCs), perfluorocarbons (PFCs), and sulfur hexafluoride ($SF_6$) (Bera et al., 2009). While $CO_2$ is present at the highest concentration of all greenhouse gases in the atmosphere, other less abundant gases such as $N_2O$, $CH_4$, HFCs, PFCs and $SF_6$ have much higher potency of global warming due to their enhanced ability to absorb infrared wavelengths. The Intergovernmental Panel on Climate Change (IPCC) has stated that "Most of the observed increase in global average temperatures since the mid-20$^{th}$ century is very likely due to the observed increase in anthropogenic greenhouse gas concentrations" (Forster et al., 2007).

Global atmospheric concentrations of $CO_2$, $CH_4$ and $N_2O$ appear to have increased markedly as a result of human activities. Theorists on both sides of the climate change debate attempt to study changes in the heat budget of the atmosphere to see if it could lead to an increase in the average surface temperature of the earth (Guirguis et al., 2011; Kalnay et al., 1996). These emissions are increasing GHG concentrations in the atmosphere and enhancing

the natural 'greenhouse effect', which is causing global warming. Key indicators of change such as increased mean temperatures, changes in patterns of precipitation, increased cloud cover, more frequent floods and other extreme events may be due, at least in part, to the radiative-forcing effects of increased concentrations of GHGs.

### *1.1.3 Rate of change*

There is increasing evidence that the world's climate is changing and the rate of change since the onset of the industrial revolution is greater than would be expected from natural variability alone. Levels of atmospheric greenhouse gases were all relatively constant over the 2000 years prior to 1850 (White, 2009). However, this pattern has dramatically changed since 1850. Substantial quantities of GHGs have been released into the atmosphere as a result anthropogenic activities. The Fourth Assessment Report of IPCC (2007) details the extent of recent global change. In 2007, atmospheric concentrations of the three most important greenhouse gases ($CO_2$, $CH_4$, $N_2O$) reached new highs, 37%, 156% and 19% above pre-industrial levels, respectively, according to the most recent analysis by the World Meteorological Organization (WMO). The global atmospheric concentrations of $CO_2$, $CH_4$, $N_2O$ rose to 383 parts per million (ppm), 1789 parts per billion (ppb) and 321 ppb.

### *1.1.4 Agriculture's impact on greenhouse gas emissions*

Agricultural activities are significant producers of $CH_4$ and $N_2O$. For example, in 2005, $CH_4$ and $N_2O$ emissions from the agricultural sector were responsible for about 10–12% of the total global anthropogenic emissions of GHGs, including 3.3 $GtCO_2eq$/year of

$CH_4$ (billion tonnes of carbon dioxide equivalent) (50% of total $CH_4$ anthropogenic emissions) and 2.8 $GtCO_2eq$/ year $N_2O$ (60% of total $N_2O$ anthropogenic emissions) (Bessou et al., 2011). The greatest contributions to $CH_4$ emission are enteric fermentation (27% of agricultural greenhouse gases), manure management (7%) and minor contributions from rice (*Oryza sativa L.*) cultivation (10%) and agricultural burning. The greatest contributions to $N_2O$ emission are the microbial nitrification and denitrification of microorganisms in soils (40%) (Bessou et al., 2011). Annual $CO_2$ emissions by agricultural lands (0.04 $GtCO_2eq$/year) are low compared with overall anthropogenic $CO_2$ emissions (Smith et al., 2007).

## 1.2 The nitrogen cycle

### *1.2.1. Current status of nitrogen in the environment*

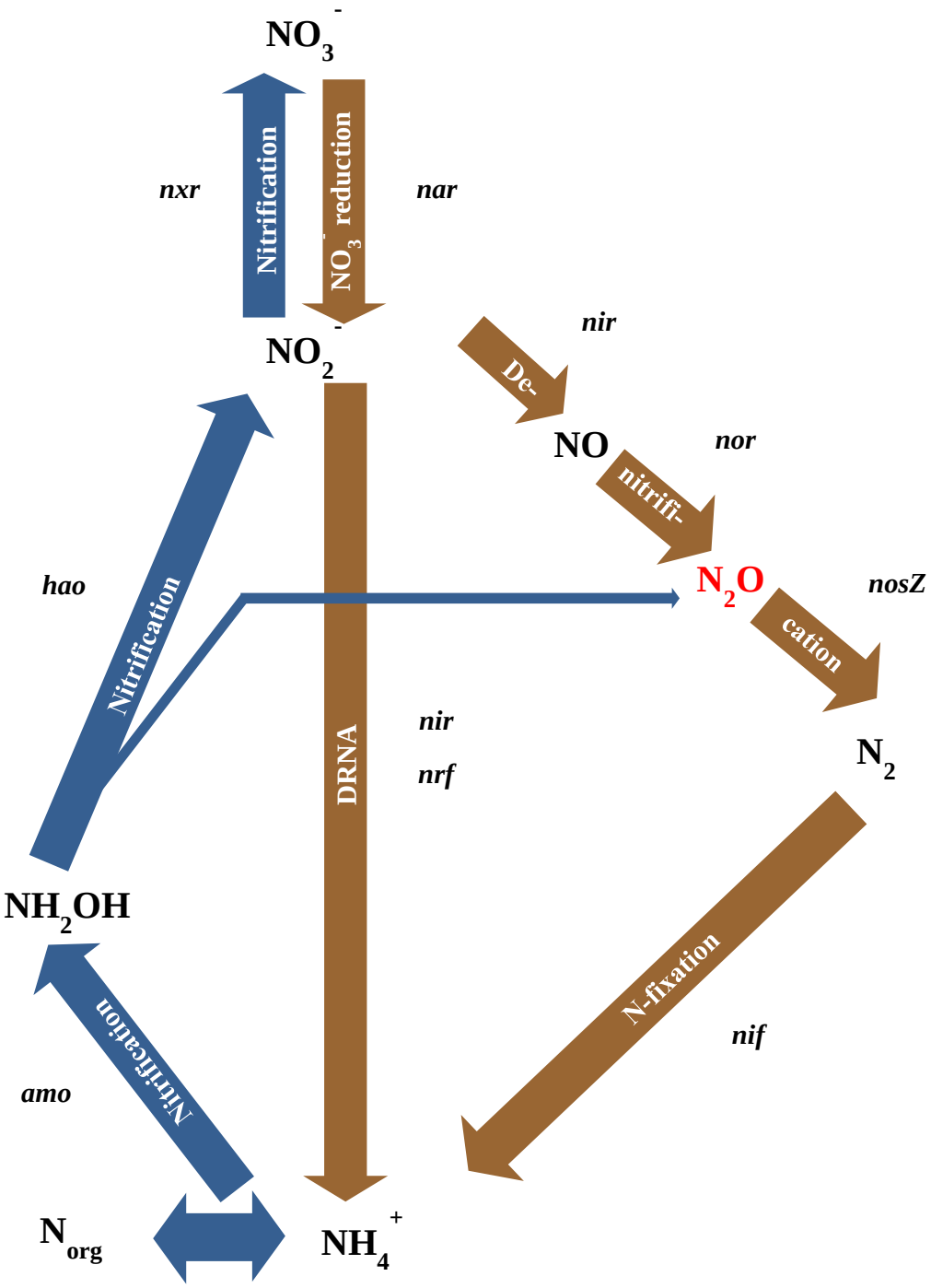
The development of new agricultural practices to feed the world's growing population has drastically disrupted the nitrogen cycle. For example, the use of nitrogen fertilizers increased by about 800% from 1960 to 2000 (Fixen and West, 2002). For the main crops (wheat, rice, and maize) accounting for about 50% of current fertilizer use, the nitrogen use efficiency is typically less than 40% since most of the applied fertilizer is leached out of the root zone or is lost to the atmosphere by denitrification before biomass accumulation (Canfield et al., 2010). The large acceleration of the global nitrogen cycle as a result of the addition of bioavailable nitrogen by farmers has led to many environmental problems, such as extensive eutrophication of fresh waters and coastal zones, including increased concentrations of the potent greenhouse gas $N_2O$. Before the end of the nineteenth century, the fixation of nitrogen ($N_2$), the reduction of $N_2$ to ammonium ($NH_4^+$) by symbiotic

bacteria in legumes, and the limited amount of nitrogen in manure are the main agricultural sources of reactive nitrogen. However, the developing of the Haber–Bosch process, producing cheap ammonia ($NH_3$) on an industrial scale from $N_2$ and energy, successfully changed the world in terms of crop yields, without anticipating downstream geophysical consequences such as radiative forcing by nitrous oxide. The fertilizers make it possible to sustain the growing population, without them about half of humanity would not be sufficiently nourished. The acceleration of the global nitrogen cycle is thought by many scientists to be of grave concern for the environment (Nojiri et al., 2009). Most of the nitrogen in fertilizers and manures added to farm fields is lost as pollution (such as $NH_3$ and NOx, $N_2O$) (Sutton et al., 2011). Some of the nitrogen (primarily as nitrates) flows onward to river and enter marine ecosystems. Fertilizer run-off can also cause toxic algal blooms and fish kills, which are the results of hypoxia (shortage of oxygen) or anoxia (total loss of oxygen). Gulf of Mexico, the most famous "dead zone", is thought to result from excess of nitrogen from farm fertilizers, sewage and emissions from vehicles and factories entering the gulf and triggering the proliferation of plankton.

### *1.2.2. Nitrifying and denitrifying cycles*

A large part of the nitrogen fertilizer, neither taken up by plants nor lost via leaching, ends up as the atmospheric gas $N_2O$ through bacterial nitrifying and denitrifying process (Figure 1.1). In the presence of oxygen, $NH_4^+$ is sequentially oxidized to nitrate ($NO_3^-$) via hydroxylamine ($NH_2OH$), nitrite ($NO_2^-$), and $NO_3^-$ through the bacterial nitrification pathway. In this process, $N_2O$ is produced at low rates as a by-product, which is considered as an important source of atmospheric $N_2O$ (Canfield et al., 2010). In the absence of oxygen,

**Figure 1.1. The nitrogen cycle showing the major biological nitrogen transformation pathways**. The major genes encoding enzymes that catalyze the transformations are indicated: nitrate reductase (*nar*), nitrite reductases (*nir*, *nrf*), nitric oxide reductase (*nor*), nitrous oxide reductase (*nosZ*), nitrogenase (*nif*), ammonium monooxygenase (*amo*), hydroxylamine oxidoreductase (*hao*), and nitrite oxidoreductase (*nxr*). (Adapted from Canfield et al., 2010)


NO3-
Nitrification
nxr
NO3- reduction
nar
NO2-
nir
De-
NO
nor
nitrifi-
N2O
nosZ
cation
hao
Nitrification
DRNA
nir
nrf
N2
NH2OH
N-fixation
nif
Nitrification
amo
Norg
NH4+

$NO_3^-$ can be reduced to $NH_4^+$ or $N_2$ by microbes through dissimilatory nitrate reduction to ammonium (DNRA) or denitrification process. The denitrification pathway is the more common route taken by the denitrifiers, representing 60 genera of bacteria and some archaea and eukaryotes (e.g., fungi, protozoa, and benthic Foraminifera and Gromiida) (Canfield et al., 2010; Demaneche et al., 2009). Denitrification is a microbial respiratory process within the nitrogen cycle responsible for the return of fixed nitrogen to the atmosphere, via four metalloenzymes: nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductases. The microbial denitrification process is regarded as an important source of the greenhouse gas $N_2O$, since $N_2O$ is an obligate intermediate, and some of it ultimately diffuses out of soil into the atmosphere. It has been estimated that the current global denitrification rate is about 573 TgN/yr, with 124 TgN/yr (22%) derived from the land surface and 110 TgN/yr (19%) from wetlands (Schlesinger, 2009).

### *1.2.3. Strategies to mitigate nitrogen emissions*

Since excess reactive nitrogen could affect the quality of water, soil and especially air, how to reduce nitrogen emissions is thought by many to be the pivotal environmental challenge for the 21$^{st}$ century (Sutton et al., 2011). To satisfy a growing global demand for food, it may be possible to develop climate-smart agriculture to tackle climate change. Clearly more efficient ways of controlling the loss of nitrogen from soils begs urgent attention. Some potential ways have already been found, such as more scientific approaches for managing fertilizer application to achieve cost and environmental goals. Since the overuse of nitrogen fertilizer is poisoning air, soil and water, farmers should ideally strive to cut the use of the fertilizers in such a way as not to reduce crop yields. Most farmers believe

that the crop yields could be increased by applying more fertilizer. However, recent report presents that the more fertilizer is applied, the less efficient of plants take up nitrogen and more nitrogen is lost into the environment (Ju et al., 2009). Secondly, researchers found that more $N_2O$ could be released from soil if the soils are dry and then suddenly become wet. It is recommended the farmers could keep $N_2O$ emissions down by keeping soil moist (Gilbert, 2011). Furthermore, soil chemist Keith Goulding and his colleagues are researching the methods of mitigating $N_2O$ emissions from agriculture by managing the microbes, or their genes to less the loss of nitrogen from the soil (Gilbert, 2011).

## 1.3 Nitrous oxide

### *1.3.1 The properties of nitrous oxide*

As the third most important GHG, $N_2O$ is present at a concentration of about 321 ppb in the atmosphere compared to a pre-industrial high of 275 ppb. Recent reports suggest that the atmospheric concentration of $N_2O$ is now increasing at a rate as high as 0.3% per year and it is anticipated that this trend is set to continue (Wuebbles, 2009). Special attention has increasingly been paid to $N_2O$, because of its global warming potential (GWP) value and atmospheric life-time. Its GWP is 298 over a 100-year time span – indicating that 1 kg of $N_2O$ released into the atmosphere has a global warming effect equivalent to 298 kg of $CO_2$ over a 100 year period (Repo et al., 2009). And further exacerbating the negative effects of such accumulation is the chemistry of laughing gas itself, that $N_2O$ is very stable in the troposphere. $N_2O$ has important effects both on climate change and on stratospheric ozone depletion (Dyominov and Zadorozhny, 2008), and it remains aloft for 114 years before

undergoing reactions resulting in its destruction (Billings, 2008). $N_2O$ absorbs infrared radiation in the atmosphere, causing atmospheric warming. $N_2O$ gradually diffuses into the stratosphere where it is degraded by photolysis (90%) (a). The remaining ten percent of $N_2O$ reacts with excited atomic oxygen O ($^1D$) releasing active chemicals (NO) into the stratosphere (b) and destroying the ozone layer through the nitrogen oxide–catalyzed processes (c) (Rahn and Wahlen, 1997; Ravishankara et al., 2009):

$$N_2O + h\nu \rightarrow N_2 + O(^1D) \quad \text{(a)}$$

$$N_2O + O(^1D) \rightarrow 2NO \quad \text{(b)}$$

$$N_2O + O(^1D) \rightarrow N_2 + O_2$$

$$NO + O_3 \rightarrow NO_2 + O_2 \quad \text{(c)}$$

$$O + NO_2 \rightarrow NO + O_2$$

### *1.3.2 The effects of nitrous oxide*

In the past, policy-makers, regulators and atmospheric scientists have focused on the more potent chlorofluorocarbons (CFCs) and ignored the ozone-destroying abilities of $N_2O$. Until recently, $N_2O$ has been heralded as the main cause of stratospheric ozone depletion and is expected to remain the largest agent throughout the 21$^{st}$ century (Ravishankara et al., 2009). The ozone-depleting potential (ODP) of a gas is the value of how much ozone is depleted by a particular gas, relative to that destroyed by the same amount of chlorofluorocarbon 11 (CFC-11, also known as trichlorofluoromethane $CCl_3F$, which is one of the most significant ozone-depleting substances). The ODP of $N_2O$ is about one-sixtieth of that of CFC-11. Since the ODP alone cannot fully quantify the impact of $N_2O$, the emission history and even the future emission potential should perhaps also be considered.

The atmospheric chemist A.R. Ravishankara and his colleagues found that $N_2O$ has the greatest impact on ozone depletion, when the large scale of human-related emissions of $N_2O$ was taken into account (Buchen , 2009; Ravishankara et al., 2009).

Any depletion in the stratospheric ozone layer leads to increase in the solar UV-B radiation at the surface of the Earth. This change is likely to have some consequential detrimental effects on human health, primarily on the skin, the eye and the immune system, although some degree of it is needed to promote the synthesis of vitamin D. Over the past five decades, reduction in the global ozone layer has dramatically boosted skin cancer cases, including melanoma and the non-melanoma skin cancers, basal cell carcinoma and squamous cell carcinoma (Bikle, 2008; Diffey, 2004). For example, in Sweden, the incidence of squamous cell carcinoma has become four times greater in both men and women over the past 40 years (Andrady et al., 2009). Solar UV radiation is also recognised as a risk factor for cataract and pterygium, pinguecula and squamous cell carcinoma of the cornea and conjunctiva (Norval et al., 2011). Furthermore, the overexposure to UV radiation is identified as an environmental risk factor suppressing some aspects of immunity. The incidence of these adverse effects continually increases, thus posing a significant world-wide health burden.

### *1.3.3 Agriculture and nitrous oxide emissions*

The concentrations of atmospheric $N_2O$ have been rising as a result of increased emissions from agricultural fertilizers, animal waste and biomass burning. Since pre-industrial times, atmospheric concentrations of $N_2O$ have increased markedly due to anthropogenic perturbation of the global nitrogen cycle (Wolf et al., 2010 ). At present, the

annual emissions of $N_2O$ are approximately 17.7 TgN, but estimating each individual source of $N_2O$ is accompanied with large approximations (Wuebbles, 2009).

Professor John Beddington, the UK government's chief scientific adviser, told a meeting in London (28 February, 2011) that agriculture must become a key topic in future climate-change discussions because it contributes significantly to global $CO_2$ and $N_2O$ emissions. The agricultural sources present a large challenge because the growing global demands for food affect the ability to reduce $N_2O$ emissions from the use of fertilizers. Newer technologies for increasing agricultural efficiency and potential methods that may help mitigate $N_2O$ are clearly needed. This thesis set out to explore one such potential strategy of reducing $N_2O$, that of increasing the amount of available enzyme catalyst in agri-system environments: soil microbiota, rhizosphere, roots, crop plants themselves and their crop residues or detritus.

## 1.4 Nitrous oxide reductase

Nitrous oxide reductase ($N_2OR$ or NosZ, EC 1.7.99.6) is a multi-copper enzyme that catalyzes the last step of bacterial denitrification, the conversion of $N_2O$ to $N_2$ (Zumft, 2005). The chemical reaction is in the equation below:

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$$

$N_2OR$ is the only known enzyme capable of reducing potent $N_2O$ into inert $N_2$ (Richardson et al., 2009). Therefore, a better understanding of $N_2OR$ may have a positive impact on both the environment and biotechnological interventions to ameliorate cropping systems.

Since $N_2OR$ was first identified in *Pseudomonas stutzeri* (formerly *Pseudomonas perfectomarina*), it has been studied extensively (Coyle et al., 1985; Riester et al., 1989). Highly conserved, this enzyme has been identified and characterized in a wide range of microorganisms: *Rhodobacter capsulatus* (formerly *Rhodopseudomonas capsulate*) (McEwan et al., 1985), *Paracoccus denitrificans* (Snyder and Hollocher, 1987), *Wolinella succinogenes* (Teraguchi and Hollocher, 1989), *Achromobacter cycloclastes* (Hulse and Averill, 1990), *Pseudomonas aeruginosa* (Soohoo and Hollocher, 1991), *Flexibacter canadensis* (Jones et al., 1992), *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*) (Berks et al., 1993), *Thiobacillus denitrificans* (Hole et al., 1996), *Rhodobacter sphaeroides f. sp. Denitrificans* (Sato et al., 1998), *Alcaligenes xylosoxidans* (Ferretti et al., 1999), *Pseudomonas nautica* (Prudencio et al., 2000), and in *Hyphomicrobium denitrificans* A3151 (Yamaguchi et al., 2003).

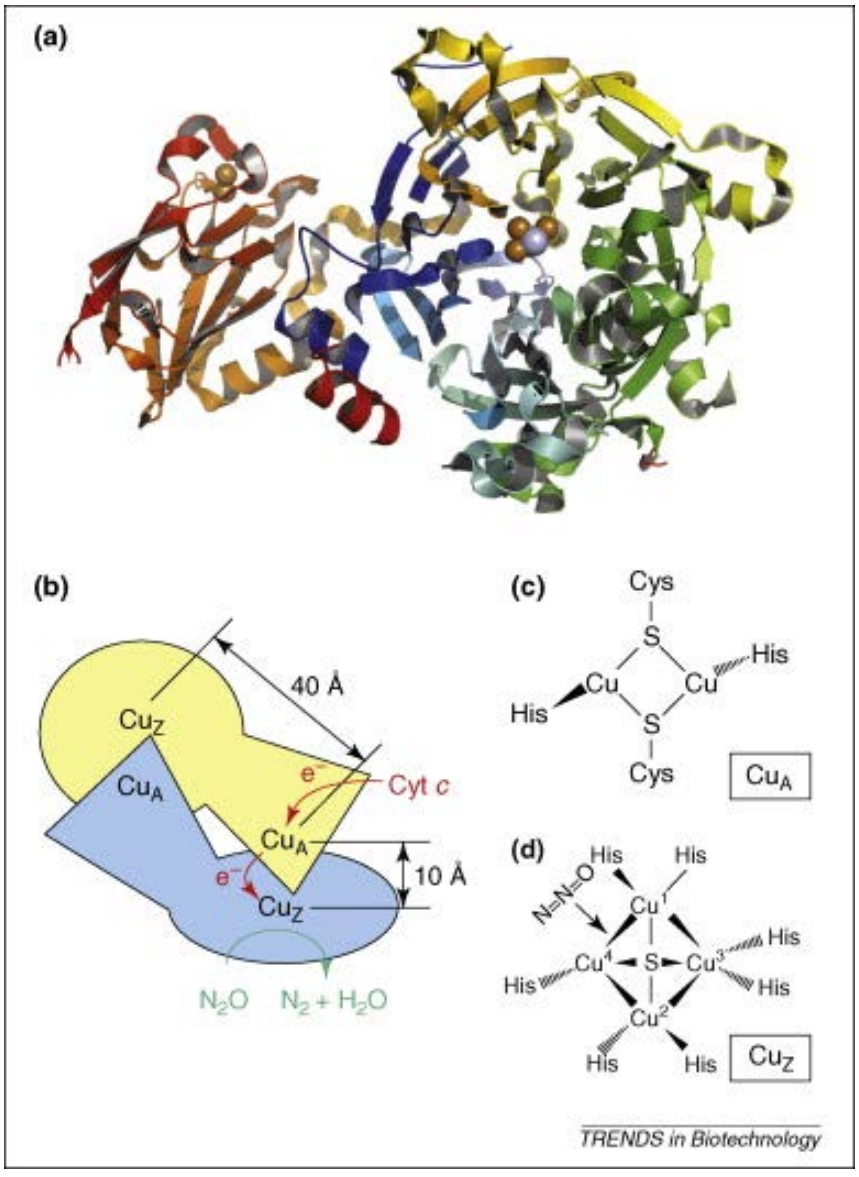
### *1.4.1 The structure of nitrous oxide reductase*

$N_2OR$ is a homodimeric (monomer mass about 66 kDa) multicopper protein (Zumft, 2005) (Figure 1.2, a). Each monomer is composed of two distinct domains: a C-terminal domain with a cupredoxin fold that carries the binuclear copper centre A ($Cu_A$) and an N-terminal seven-bladed β -propeller domain which contains the unique tetranuclear copper centre Z ($Cu_Z$) (Haltia et al., 2003). This copper-containing fold of cupredoxin is quite distinct from the iron-sulfur fold of ferredoxins (Altosaar et al., 1977). In $N_2OR$ the C-terminal domain consists of about 130 amino acids and is connected with the N-terminal domain that comprises about 450 amino acids by a short linker region of low positional conservation (Charnock et al., 2000).

Over the past twelve years, the three-dimensional structure was unveiled and the arrangement of the coppers in $N_2OR$ was elucidated. The carboxy-terminal portion of one monomer in a dimer opposes the amino-terminal domain of the second monomer (Figure 1.2, b). There are two calcium sites located at the monomer-monomer interface surface and one chloride site associated with $Cu_Z$ in the $N_2OR$ monomer (Brown et al., 2000; Zumft, 2005). The head-to-tail homodimer is formatted and stabilized by numerous interactions across the dimer interface including two calcium sites and a number of Cu-binding residues at the intermonomeric interface (Haltia et al., 2003). Within the same monomer, the distance between the $Cu_A$ and $Cu_Z$ is around 40 Å. The intra-monomer distance is too long for efficient electron transfer. However, domain exchange brings the two monomers into contact and results in the $Cu_A$ and $Cu_Z$ of opposing monomers being within approximately 10Å (i.e. much closer than the intra-monomer distance) (Dell'Acqua et al., 2011). The inter-monomer distance is thought by most structuralists to be suitable and sufficient for efficient electron transfer. The electrons are believed to be transferred from the $Cu_A$ in one monomer to the $Cu_Z$ in the other monomer (Ghosh et al., 2007).

There are two distinct copper sites in $N_2OR$ (Figure 1.2, c, d). The $Cu_A$ centre is the electron-transfer site similar to that found in cytochrome *c* oxidase. In the $Cu_A$ centre, the two Cu atoms of $Cu_A$ are bridged by two cysteine residues. The $Cu_Z$ centre is the catalytic site, with a novel $\mu_4$-sulfide-bridged structure. The four Cu atoms of $Cu_Z$ are ligated by seven histidine residues: two histidines coordinated to $Cu^1$, $Cu^2$ and $Cu^3$, and only one His is seen to be coordinated to $Cu^4$. The nature of the bridging ligand between $Cu^1$ and $Cu^4$, the proposed binding site for the substrate $N_2O$, is still unclear. It has been proposed that the substrate $N_2O$ binds to $Cu^4$ of $Cu_Z$ through oxygen, the most accessible because of its single histidine ligand (Moura and Moura, 2001).

**Figure 1.2. The molecular structure of nitrous oxide reductase ($N_2OR$).**
**(a)** The crystal structure of $N_2OR$ monomer from *Pseudomonas denitrificans*.
**(b)** Schematic illustration the electron transfer within the dimeric $N_2OR$. Electron transfers from the donor cytochrome *c* via $Cu_A$ to $Cu_Z$.
**(c)** Structure of the binuclear $Cu_A$ centre, representing the electron transfer site.
**(d)** Structure of the tetranuclear $Cu_Z$ centre, representing the catalytic site. $N_2O$ is proposed to bind to $Cu_Z$ sidewise between $Cu^1$ and $Cu^4$. (Adapted from Richardson et al., 2009 with permission)


(a)
(b)
40 Å
$Cu_Z$
$Cu_A$
e⁻
Cyt *c*
$Cu_A$
e⁻
10 Å
$Cu_Z$
$N_2O$
$N_2 + H_2O$
(c)
Cys
S
His
Cu
Cu
His
S
Cys
$Cu_A$
(d)
His
His
N=N=O
$Cu^1$
His
$Cu^4$
S
$Cu^3$
His
His
$Cu^2$
His
His
$Cu_Z$
TRENDS in Biotechnology

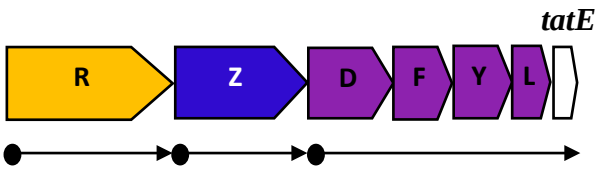
The mechanism of $N_2O$ reduction by the $Cu_Z$ centre has also been investigated. The electron-rich $Cu_Z$ site with four reduced $Cu^1$ gives two electrons to the substrate, $N_2O$, and reduces it to $N_2$ and $H_2O$. The $Cu_A$ accepts electrons from a reducing substrate. Two electrons are transferred crossing the subunit interface to the $Cu^2$ and $Cu^4$ for the reduction of oxidized $Cu_Z$ site and completion of the catalytic cycle (Chen et al., 2002; Zumft, 2005). This thesis therefore set out to see if, rather than function only in bacterial cells, whether this enzyme could also be biosynthesized in a plant cell environment where similar electron-transfer reactions occur albeit mediated by different electron-transfer proteins e.g. where ferredoxin is known to function (Altosaar et al., 1977).

### *1.4.2 The* nos *genes and their products*

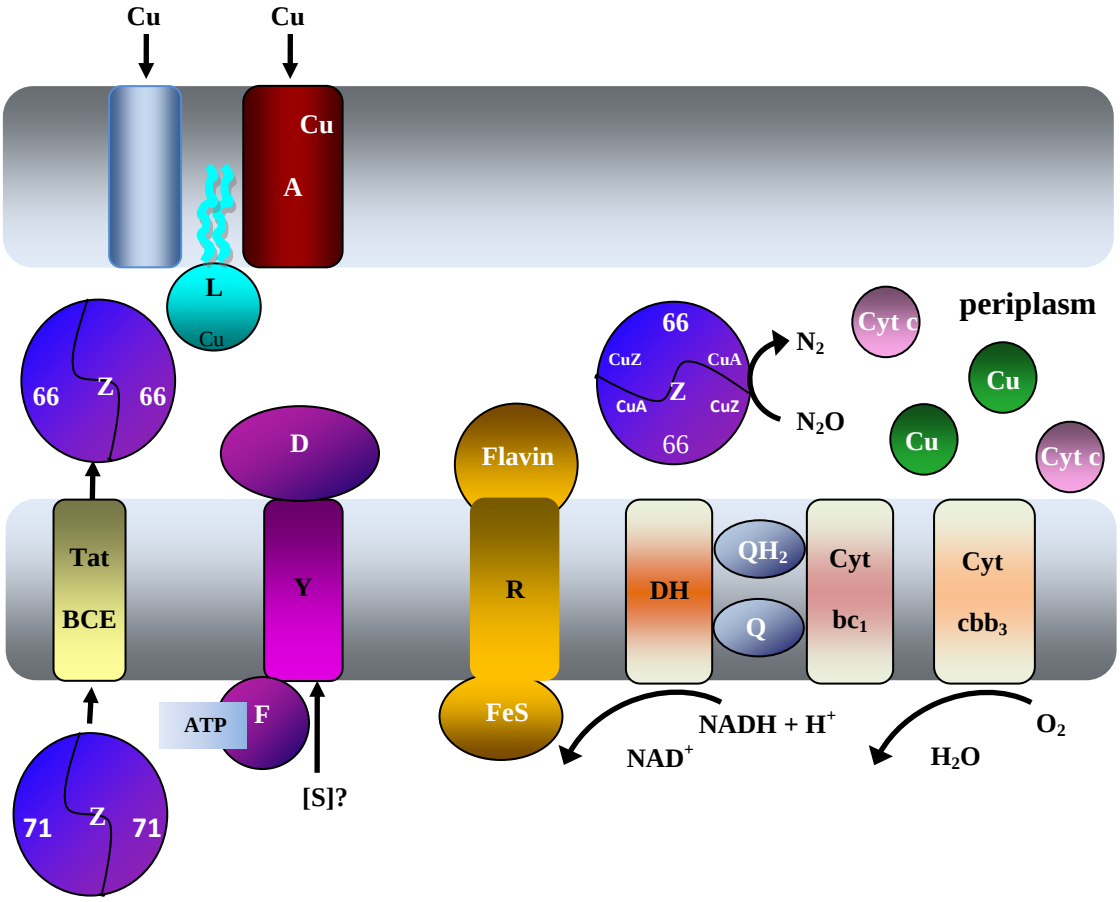
Nitrous oxide reductase was first purified from *Pseudomonas stutzeri* Zobell and the nucleotide sequence of the structural gene (*nosZ)* was also the first determined in *Pseudomonas stutzeri* Zobell (Holloway et al., 1996). $N_2OR$ is encoded by the *nosZ* gene, which is located within the 8 kb *nos* cluster on the 4.3 Mb chromosome of *P. stutzeri* (Jungst et al., 1991). The *nos* genes are clustered within a single locus and consist of three transcriptional units: *nosR*, *nosZ*, and *nosDFYLtatE* operon (Cuypers et al., 1992; Honisch and Zumft, 2003; Vollack and Zumft, 2001; Zumft, 2005) (Figure 1.3, a). The *nosZ* sequence is 1917 bp including a 150 bp 5' signal sequence that targets the mature peptide to the periplasmic space (GenBank accession X53676) (Viebrock and Zumft, 1988). An intriguing feature of the NosZ precursor is that it contains an unusually long signal peptide of fifty amino acids rather than the more usual twenty-five or so. Furthermore, the sequence analysis indicated that a conserved sequence motif with two arginines (Arg20-Arg21) is present at the

**Figure 1.3. Proposed $N_2O$-reducing respiratory module**.
**(a)** Genetic organization of the *nos* gene cluster of *P. stutzeri*. The dots represent the promoters that can be activated by the transcription factor DnrD.
**(b)** Proposed membrane topology of the components for $N_2OR$ biogenesis and $N_2O$-reduction. Single uppercase letters A, D, F, L, R, Y, and Z indicate the proteins NosA, NosD, NosF, NosL, NosR, NosY, and NosZ, respectively. Copper may enter the periplasm through the NosA or another porin. NosD, NosY and ATP-hydrolyzing protein NosF together form an ABC-type transporter that is thought to be involved in the delivery of either Cu or S to NosZ. The Cu-containing lipoprotein NosL may provide Cu for NosZ in the periplasm. The membrane-bound NosR acts as a transcriptional regulator for the expression of NosZ. The NosZ precursor is exported to the periplasmic space by a Tat translocon. The $N_2O$-reducing respiratory chain consists of NADH dehydrogenase (DH), the Q-cycle ($QH_2$/Q), cytochrome *bc*1 (Cyt *bc*1), and a terminal oxidase (Cyt *cbb*3). The pink icon Cyt *c* is periplasmic *c*-type cytochrome, the green icon Cu is cupredoxin, both accepting electrons from cytochrome *bc*1 complex. (Adapted from Zumft and Kroneck, 2007)

(a)


tatE
R
Z
D
F
Y
L

(b)


Cu
Cu
Cu
A
L
Cu
66
Z
66
71
Z
71
Tat
BCE
D
Y
ATP
F
[S]?
Flavin
R
FeS
66
CuZ
CuA
Z
CuA
CuZ
66
N2
N2O
Cyt c
periplasm
Cu
Cu
Cyt c
DH
QH2
Q
Cyt
bc1
Cyt
cbb3
NADH + H+
NAD+
O2
H2O

beginning of the signal sequence, directing the protein to the twin-arginine translocation (Tat) pathway for folded protein (Hoeren et al., 1993; Palmer et al., 2005; Philippot, 2002; Philippot et al., 2001). Such "double-arginine" signal sequences are conserved in other periplasmic proteins containing complex redox cofactors. Berks (Berks, 1996) identified a novel Sec-independent membrane targeting and translocation system of cofactor-containing proteins wherein they have a long signal peptide and a twin-arginine motif serving as the addressing sequence. It indicates that NosZ is translocated by this novel Tat export system (Philippot, 2002; Weiner et al., 1998). In *P. stutzeri*, the *tat* locus consists of *tatABC* and a *tatE* gene is found as part of the *nos* gene cluster suggesting a Nos-related role for TatE (Zumft, 2005).

The transcription of *nosZ* depends on the *trans*-acting component NosR and DnrD that is a homologue of the Crp-Fnr family of transcription factors (Vollack and Zumft, 2001). In most $N_2O$-respiring bacteria, the *nosR* gene is located upstream of the *nosZ* and functions as a *trans*-acting component with *nosZ* as the regulatory target. In *P. stutzeri*, *nosR* consists of a single open reading frame (ORF) of 2172 nucleotides with a 105 bp signal sequence (Cuypers et al., 1992). The NosR has a signal peptide directing it to the Sec pathway. The membrane-bound regulator NosR has a transmembrane core of five helices with the periplasmic flavin domain and iron-sulfur (Fe-S) group in its cytoplasmic domain. As a regulatory protein, NosR is essential for the expression of the NosZ and for maintaining or sustaining cellular NosZ activity (Wunsch and Zumft, 2005). The transcription of the *nosD* operon is also activated by NosR (Honisch and Zumft, 2003). The signal regulatory pathway may function according to the following pattern: the nitric oxide (NO) derived from nitrate denitrification activates *nosR* promoter via the transcription factor DnrD, which in turn results in *nosR* expression and activation of *nosZ* and *nosD* operon (NO → DnrD →

*nosR*/NosR → *nosZ*, *nosD* operon) (Zumft, 2005).

The *nosDFYLtatE* operon is located immediately downstream of the $N_2$OR structural gene *nosZ.* A total of 35 denitrifiers and genomes were analyzed and showed a conserved *nosDFYL* gene pattern, representing approximately 75% of the relevant entries in the data pool at that time (Zumft, 2005). It was suggested that the *nosDFYL* genes play an important role. The *nosDFY* genes were identified as sulphur-provision genes for the assembly of the catalytic site in NosZ, encoding a periplasmic protein, an ATPase, and an inner membrane protein, presumably forming a ABC transporter (Zumft, 2005). Analysis of the sequence showed *nosD* has a 1,311-nucleotide ORF. The NosD precursor protein contains a 27 amino-acid leader peptide cleaved during export to its periplasmic location. NosD is envisaged as the binding protein of the ABC transporter. Sequence analysis predicts that *nosF* (927 bp) encodes an ATPase, capable of providing energy for metal or small molecule transfer (Zumft et al., 1990). The 831 bp *nosY* gene, containing a secretion signal sequence, encoded a five-span, 29.4 kDa membrane protein. NosY is presumed to interact with NosF and to represent the transport protein.

In *P. stutzeri*, *nosL* is cotranscribed with *nosDFY*, indicating an important role for NosZ biogenesis. The *nosL* gene is 573 bp and has a 69 bp signal sequence that targets expression to the outer membrane in *P. stutzeri* (McGuirl et al., 2001). It has been shown to bind copper. The role of NosL is presumed as a metallochaperone to guide Cu from the site of periplasmic entry to NosZ (McGuirl et al., 2001).

### *1.4.3 NosZ biogenesis*

NosZ or $N_2OR$ as it is otherwise called is a complex metalloenzyme. The assembly of its essential metallocluster requires several accessory, transport, and cofactor insertion proteins (Figure 1.3, b) (Taubner et al., 2006). The biogenesis of the $Cu_A$ centres of NosZ has been studied extensively. NosA, a Cu porin of the outer membrane, is the key player of this Cu-uptake process.

Although the biogenesis of NosZ has been studied by Zumft and coworkers (1990), relatively little is known about the biogenesis of the Cuz centres. The biogenesis of the unique catalytic cluster (Cu-S) of NosZ requires the co-expression of a multiprotein assembly apparatus (encoded by *nosDFYL*) to provide copper and sulfur insertion into the $Cu_Z$ cluster of $N_2OR$ (Taubner et al., 2006; Wunsch et al., 2003). NosZ assembles its Cu centres posttranslocationally within the periplasm. Apoenzyme NosZ is transported to the periplasm prior to and independent of metal cofactor insertion. The pool of exogenous Cu appears to provide the essential metal co-factor from the periplasm. As a member of such a metallochaperone process, NosL would delivery Cu to the metalloenzyme target, and the NosDFY may be needed for acquisition of Cu and involved in metallocluster assembly. The precise chemical form of sulfur supporting $Cu_Z$ biogenesis remains unclear (Zumft, 2007). Such dissection of the enzymatic machinery participating in reduction of nitrous oxide in bacterial cells is clearly much more advanced. In the more complicated eukaryotic intracellular 'systems' where no evidence of $N_2O$ reduction has ever been reported, it would correspondingly be that much more complicated to detect or identify copper translocation partners or chaperones. One of the goals therefore at the outset of this thesis was to express parts of the $N_2OR$ biogenesis pathway *in planta* to see if the reductase itself could be reconstituted or complemented with copper. If such proof-of-principle experiments were to

be positive then one can envisage deploying such $N_2$OR-enabled plants for remediation of the atmosphere.

## 1.5 Phytoremediation

### *1.5.1 Phytoremediation technologies*

Phytoremediation, the use of plants and their associated microbes to clean up contaminated soils, sediments, and water, is accepted as a cost-effective, non-invasive alternative or complementary technology for engineering-based remediation methods (Cherian and Oliveira, 2005; Pilon-Smits, 2005).

Phytoremediation uses the naturally occurring plant processes and mechanisms to degrade or sequester a broad range of organic and inorganic pollutants. Organic pollutants are typically manmade and xenobiotic to a plant's system. There are a few major categories of organic contaminants that have successfully been degraded by a number of different plant species, including organic solvents such as trichloroethylene (TCE), herbicides such as atrazine, pesticides such as lambda cyhalothrin, explosives such as trinitrotoluene (TNT), and petroleum products such as oil (Aprill and Sims, 1990; Bouldin et al., 2006; Burken and Schnoor, 1997; Hughes et al., 1997). The phytoremediation of organic pollutants has been termed as a 'green liver model', because the liver of mammalians is also heavily involved in the detoxification of many foreign or xenobiotic compounds. Fortunately, some plants do already actually contain several "liver-like" enzymes, catalysts that are responsible for the degradation of many varieties of organic compounds (Schaffner et al., 2002). The term "green-liver concept" has been introduced in reference to the use of plant cytochrome P450

molecules in the degradation of organics (Sandermann, 1994). The plant cytochrome P450 proteins, the largest family of plant proteins, with over 1000 known sequences (Morant et al., 2003), convey plant herbicide tolerance directly (Werck-Reichhart et al., 2000). Furthermore, plants with mammalian genes encoding cytochrome P450 overexpression increase the metabolism and removal of a variety of organic pollutants and herbicides from the environment.

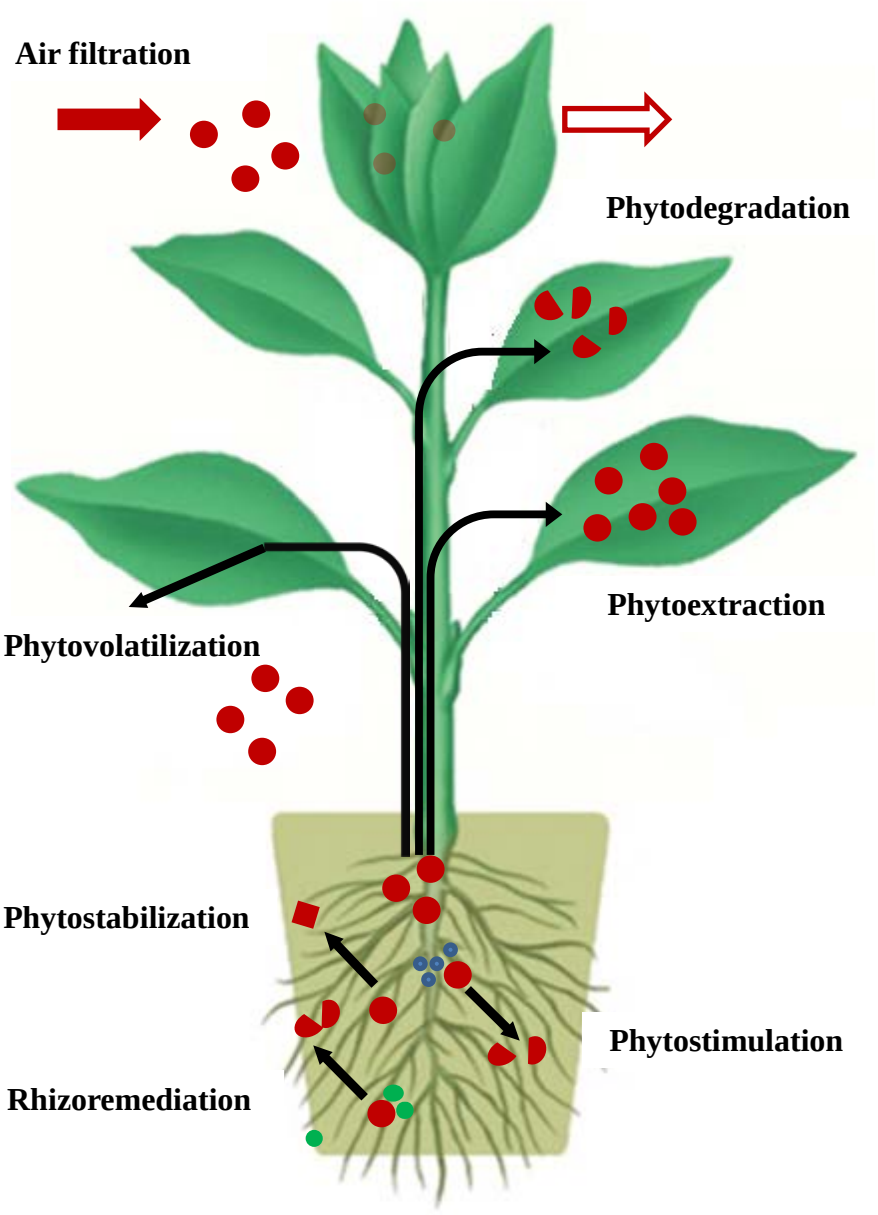
Inorganic pollutants, in contrast, are mostly natural elements in the earth's crust, atmosphere, or released from anthropogenic activities such as mining, transport, industry, modern agriculture, waste disposal and nuclear technology or military activities (Smits and Freeman, 2006). Inorganics cannot readily be degraded, but they can be phytoremediated via phytostabilization, phytoextraction, volatilization or sequestration in harvestable plant tissues. Inorganic pollutants, which can be phytoremediated, include the excess plant macronutrients such as nitrate and phosphate, micronutrients such as Chromium (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Molybdenum (Mo), and Zinc (Zn), and nonessential elements such as Arsenic (As), Cadmium (Cd), Cobalt (Co), Fluorine (F), Mercury (Hg), Selenium (Se), Lead (Pb), Vanadium (V), and Tungsten (W), and radionuclides such as Uranium-238 ( $^{238}$U), Cesium-137 ($^{137}$Cs), and Strontium-90 ( $^{90}$Sr) (Jadia and Fulekar, 2008; Nwoko, 2010).

### *1.5.2 Phytoremediation strategies and their uses*

Plants can contribute to remediation in several different ways (Figure1.4).

**Phytostabilization** Phytostabilization refers to the practice of establishing a vegetative cover on the sites of the contaminated soil or sediment, reducing the mobility and toxicity of pollutants, and preventing the exposure of the soil or sediment to humans

**Figure 1.4. Schematic overview of some phytoremediation technologies used for remediating pollutants.** Several clean-up strategies are indicated herein, namely phytodegradation, phytoextraction, phytostimulation, rhizoremediation, phytostabilization, and phytovolatilization. The possibility of Air Filtration (top arrow) has been mentioned in the literature, even in the patent literature, but this thesis is the first experimental evidence of air filtration applied to nitrous oxide reduction ("cracking" or "scrubbing"). The red dots (●) represent troublesome pollutants. The blue dots (●) represent rhizospheric microorganisms. The green dots (●) represent secreted enzymes. (Adapted from Pilon-Smits, 2005)


Air filtration
Phytodegradation
Phytoextraction
Phytovolatilization
Phytostabilization
Phytostimulation
Rhizoremediation

or animals. This technique primarily aims to stabilize pollutants (metals and radionuclides) in the soil through absorption and accumulation by roots, adsorption onto roots or precipitation in the rhizosphere, and prevent pollutant migration via erosion or leaching. For large areas contaminated with high and multi-elemental pollutants, phytostabilisation is a good remediation option.

**Phytostimulation** (sometimes called rhizodegradation) Plants can biodegrade organic pollutants by stimulating appropriate rhizospheric microorganisms. Phytostimulation is mainly suitable for immobile hydrophobic organics such as polychlorinated biphenyl (PCB), polycyclic aromatic hydrocarbon (PAH) or dichlorodiphenyldichloroethane (DDD) (Hutchinson et al., 2003). Although these contaminants cannot be taken up by plants, they can be degraded into non-toxic forms by soil organisms, such as fungi or bacteria associated with the plant roots, or via enzymes exuded from microorganisms or plants. Plants species that can produce specific exudate compounds to promote rhizodegradation via specific plant-microbe interactions have become common choices for phytostimulation in some scenarios. For example, mulberry trees can degrade PCB and PAH by exuding phenolic compounds that enhance the expression of microbial genes involved in the biodegradation of recalcitrant PCB and PAH (Olson et al., 2003). Furthermore, a positive plant-type for phytostimulation is a large, dense root system, because it can promote microbial growth.

**Rhizoremediation** Rhizoremediation harnesses the plant's ability to secrete pollutant-degrading enzymes into the area near the roots of plants (a zone known as the rhizosphere). The chief advantage of rhizoremediation is that it does not require the plant to take up the pollutant, which could be degraded extracellularly by the secreted enzymes. This secretion-enzyme strategy is used to degrade the less bioavailable pollutants, such as aromatic pollutants. For example, an extracellular laccase gene (*LACIII*) of fungal origin was

expressed in tobacco plants. The transgenic plants secreted the laccase into the rhizosphere, and removed the phenolic pollutants, bisphenol A and pentachlorophenol, with high efficiency (Doty, 2008; Sonoki et al., 2005).

**Phytodegradation** (also called phytotransformation) Plants can also accumulate and degrade the organic pollutants directly by the enzymes produced by plants (McCutcheon and Schnoor, 2003). For phytodegradation, the contaminants are taken up by plants and are degraded into breakdown products by enzymes within the plants (Arthur et al., 2005). This technique primarily targets organics that are mobile in plants, such as herbicides, TCE, TNT, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX), and methyl-tert-butylether (MTBE) (Newman and Reynolds, 2004). For example, poplar trees have been shown to take up TCE and degrade it to several metabolic components (trichloroethanol, trichloroacetic acid, and dichloracetic acid) (Newman et al., 1997).

**Phytovolatilization** Phytovolatilization involves the uptake of soluble organic contaminants by the plant roots, transport of contaminants from the roots to the leaves and transpiration into the atmosphere through the stomata in volatile form. Phytovolatilization has been successfully used for the removal of inorganics, such as Hg, Se. The mercuric ion was transformed into less toxic volatile elemental mercury by transgenic Arabidopsis and yellow poplars containing bacterial mercuric reductase (Rugh et al., 1998; Rugh et al., 1996). Phytovolatilization is used to percolate organic compounds, such as TCE and MTBE out of the soil. It was found that the poplar tree is the most-used and efficient species for phytovolatilization of volatile organic compounds, due to its high transpiration rate (Pilon-Smits, 2005).

**Phytoextraction** (or phytoaccumulation) Phytoextraction refers to the removal of pollutants, with the uptake of pollutants by plant roots and their accumulation in harvestable

organs (shoots or leaves) of the plants. Then the pollutants accumulated in stems and leaves can be removed from the site by harvesting the plants. The technique is mostly applied to trap heavy metals (Pb, Zn) and other toxic inorganics (Se, As and radionuclide $^{137}Cs$) in soil, sediment, and sludges (Blaylock and Huang, 2000). Some hyperaccumulators, which take up particularly high amounts of one or more toxic substances in their shoots during normal growth and reproduction, have been reported for As, Co, Cu, Mn, nickel (Ni), Pb, Se, and Zn (Pilon-Smits, 2005). Furthermore, chelators like ethylene diamine tetra-acetic acid (EDTA) enhance the uptake, translocation and accumulation of Pb, Cd, Cr, Cu, Ni, and Zn in *Brassica juncea* (Indian mustard) and *Helianthus anuus* (sunflower) (Turgut et al., 2004). Indian mustard and sunflower have become popular species for phytoextraction of inorganics, due to their favourable properties, such as fast growth, high biomass, high tolerance to the pollution, and high rates of plant uptake, translocation, and accumulation in harvestable organs (Salt et al., 1995).

Phytoremediation not only can be used for remediating solid and liquid pollutants, but also can be used to filter gaseous pollutants. Air filtration plants can be used to filter gaseous pollutants, such as NOx, $CO_2$, volatile halogenated hydrocarbons (methyl bromide, methyl chloride), nerve gases, sulphur dioxide, and ozone (Yunus et al., 1996; Jeffers and Liddy, 2003; Morikawa et al., 2003). The present thesis is an attempt to broaden the applicability of such atmospheric bioremediation by enabling plants to reduce $N_2O$ *in situ*.

### *1.5.3 Phytoremediation: advantages, limitations, present status*

Phytoremediation is an attractive alternative to existing remediating technologies and has attracted considerable attention in the past decade, since it is much more low-cost

and much less invasive compared to the physical and chemical methods used to remediate contaminated sites (Horikoshi et al., 2011; Kramer, 2005; Kramer and Chardonnens, 2001; McGrath and Zhao, 2003). Phytoremediation also has advantages over traditional methods of bioremediation using microorganismal metabolism to breakdown pollutants into harmless products. Phytoremediation does not require high microbial density nor a continuous nutrient supply as plants acquire their own nourishment from soil nutrients and photosynthesis.

According to statistical data, approximately $25-50 billion are spent worldwide every year for environmental clean-up (Pilon-Smits, 2005; Tsao, 2003). So, it is important and urgent to look for cheaper and more effective remedial measures. It has been estimated that the cost of remediation per contaminated hectare of soil using conventional, civil-engineering techniques (such as pollutant filtration, concretion/stabilisation, size selection and pyrometallurgical processes, electrokinetical treatment, chemical oxidation/reduction of pollutant, excavation) ranges between $0.27 and $1.6 million, while phytoremediation costs about 10–1000 times less (Kidd et al., 2009; McGrath and Zhao, 2003; Mulligan et al., 2001).

Over the last decade these plant-based technologies have been accepted by the public and gained regulatory support. Until now, the performance of most transgenic plants generated so far is not yet sufficient for commercial applications in the phytoremediation sphere. The phytoremediation market in the USA is about 0.5% of the total remediation market (about $100-150 million per year)(Pilon-Smits, 2005). As far as can be inferred from the published data, the development of phytoremediation technologies has largely been confined to bench- and greenhouse-scale. Although various plant species have been shown to be able to phytoremediate a large number of both organic and inorganic contaminants, widespread utilization of phytoremediation is limited by the characteristics of a phytoremediator: rapid growth, high biomass, and sufficient ability to tolerate, detoxify, and

accumulate contaminants (Kramer and Chardonnens, 2001). If studied more closely at the biochemical level, phytoremediation processes may be enhanced and empowered further in terms of environmental benefits. The more scrutiny given to key steps like pollutant availability, rhizosphere processes, pollutant uptake, translocation, chelation, degradation, and volatilization, the higher the likelihood that phytoremediation steps can be better catalyzed (Pilon-Smits, 2005). Furthermore, the development rate of phytoremediation technologies can be improved the more we know about underlying genetic, molecular, biochemical, physiological and agronomic processes (Kramer, 2005).

The use of genetic engineering technology to create transgenic plants has opened up new possibilities and has great potential in phytoremediation. This technology may someday be able to play a substantial role in improving the ability of plants to remove environmental pollution and thereby increase phytoremediation efficiency. Overexpression of genes from microbes, plants, and animals are increasingly being used to enhance the ability of plants to tolerate, uptake, transport, sequester and degrade pollutants (Doty, 2008). For example, the mammalian cytochrome P450 gene was introduced into plants, as an alternative to native plant P450 proteins, resulting in the increase of metabolism and removal of a variety of organic pollutants and herbicides. Mammalian enzyme, cytochrome P450 2E1, has been overexpressed successfully in transgenic poplar (Doty et al., 2007) in an attempt to achieve such amelioration of the environment. The transgenic poplar had greatly increased rates of metabolism of several volatile hydrocarbons, including TCE, vinyl chloride, carbon tetrachloride, benzene, and chloroform. Phytoremediation efficiency of plants might be substantially improved by directing transgene expression to specific tissues or cell types.

## 1.6 Hypothesis and Objectives

### *1.6.1 Rationale*

Therefore, taking this historical perspective of reactive nitrogen in earth sciences and life sciences, it may prove useful to explore the potential of nosZ, its adaptability to biotechnological applications, to make it as amenable as possible for exploitation or test its 'plasticity'. Plants engineered to express bacterial $N_2OR$ may help to produce a "green gene" system that may prove to have a substantial impact on rates of $N_2O$ emissions from agricultural soils on which the transgenic plants are eventually grown. Furthermore, the more complex biochemical environment of eukaryotic higher plant cells may be able to complement the activation of the prokaryotic $N_2OR$ protein moiety itself. For example, assuming such a 'systems biology' strategy, the proteome of eukaryotic plant cells, being more complex than that in prokaryotes, may be able to fulfill the need for an ABC-type transporter in the assembly of the catalytic center of $N_2OR$ *in planta*. Furthermore, plant tissues assessed over a 24-hour light-dark cycle may be anoxic enough to allow the oxygen sensitive enzyme nosZ to function.

### *1.6.2 Hypothesis*

1. Higher plant cytoplasm has enough available copper in its cellular pool(s) to insert Cu into the nosZ protein and render it active; (Chapter 2)
2. Eukaryotic higher plant cell proteome is complex enough to obviate the need for the assembly apparatus (nos D, F, Y, and L proteins); (Chapter 3)

3. Foreign enzyme $N_2OR$ can be secreted from transgenic roots and accumulates in the rhizosphere using a tissue (root)-specific promoter. (Chapter 4)

### *1.6.3 Objectives*

1. Atmospheric phytoremediation (Air filtration) - removing $N_2O$ from air by constitutively expressing $N_2OR$ in plants. Since $N_2O$ is reasonably soluble in water and hydrophobic materials, it is already entering plants through the stomata of the leaves (as does $CO_2$) and so should be rapidly reduced to $N_2$ as a result of high expression of $N_2OR$ in leaf tissues such as mesophyll cells.

2. Soil detoxification (Rhizoremediation) - removing $N_2O$ from soils by producing a catalytic sink of $N_2OR$ in soils. The recombinant bacterial $N_2OR$ is to be expressed in plant roots and secreted into soils to catalyze the conversion of $N_2O$ to the inert gas $N_2$. By expressing this enzyme in plant roots, and by secreting it into soil, it may enable an enzyme-suspension environment to be created within and around the rhizosphere of crop plant roots. This novel transgenic system may lead to a measurable lowering of $N_2O$ emissions from soils.

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# Chapter 2

## Active Bacterial Nitrous Oxide Reductase Expressed in Transgenic Plants

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## 2.1 Author contributions

Shen Wan, author of the present Ph.D. Thesis (2006-2011), personally wrote more than 50% of this chapter. S. Wan was also the direct supervisor of the fourth year BSc undergraduate research assistant, Kagami Goto (2009-2010). K. Goto was diligent enough to achieve exclusion of oxygen from the anaerobic chamber experimental installation. S. Wan and K. Goto contributed equally to this essential requirement of the study, and their names are presented in alphabetical order. Illimar Altosaar and Julianne Staebler conceived the study. J. Staebler, Yaseen Mottiar, and Mohsin Zaidi designed, generated and analysed the transgenic plants. To have access to some control bacterial enzyme, Anastassia Voronova purified $N_2OR$ from *P. stutzeri*. David Blais assisted with the western immunoblot studies. S. Wan, K. Goto, Y. Mottiar and Amanda Johnson were involved in the methyl viologen-linked enzyme assays. S. Wan, K. Goto, Y. Mottiar and I. Altosaar wrote the manuscript. Y.M., A.J. and K.G. were all fourth year BSc undergraduate research assistants.

## 2.2 Abstract

The abundant and global usage of agricultural fertilizers greatly increases nitrous oxide ($N_2O$) emissions, a potent greenhouse gas. Soil $N_2O$ release is mainly attributed to the inefficient last step of the microbial denitrification pathway responsible for $N_2O$ conversion to dinitrogen ($N_2$) catalyzed by the nitrous oxide reductase enzyme ($N_2OR$). Mitigation of this inefficient global denitrification may be approached by manipulating $N_2OR$ to reduce $N_2O$ more efficiently, preventing its release into the atmosphere. We have thus transferred the *nosZ* gene encoding $N_2OR$ from *Pseudomonas stutzeri* into tobacco plants and observed $N_2O$-reducing capabilities in extracts of transgenic leaves. Constitutive heterologous $N_2OR$ expression yielded some functional holoenzyme despite the plants lacking the complete native bacterial operon and periplasmic localization. If scaled up, such atmospheric phytoremediation of $N_2O$ by plants harboring $N_2OR$ could be invaluable in efforts to reduce greenhouse gas emissions.

## 2.3 Introduction

Amid increasing evidence that anthropogenic greenhouse gas emissions are directly implicated in climate change[1], innovative solutions for emissions mitigation are urgently needed. Among the primary greenhouse gases, $N_2O$ has a significant global warming potential (GWP) of 310 and an atmospheric lifetime of 114 years, which is significantly higher than that of $CO_2$ (GWP of 1 and lifespan of 100 years). $N_2O$ is also known to participate in stratospheric ozone depletion.[2] Atmospheric levels of $N_2O$ have increased from

270 parts per billion in pre-industrial times to 319 parts per billion by 2005, an increase of 0.26% per year.[1] The primary driving force for these increased emissions has been the adoption of soil nitrogen enriching strategies associated with modern agriculture.[3,4] If evolving agricultural practices can mitigate greenhouse gas emissions and climate change, $N_2O$ should be the primary target.[5]

Although agriculture is responsible for just 10-12%[6] of all anthropogenic greenhouse gas emissions globally, it is implicated in at least 58%[6] and as much as 84%[7] of all anthropogenic $N_2O$ emissions.[8] Agricultural intensification during the last century saw global consumption of nitrogen fertilizers increase from 31 million metric tonnes in 1961 to 165 million metric tonnes in 2005[9] concomitant with a 10% increase in atmospheric $N_2O$ concentrations.[6] Excess nitrogen present in agricultural soils boosts microbial nitrification and denitrification to elevated levels. The oxidation of ammonium by nitrifying bacteria and the reduction of nitrate by denitrifying bacteria can result in the production and release of $N_2O$[10], as the bacterial population as a whole does not always carry out the final denitrification step either efficiently or in synchrony with upstream parts of the pathway.[11] Therefore, enhancing the final step of denitrification to transform $N_2O$ into $N_2$ may become a beneficial strategy in mitigating $N_2O$ emissions.

Until now, nitrous oxide reductase ($N_2OR$) is the only known biocatalyst capable of transforming potent $N_2O$ into inert $N_2$.[5] To date, this enzyme has been detected in Gram-negative denitrifying bacteria such as *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Bradyrhizobium japonicum* and *Paracoccus denitrificans* as well as in Gram-positive *Wolinella succinogenes*.[12] The most studied form of $N_2OR$ is that of *P. stutzeri*. The holoenzyme contains two identical subunits (65.8 kDa each) with six copper (Cu) atoms

each. Each monomer of $N_2OR$ has two domains, one having a dinuclear $Cu_A$ centre that acts as the electron transfer site, and the other having a tetranuclear $Cu_Z$ centre that acts as the catalytic site. Two-electron redox chemistry localised at each $Cu_Z$ site yields the following stoichiometry: $2\ H^+ + 2\ N_2O + 2\ e^- \rightarrow N_2 + H_2O$.[13,14] In denitrifying bacteria such as *P. stutzeri*, $N_2OR$ is encoded by the *nosZ* gene which forms part of the conserved *nosRZDFYL* operon.[14] Three of these genes, *nosD, nosF* and *nosY*, encode an ABC-type transporter complex. A copper chaperone that presumably aids in cofactor assembly is encoded by *nosL*. Finally, *nosR* encodes a transcriptional regulator.[14]

Since higher plants do not possess a native $N_2OR$, one approach to reduce $N_2O$ emissions would be to introduce into plants the bacterial $N_2OR$ gene involved in the final step of denitrification where $N_2O$ is transformed into $N_2$. In this study, *Nicotiana tabacum* (tobacco) plants were engineered to express the *nosZ* gene with two objectives in mind: to test for copper insertion into the apoprotein, and to see if the plant tissues are anoxic enough to permit the activated enzyme to function. Our results demonstrate the bacterial $N_2OR$ expressed in transgenic plants can catalyze the conversion of $N_2O$ to $N_2$. Those findings present a novel system with the potential to reduce $N_2O$ release into the environment.

## 2.4 Experimental Methods

### *2.4.1 Construction of binary expression vector pd35S*-nosZ

All construct components (d35S promoter[15], *extensin* signal sequence[16], and *nosZ* gene sequence) were amplified by PCR using gene-specific primers (Supplementary Table 2.1)

and cloned into the pPCR-Script vector (Agilent Technologies, CA, USA). Following construct assembly, the full cassette was introduced into the binary vector pRD400 as a *Kpn*I-*Eco*RI fragment (Supplementary Figure 2.1). The full-length coding sequence of binary vector pd35S-*nosZ* was sequenced to confirm the inserted ex::*nosZ* fusions were in-frame and without mutations. Then, the pRD400 expression vector was introduced into the *A. tumefaciens* strain LBA4404, which was used to generate transgenic *Nicotiana tabacum* cv. Xanthi nc. plants following standard *Agrobacterium*-mediated transformation methods.[17] Putative pd35S-*nosZ* transformed lines (31 plant lines) were successfully rooted and transferred to soil. Seeds were collected from randomly selected $T_0$ plants, germinated on MS medium containing 100 mg/L kanamycin, and PCR-positive $T_1$ plants were grown in a greenhouse.

### *2.4.2 PCR analysis of transgenic plants*

Young leaf tissue was harvested from tobacco seedlings during the transfer of plants from rooting media to soil. Tissue was frozen and ground to a fine powder in liquid nitrogen prior to genomic DNA extraction using the DNeasy Plant Mini kit (Qiagen, ON, Canada). The purity of DNA samples was 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 PCR-amplification of a segment of DNA starting within the d35S promoter region and extending into the *nosZ* coding region. DNA from transformed plants was amplified with primers d35SF2 5’ GCACAATCCCACTATCCTTCGC and nosS1NC 5’ ATCGTTCGGGTGCGGGATGATG

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, CA, USA), 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 a non-transformed tobacco plant. Positive control reactions were also performed under the same conditions using 1 ng of pPCR-Script-d35S-ex-nosZ-NOSter plasmid.

### *2.4.3 RT-PCR analysis of transgenic plants*

To confirm that the bacterial gene was actually transcribed in plants, reverse transcriptase-PCR (RT-PCR) was performed on extracts from leaf tissue. Extraction of total RNA from leaf tissue was performed with the RNeasy Plant Mini kit (Qiagen, ON, Canada). 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 performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA). 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 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 included 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 that the cDNA template was replaced with 1 ng of pPCR-Script-d35S-ex-nosZ-NOSter 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.

### *2.4.4 Western blot analysis of protein extracts from transgenic plants*

To assess presence of the bacterial protein, leaf 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 removed by centrifugation for 10 min at 3000g, the 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., IL, USA).

Protein samples (175 μg leaf protein and 200 ng *P. stutzeri* protein for the positive control) were heated at 95°C for 10 min in 2x protein sample buffer (as above and containing 0.1% bromophenol blue, 5% β-mercaptoethanol) and loaded onto a 10% SDS polyacrylamide gel with a 5% stacking gel. Gels were electrophoresed at maximum voltage

(300 V) and 16 mA in a Mini-PROTEAN 3 System (Bio-Rad, CA, USA) until the bromophenol blue had exited the gel.

Proteins were transferred onto a nitrocellulose membrane using the Trans-Blot transfer cell (Bio-Rad, CA, USA). Transfer conditions were 10 V and maximum current (150 mA) for 1 h. Following transfer, the nitrocellulose membrane was placed in a 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-$N_2$OR serum (W.G. Zumft, Karlsruhe Institute of Technology) in fresh blocking solution at a concentration of 1:5000 for an additional hour followed by four 5 min washes with TBS-Tween buffer. A one-hour incubation with the secondary antibody, biotin-SP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., PA, USA), at a concentration of 1:25000 in TBS-Tween was also followed by four 5-min washes with TBS-Tween buffer. The final antibody incubation with the tertiary antibody, anti-goat biotin horseradish peroxidase linked antibody (Cell Signalling Technology Inc., MA, USA), at a concentration of 1:1000 in TBS-Tween was carried out overnight at 4$^{o}$C with shaking. To remove excess antibody, the membrane was washed four times with TBS-Tween 5 min periods each. Detection of proteins was carried out using the ECL Western blotting chemiluminescent detection reagents (Amersham Biosciences, QC, Canada) according to the manufacturer's protocol.

### *2.4.5 Extraction of crude protein from transgenic tobacco leaves*

The $T_1$ progeny tobacco plants were grown in a rooftop glasshouse with 16 hours of daylight at 400 W/m$^2$ and 25ºC and 8 hours of dark at 21ºC. Plants were watered daily and

fertilized twice weekly. Mature leaves were harvested roughly 12 weeks after germination and were immediately flash frozen and ground with a mortar and pestle using liquid nitrogen. Leaf extracts were prepared by adding 2 mL of 0.1 M phenylmethanesulfonylfluoride in 10 mM phosphate buffer to 100 mg of ground leaves in a small glass vial that was capped with a rubber septum and bubbled gently with argon prior to room temperature centrifugation at 1000g for 10 seconds. The supernatants were removed, degassed by vacuum and bubbled gently with argon.

### *2.4.6 Purification of $N_2OR$ from* P. stutzeri

To serve as a positive control for enzyme assays, $N_2OR$ protein was purified from *P. stutzeri* according to previously reported procedures with slight modifications.[18] Briefly, 1 g/L sodium nitrate was used to induce $N_2OR$ expression in *P. stutzeri* which was grown in a defined medium under low shaking conditions (120 rpm). After two scale-up inoculations and 48 hours of total incubation, 12 L of culture was pelleted by centrifugation and washed with 50 mM $MgCl_2$ in 25 mM Tris pH 7.5. The pellet was resuspended in 25 mM Tris pH 7.5 and disrupted by sonication. The supernatant was applied to a DEAE Sepharose ion-exchange column. Elution was performed at a flowrate of 2 mL/min using 25 mM Tris pH 7.5 (buffer A) and 25 mM Tris pH 7.5, 0.3 M NaCl (buffer B) at 4ºC over 5 column volumes with a linear gradient of 0 to 100% for buffer B. SDS-PAGE was used to identify $N_2OR$-containing fractions which were subsequently pooled and concentrated by dialysis using a 14 kDa membrane. Hydroxyapatite chromatography was then performed using 10 mM and 400 mM $NaH_2PO_4$ pH 7.2 buffers with a 0 to 80% linear gradient of the second buffer and 10

column volumes. $N_2OR$ protein eluted as a single peak between 35 and 50% buffer B. Finally, $N_2OR$-containing fractions were flash frozen and stored at -80ºC. SDS-PAGE was used to verify purity while the Bradford assay was used to evaluate the concentration (Supplementary Figure 2.2). All chromatography reagents were degassed and all manipulations were performed under argon.

### *2.4.7 Methyl viologen-linked assay of $N_2OR$*

The specific activity of $N_2OR$ in four independent transgenic plant lines (d35S: *nosZ*-6, 14, 16, 18) was studied. Leaf extracts were evaluated using the methyl viologen-linked assay described previously.[19] All reagents were degassed by vacuum and all manipulations were performed inside an anaerobic chamber maintained at 37ºC, 10% $H_2$, 5% $CO_2$ and 85% $N_2$. Briefly, 200 µL each of 10 mM reduced methyl viologen dye and 5 mM sodium dithionite were added to 100 mg portions of leaf extract in a cuvette. The absorbance was continuously monitored at a wavelength of 600 nm using a spectrophotometer. $N_2OR$ protein purified from *P. stutzeri* served as a positive control (5 µL, 0.956 µg/µL) and leaf extract from a non-transformed plant was used as a negative control. Once the background oxidation rate was stable and below 3.3 µmol $O_2$ reduced $min^{-1}$, 25 µL of 25 mM $N_2O$-saturated water was added and the absorbance was monitored at ten second intervals for another four minutes to obtain the total oxidation rate. Since methyl viologen ($\varepsilon$ = 11400 $M^{-1}$ $cm^{-1}$) oxidation is coupled to $N_2O$ reduction in a 2:1 molar ratio, the activity of $N_2OR$ was evaluated using the Beer-Lambert law (Supplementary Method 1), and the oxidation rate was corrected for background.[19]

## 2.5 Results

### *2.5.1 Transgenic tobacco plants expressing* nosZ

We generated 31 transgenic *Nicotiana tabacum* cv. Xanthi nc. plants expressing the *nosZ* gene from *P. stutzeri* under the transcriptional control of a constitutive promoter. The *extensin* signal sequence was used to direct enzyme transport into the extracellular matrix. The overall growth and developmental traits, such as plant height, leaf width, and flower size and color of transgenic plants were similar to those of non-transformed plants. $T_1$ plant lines were grown from the seeds of the first generation.

### *2.5.2 Molecular characterization of transgenic tobacco plants*

To determine the presence of the *nosZ* gene, $T_0$ transgenic tobacco plants regenerated under kanamycin selection were screened by PCR using sequence-specific primers. A 637 bp fragment of the recombinant DNA corresponding in size to the positive control was amplified from plant genomic DNA (Supplementary Figure 2.3). A negative control was included and no amplification of recombinant DNA from a non-transformed plant sample was observed. Through PCR screening, the transformation success rate was found to be over 96%.

Reverse transcription PCR analysis was used to assess expression of the mRNA. Six plant lines (d35S: *nosZ*-6, 14, 16, 18, 22 and 29) were selected. Total RNA was reverse transcribed using random primers and the full-length cDNA was PCR-amplified with primers

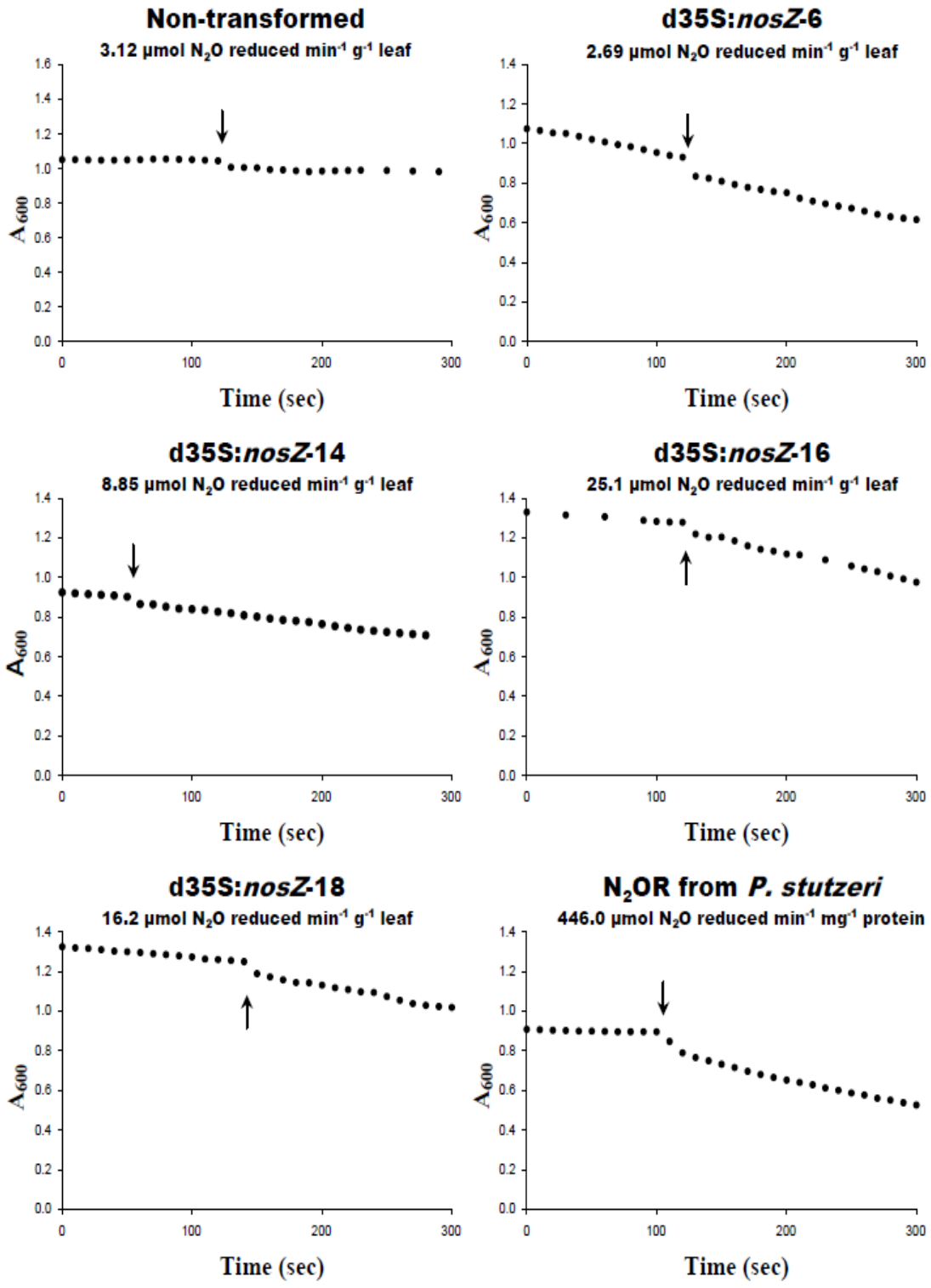
specific to the *extensin* signal sequence and *nosZ* (Supplementary Figure 2.4). The expected 1869 bp full-length cDNA corresponding in size to the positive control was detected in each of the leaf samples. Notable differences in transcript abundance were detected among the different transgenic lines and no observable product was amplified from the cDNA of the non-transformed plant.

Recombinant $N_2OR$ protein produced by five $T_0$ transgenic tobacco lines (d35S: *nosZ*-6, 14, 16, 18 and 29) was detected using SDS-PAGE followed by Western immunoblotting. A band corresponding to $N_2OR$ was detected at 72 kDa in the extract from *P. stutzeri*. Protein bands slightly larger in size, 81-84 kDa, were detected in the transgenic tobacco leaf extracts (Supplementary Figure 2.5). No similar protein was detected in the non-transformed plant extract. The small size difference of the recombinant protein among plant lines could be the result of differential patterns of glycosylation or varying signal peptide cleavage. Based on chemiluminescent signal strength, plants d35S: *nosZ*-16, 14, 6, and 18 (medium expresser) contained the highest observable levels of $N_2OR$ protein.

### *2.5.3 The activity of recombinant $N_2OR$*

The $N_2O$-reducing capacity of the recombinant $N_2OR$ expressed in $T_1$ *nosZ*-transgenic tobacco plants was assessed, using the standard methyl viologen-linked assay.[19] Crude protein extracted from non-transformed tobacco served as the negative control and purified $N_2OR$ from *P. stutzeri* was used as a positive control. Figure 2.1 shows absorbance trace plots for transgenic leaf extracts from d35S: *nosZ*-6, 14, 16, 18 alongside that from a non-

**Figure 2.1. Trace plots monitoring 600 nm absorbance as a function of time**. The arrows depict when $N_2O$-saturated water was added as enzyme substrate. The immediate sharp drop in absorbance is due to dilution of the reaction mixture in the cuvette. The change in slope after $N_2O$ addition was used to calculate the specific activities shown above each curve.


Non-transformed
3.12 µmol N2O reduced min-1 g-1 leaf
d35S:nosZ-6
2.69 µmol N2O reduced min-1 g-1 leaf
d35S:nosZ-14
8.85 µmol N2O reduced min-1 g-1 leaf
d35S:nosZ-16
25.1 µmol N2O reduced min-1 g-1 leaf
d35S:nosZ-18
16.2 µmol N2O reduced min-1 g-1 leaf
N2OR from P. stutzeri
446.0 µmol N2O reduced min-1 mg-1 protein
A600
Time (sec)

transformed control. The activity of $N_2OR$ was calculated using the Beer-Lambert law and the values are shown in Figure 2.1. In all cases, the background rate of oxidation was less than 3.3 μmol $O_2$ reduced $min^{-1}$ and was subtracted for specific activity calculations. The change in the slope before and after the addition of $N_2O$ reflects the oxidation of methyl viologen and indirectly the reduction of $N_2O$. The $N_2O$ reducing capability of the extracts was determined by comparing the magnitude of this change to that of the non-transformed control plant.

Of all transgenic plants tested, extracts from plant line d35S:*nosZ*-16 showed the highest $N_2OR$ activity with $N_2O$ reduction rates (25.1 μmol $O_2$ reduced $min^{-1}$ $g^{-1}$ leaf) more than eightfold higher than levels in the non-transformed plant (Figure 2.1). Similarly, extracts from plant d35S:*nosZ*-18 showed $N_2O$ reduction rates (16.2 μmol $O_2$ reduced $min^{-1}$ $g^{-1}$ leaf) more than five times greater. Finally, d35S:*nosZ*-14 extracts had $N_2O$-reducing capabilities (8.85 μmol $O_2$ reduced $min^{-1}$ $g^{-1}$ leaf) about 3 times greater than that of the non-transformed plant, while d35S:*nosZ*-6 showed lower activity than the negative control. Taken together, the d35S:*nosZ* plants showed $N_2O$-reducing patterns similar to that of the purified enzyme (positive control). Despite such leaf-to-leaf sample variability, the data confirms that extracts from plants expressing the transgene were actively oxidising the viologen dye.

## 2.6 Discussion

As theoretical and potential ways to mitigate greenhouse gas, Richardson *et al* have proposed heterologous expression of bacterial $N_2OR$ in plants as a means of mitigating $N_2O$ emissions.[5] But successful expression and assembly of the complex metalloenzyme $N_2OR$ in

plants is challenging. Although $N_2OR$ is encoded by the *nosZ* gene, the biosynthesis and assembly of the enzyme in bacteria seems to require the products of several *nos* genes, including a putative ABC-type transporter encoded by *nosDFY,* and the Cu chaperone NosL for biogenesis of the metal centre.[20] We suspected that the more sophisticated proteome of an eukaryotic plant cell could fulfill the need for an ABC-type transporter, a variety of cytochrome-based electron donor(s) as well as a copper chaperone(s), and therefore we set about to test whether such cryptic complementation could indeed activate $N_2OR$ in single-gene transformants. An analysis of various plant genomes reveals that they encode two to three times more ABC transporters than the genomes of bacteria and animals.[21] For example, the number of genes encoding ABC transporters in rice (153 genes) is more than twice that in *E. coli* (67 genes).[22] These observations together with the characteristics of plant cells including the presence of cytochrome *c* oxidase and the frequent expression of ABC transporters more strongly supports the viability of our hypothesis of cryptic complementation.

A second major challenge to achieving successful deployment of bacterial $N_2OR$ in transgenic plants involves the redox state *in planta*. It is widely held that the presence of oxygen has a fatal effect on the functionality of $N_2OR$.[20] It is, however, now known that the occurrence of highly reduced anoxic tissues (hypoxia) in plants is a more widespread phenomenon, one relatively ignored over the past decades of photosynthetic physiology.[23] Since plants actually lack efficient systems for oxygen delivery, the movement of oxygen through the plant tissues, driven by the diffusion gradient, does not satisfy the rate of oxygen consumption.[24] The oxygen in plant tissues may fall to low concentrations, even so low as to make the tissues highly anoxic. We therefore argued that since plant tissues have the

tendency to fall into anoxia, this could allow enzymes normally classified as anaerobic, like $N_2OR$, to function well enough to modify $N_2O$ flux in the field.

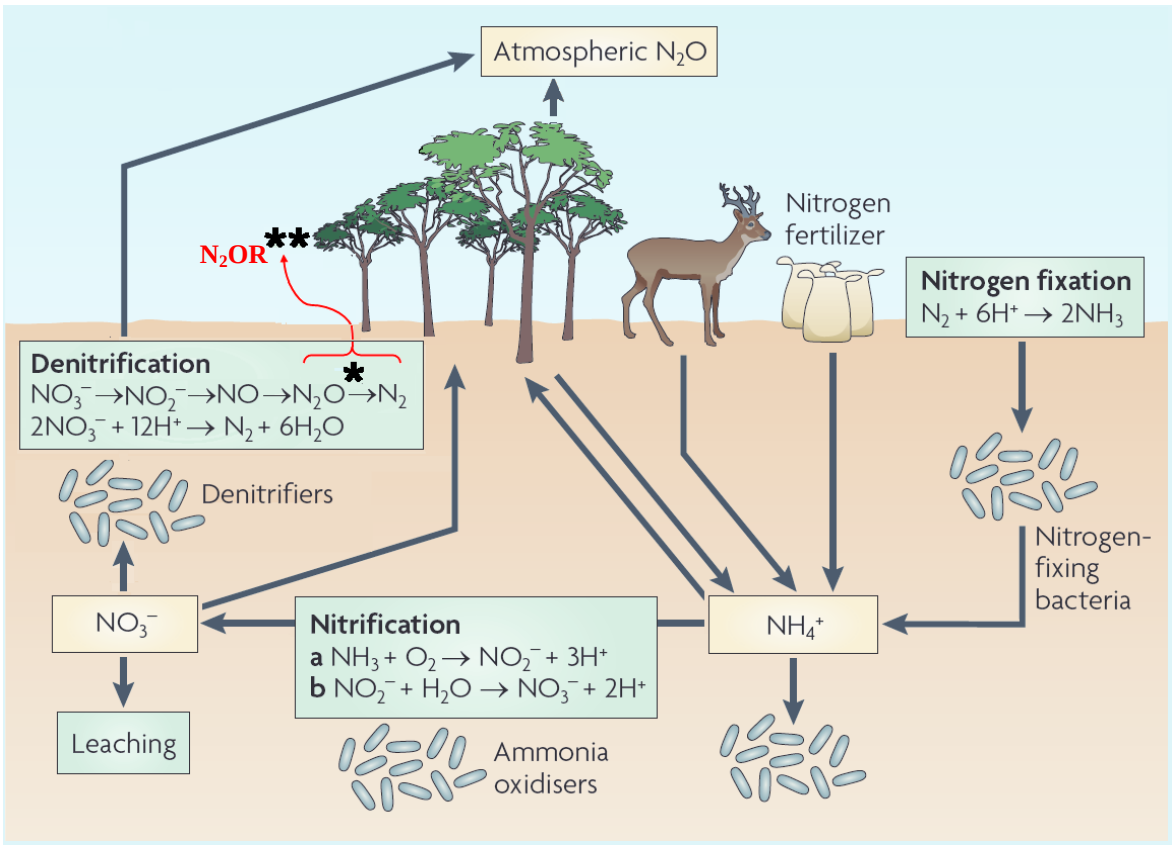
In the laboratory we were able to generate 31 transgenic tobacco plants expressing the *nosZ* gene, from soil bacterium *P. stutzeri*, under the transcriptional control of a constitutive promoter. The transgenic plant characteristics were similar to those of non-transformed plants except for the novel ability to reduce methyl viologen in the anaerobic chamber. Western blotting of the 31 plants showed that three in particular had strong protein bands, plants d35S: *nosZ*-16, 14 and 6 (Supplementary Figure 2.5). These were chosen for enzyme activity analysis along with a medium expresser, plant d35S: *nosZ*-18. The methyl viologen assay incorporated non-transgenic leaves as well as purified enzyme as negative/positive controls. All four independent transformation events (transgenic lines) showed unique activities for $N_2O$ reduction (Figure. 2.1). Enzyme activities varied as expected because of position effects from the independent T-DNA insertions sites into the host genomes.[25] The highest rate observed was from plant line d35S:*nosZ*-16, eightfold higher than background. These values were then used to extrapolate potential field mitigation effects (see below). Interestingly, the plant line that expressed a medium amount of foreign protein, d35S: *nosZ*-18 (Supplementary Figure 2.5) actually had the second highest enzyme activity (Figure. 2.1). These differences may indicate that in each plant line the micro-environment for copper insertion and/or anoxic state may be an additional factor(s) that cooperate with transgene position effect to impact net enzyme activity.

Having demonstrated $N_2O$-reducing capabilities in transgenic leaf extracts, we can conclude that properly synthesized $N_2OR$ apoprotein is amenable to copper atom insertion from the cytoplasm or apoplast of plant cells either spontaneously or through a plant-

mediated process. Although a previous study showed that heterologous expression of *nosZ* from *P. stutzeri* did not yield functional $N_2OR$ holoenzyme because of ineffective copper insertion[26], our study confirms recent evidence of $N_2OR$ activation from *Geobacillus thermodenitrificans* when expressed heterologously without the involvement of other Nos proteins.[27] Also, endogenous cytochrome(s) apparently complement the $N_2OR$ holoenzyme in plant cells in providing the electrons needed to drive the redox chemistry. Not only does the data support functional copper activation of $N_2OR$ *in planta,* but the data also show that $N_2OR$ was also free to function *in planta,* providing more evidence for the internal anoxia of plants.[24]

Our results are significant, as this is the first report of transgenic plants showing the potential of $N_2O$ bioremediation to our knowledge. We have demonstrated that transgenic plants containing $N_2OR$ may be capable of converting $N_2O$ into inert $N_2$. Expression of the prokaryotic *nosZ* gene in a eukaryotic cellular environment could endow plants with the capability of performing the final step of denitrification (Figure 2.2). Constitutive expression of bacterial $N_2OR$ in plant leaves is an innovative approach to remediation of atmospheric $N_2O$.[5] Augmenting the limiting nitrous oxide reduction pathway in fertilized soils could be further enhanced by tissue-specific expression. Transgenic plants expressing $N_2OR$ under the control of a root-specific promoter with a secretion signal could deliver $N_2OR$ into the rhizosphere and surrounding soil to prevent the release of $N_2O$ at the source. Transgenic crops like *Bacillus thuringiensis* (*Bt*) maize, and *Bt* potato are already known to deposit some of their transgene protein product like insecticidal crystal protein Cry1Ab into the soil substratum.[28] Genetically engineered crops, that produce Cry insecticidal crystal proteins from *Bacillus thuringiensis,* are known to release these proteins into soils via two routes, root

**Figure 2.2. The nitrogen cycle in soils**. Atmospheric $N_2O$ is produced and released as a by-product of microbial denitrification (adapted from Ref.10).


Atmospheric $N_2O$
$N_2OR$**
Nitrogen fertilizer
Nitrogen fixation
$N_2 + 6H^+ \rightarrow 2NH_3$
Denitrification
$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$
$2NO_3^- + 12H^+ \rightarrow N_2 + 6H_2O$
*
Denitrifiers
Nitrogen-fixing bacteria
$NO_3^-$
Nitrification
a $NH_3 + O_2 \rightarrow NO_2^- + 3H^+$
b $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+$
$NH_4^+$
Leaching
Ammonia oxidisers

exudates and upon decomposition of residues.[29] Although the fate of such recombinant proteins in the complex soil matrix is difficult to track, such prospects of applying transgenic strategies to reduce $N_2O$ emissions compelled us to initiate such studies on *in planta* expression of *nosZ*.

Transgenic plants are a new generation of genetically modified organisms with the capability to treat a polluted environment in an efficient and environment-friendly manner.[30, 31] Genetic engineering strategies have the potential of providing benefits to reduce greenhouse gas levels and radiative forcing. Commercial tobacco could be used to substantial effect since it occupies about 4 million hectares in the world.[32] Tobacco is easily transformed[33] and could be used as a full-scale test of $N_2O$ phytoremediation technology. Since $N_2OR$ is expressed in tobacco, the calculated annual conversion of $N_2O$ by genetically modified (GM) tobacco could be 28 tonnes $N_2O$/ hectare. If 4 million hectares of tobacco were to express $N_2OR$, then the annual conversion of $N_2O$ by tobacco alone would be about 112 million tonnes $N_2O$ (Supplementary Method 2), greater than the annual anthropogenic emissions of the gas in the atmosphere.

Recently, biofuel crops have undergone intensive global expansion, becoming the largest source of atmospheric $N_2O$ emissions.[34-36] Genetic engineering offers an innovative and promising approach to solve this problem, and the use of bioremediation in biofuel crops as a technology for the cleanup of $N_2O$ can be regarded as a sustainable, affordable strategy with even greater environmental benefits. Because regulatory approval would be much easier to obtain, we focus on non-grain crops not intended for human consumption. Crops grown for oil extraction, such as rapeseed, could be transformed without exposing human

populations to the transgenic protein product. Turf grass could be modified and marketed as an anti-global-warming seed.

Besides feed and ethanol corn, other crops could be similarly modified. For example, of the 90 million hectares of soybean grown globally in 2010, 81% or 73.3 million hectares were sown to biotech soybean. The dominant biotech commercial crop, herbicide tolerant soybean, occupied 50% of the global biotech crop area (148 million hectares).[37] If a $N_2O$-reducing trait was stacked in herbicide tolerant soybean, an annual conversion of $N_2O$ by GM soybean could be as high as 70000 million tonnes $N_2O$ (Supplementary Method 2). Given that 148 million hectares of arable crops already deploy bacterial proteins (e.g. the Cry protein from *Bacillus thuringiensis* in Bt plants and the *Agrobacterium*-derived 5-enol-pyruvyl-shikimate-3-phosphate synthase in Roundup Ready® crops)[37], gene-stacking this $N_2O$R capability in commercial crops is eminently feasible and may even lead to measureable reduction in atmospheric $N_2O$ levels.[38]

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## 2.9 Supporting Information

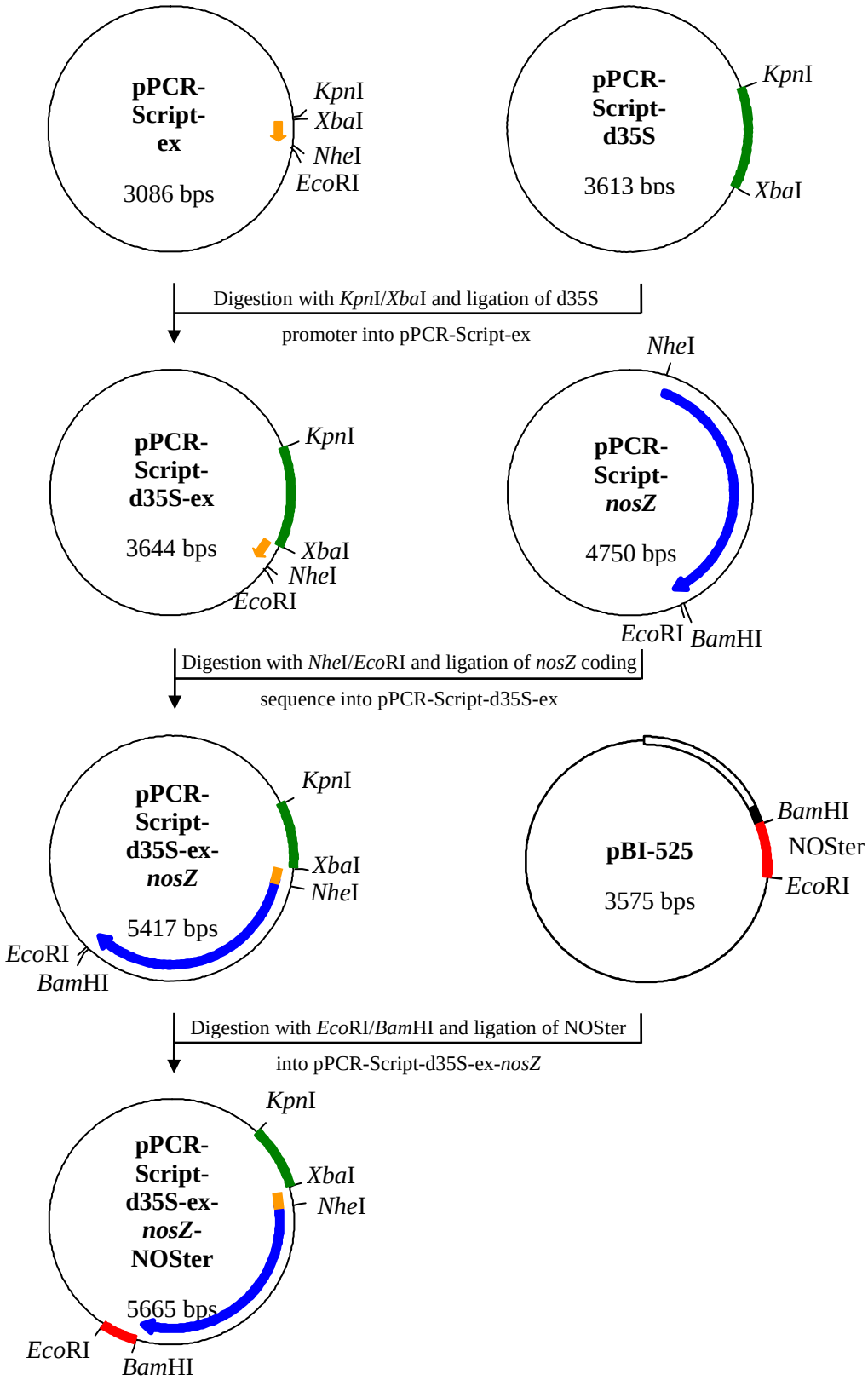
Additional supporting information on the PCR reaction conditions for amplification of expression construct components, the construction of the d35S-nosZ expression cassette, 10% SDS-PAGE analysis of $N_2$OR protein from different purification steps, molecular characterization of transgenic tobacco plants, $N_2$OR activity calculation, and the mitigation potential of $N_2$OR biotech crops is available free of charge via the Internet at http://pubs.acs.org.

**Supplementary 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 d35S promoter sequences.

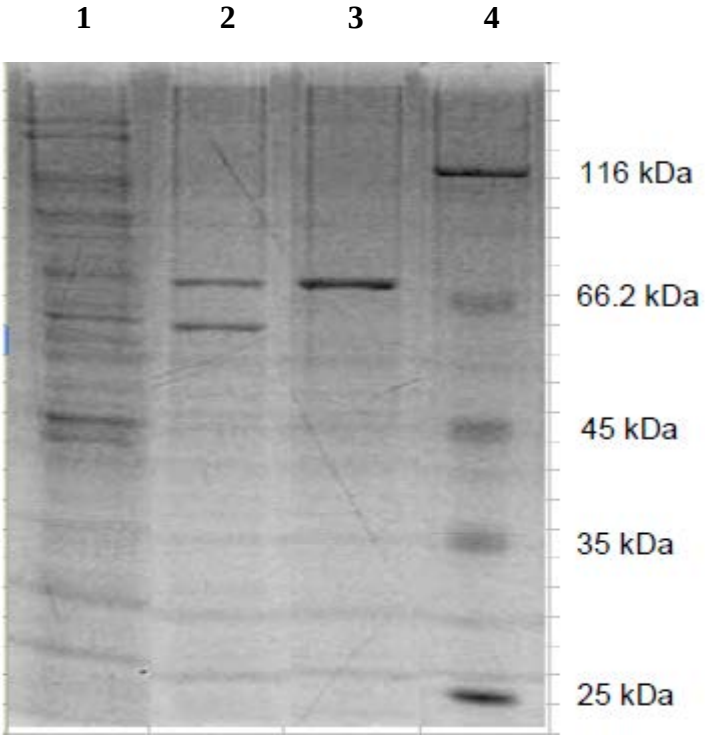
| Product | Primers | Reaction components | Cycle conditions |
|---|---|---|---|
| ***extensin* signal sequence** | exF<br>5' GGTACCTATCTAGA**ATG**GGAAGAATTGCTAGAGG<br>*Kpn*I *Xba*I<br>exR<br>5' GGAATTCATGCTAGCGGCTGTGGTTTCGGA<br>*Eco*RI *Nhe*I | 10 ng pHBV-CO DNA, 0.5 μM each primer, 1x *Pfu* buffer (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<br>5' GCGCTAGCCAGGCCGTCAAGGAGTCCAAG<br>*Nhe*I<br>nosZR<br>5' CGAATTCTTGGATCC**TTA**GGCCGGCTCGACCATCA<br>*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,<br>30c of 95°C for 1m, 58°C for 1m, 72°C for 2m and elongation at 72°C for 10m. |
| **d35S promoter sequence** | d35SF<br>5' CGGTACCCAAGCTTGCATGCCTG<br>*Kpn*I<br>d35SR2<br>5' GCTCTAGAATCCTCTCCAAATGAAATGA<br>*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.
Cycle conditions column: c = cycle, m = minute, s = second.
* DMSO was added to the reaction to facilitate denaturation of the high GC template.

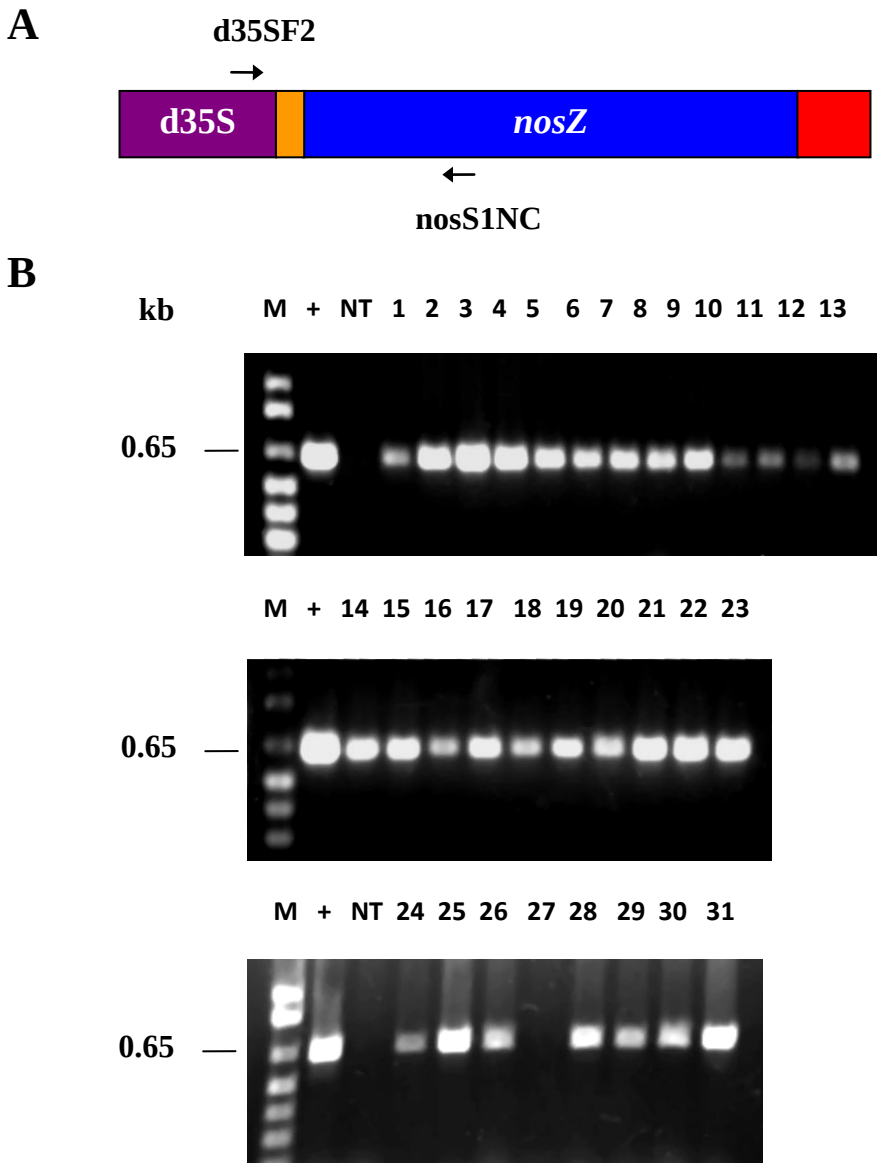
**Supplementary Figure 2.1. Construction of the d35S-*nosZ* expression cassette**. The d35S promoter sequence was subcloned from pPCR-Script-d35S into pPCR-Script-ex, containing the *extensin* signal sequence, using the *Kpn*I and *Xba*I restriction enzymes. The bacterial *nosZ* coding sequence lacking the bacterial signal sequence was then cloned into the resulting plasmid using the *Nhe*I and *Eco*RI restriction enzymes. Finally, the NOSter termination sequence was added using the *Eco*RI and *Bam*HI restriction enzymes.


pPCR-Script-ex
3086 bps
KpnI
XbaI
NheI
EcoRI
pPCR-Script-d35S
3613 bps
KpnI
XbaI
Digestion with KpnI/XbaI and ligation of d35S promoter into pPCR-Script-ex
pPCR-Script-d35S-ex
3644 bps
KpnI
XbaI
NheI
EcoRI
pPCR-Script-nosZ
4750 bps
NheI
EcoRI
BamHI
Digestion with NheI/EcoRI and ligation of nosZ coding sequence into pPCR-Script-d35S-ex
pPCR-Script-d35S-ex-nosZ
5417 bps
KpnI
XbaI
NheI
EcoRI
BamHI
pBI-525
3575 bps
BamHI
NOSter
EcoRI
Digestion with EcoRI/BamHI and ligation of NOSter into pPCR-Script-d35S-ex-nosZ
pPCR-Script-d35S-ex-nosZ-NOSter
5665 bps
KpnI
XbaI
NheI
EcoRI
BamHI

**Supplementary Figure 2.2. 10% SDS-PAGE analysis of $N_2OR$ protein from different purification steps**. Lane 1: crude clarified *P. stutzeri* lysate (1:50 dilution). Lane 2: pooled fractions from preparative anion exchange chromatography (1:50 dilution). Lane 3: pooled fractions from hydroxyapatite chromatography from half of the sample (1:25 dilution). Lane 4: marker proteins (Fermentas). Molecular weight of $N_2OR$ monomer from *P. stutzeri* is 72 kDa, and molecular weight of nitrite reductase from *P. stutzeri* is 61 kDa.

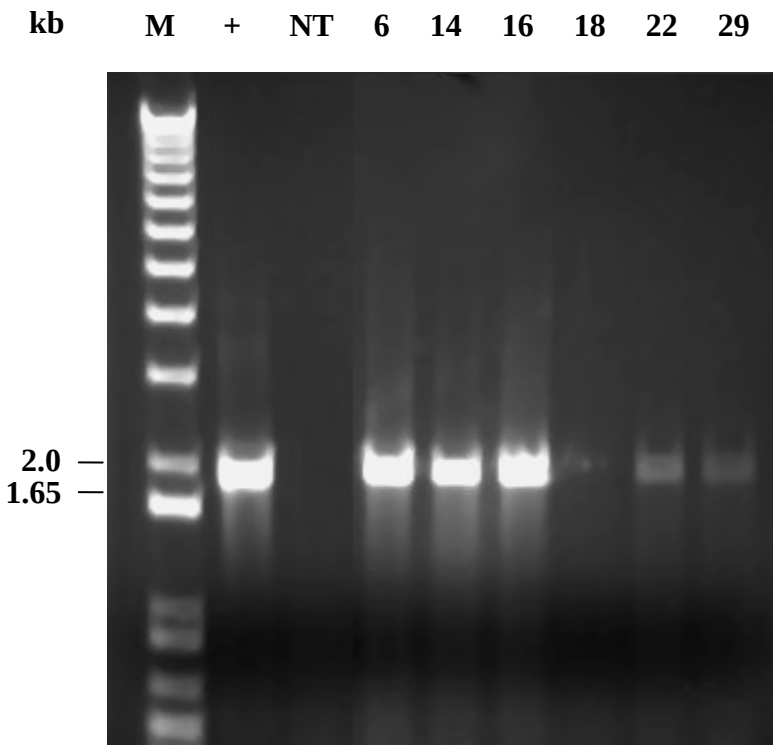

1
2
3
4
116 kDa
66.2 kDa
45 kDa
35 kDa
25 kDa

**Supplementary Figure 2.3. 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 marker 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.

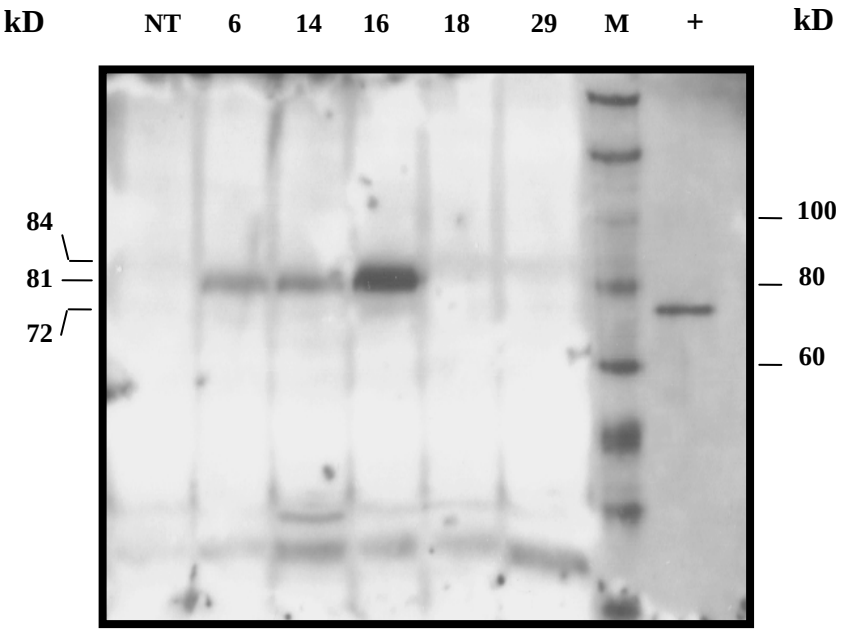

A
d35SF2
d35S
nosZ
nosS1NC
B
kb
M + NT 1 2 3 4 5 6 7 8 9 10 11 12 13
0.65
M + 14 15 16 17 18 19 20 21 22 23
0.65
M + NT 24 25 26 27 28 29 30 31
0.65

**Supplementary Figure 2.4. Expression of *nosZ* in $T_0$ d35S-*nosZ* transgenic tobacco**. RT-PCR detection of *nosZ* transcripts in leaves of transformed tobacco plants. 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 number.

.


kb
M
+
NT
6
14
16
18
22
29
2.0
1.65

**Supplementary Figure 2.5. Western immunoblot analysis of $N_2OR$ in $T_0$ leaf extracts**. Plant lines are identified by lanes 6, 14, 16, 18, and 29. NT: negative control, protein extract from a non-transformed plant; +: positive control, $N_2OR$ protein purified from *P. stutzeri*, expected size of the $N_2OR$ enzyme is 72 kDa; M: biotinylated protein ladder markers (Cell Signalling Technology Inc., MA, USA).


kD
NT
6
14
16
18
29
M
+
kD
84
81
72
100
80
60

**Supplementary Method 1.**

**$N_2$OR Activity Calculation.** The activity of $N_2$OR was evaluated using the Beer-Lambert law. The molar absorptivity (ε) of methyl viologen is 11400 $M^{-1}$ $cm^{-1}$ and the path length (ℓ) was 1 cm. A: absorbance; C: concentration; n is moles; and V is volume. By substitution and simplification, the following expression can be obtained for the rate of dye oxidised as a function of the rate of absorbance change.

$$A = \varepsilon L C$$

$$\frac{dA}{dt} = \varepsilon L \frac{dC}{dt}$$

$$\frac{dC}{dt} = \left(\frac{1}{\varepsilon L}\right)\frac{dA}{dt}$$

$$\frac{d\left(n/V\right)}{dt} = \left(\frac{1}{\left(11400\ \ M^{-1}cm^{-1}\right)\left(1\ \ cm\right)}\right)\frac{dA}{dt}$$

$$\frac{dn}{dt} = \left(\frac{mol}{11400}\right)\frac{dA}{dt}$$

$$\frac{dn}{dt} = \left(\frac{\mu mol}{0.0114}\right)\frac{dA}{dt}$$

The value used for the rate of absorbance change should be corrected for background. And since the stoichiometric ratio between methyl viologen and $N_2O$ reduced is 2:1, the activity per minute per gram of sample can be evaluated as follows where m is the mass of leaf extract or purified protein added:

$$Activity = \frac{\left(\frac{1\ \ \mu mol\ \ N_2O}{2\ \ \mu mol\ \ dye}\right)\left(\frac{\mu mol\ \ dye}{0.0114}\right)\left(dA/dt\right)}{m}$$

For example, if the background-corrected rate of absorbance change was $\left(dA/dt\right) = 0.0486 \times 10^{-4}\ \ min^{-1}$ for a purified protein sample of $m = 0.00478\ \ mg$, then the activity is calculated as follows:

$$Activity = \frac{\left(\frac{\mu mol\ \ N_2O}{0.0228}\right)\left(0.0486\ \ min^{-1}\right)}{0.00478\ \ mg\ \ protein} = 446\ \ \frac{\mu mol\ \ N_2O}{min\ mg\ \ protein}$$

**Supplementary Method 2.**

Mitigation potential of $N_2OR$ biotech crops: annual conversion of $N_2O$ if the $N_2OR$ trait were to be expressed in commercial crops.

**A: tobacco**

Using the $N_2OR$ activity of 8.85 μmol $N_2O$ reduced $min^{-1}$ gram $^{-1}$ in leaves (Fig.1),

and assuming leaf weight to be 0.5 tonne/ha[a],

active growth period to be 100 days/year,

total area of commercial global tobacco is 4 million hectares[b]; and

Given that tobacco already fluxes $CO_2$ and $O_2$ gases, then the amount of $N_2O$ could be:

= the activity of $N_2OR$ in the leaf × leaf weight × tobacco growth period × global hectarage

= 8.85 μmol $N_2O$ reduced $min^{-1}$ gram $^{-1}$ leaf × 0.5 tonne/ha × 24 hour/day × 100 days/year × $4x10^6$ ha

=$8.85x10^{-6}$ mol $N_2O$ reduced $min^{-1}$ gram $^{-1}$ leaf × 44 gram/mol × 0.5 tonne/ha × $1x10^6$ gram/tonne × 24 hour/day × 60 min/hour × 100 days/year × $4x10^6$ ha

= $1.12\times10^{14}$ g/year

=112 million tonnes $N_2O$/year

**B: soybean**

Assuming the $N_2OR$ activity of 8.85 μmol $N_2O$ reduced $min^{-1}$ gram $^{-1}$ in leaves,

leaf weight to be $1.42x10^7$ g/ha (Jagdish Kumar, Hendrick Seeds, personal communication),

active growth period to be 120 days/year,

total area of commercial biotech soybean is 73.3 million hectares[c], and

Given that tobacco already fluxes $CO_2$ and $O_2$ gases, then the amount of $N_2O$ could be:

= the activity of $N_2OR$ in the leaf × leaf weight × tobacco growth period × global hectarage

= 8.85 μmol $N_2O$ reduced $min^{-1}$ gram $^{-1}$ leaf × $1.42x10^7$ g/ha × 24 hour/day × 120 days/year × $73.3\ x10^6$ ha

=$8.85x10^{-6}$ mol $N_2O$ reduced $min^{-1}$ gram $^{-1}$ leaf × 44 gram/mol × $1.42x10^7$ g/ha × 24 hour/day × 60 min/hour × 120 days/year × $73.3\ x10^6$ ha

= $7.0\times10^{16}$ g/year

=70000 million tonnes $N_2O$ /year


[a] Andri, K.B.; Santosa, P.; Arifin, Z. An empirical study of supply chain and intensification program on Madura tobacco industry in East Java. *Int. J. Agr. Res*. **2011**, *6*, 58-66.

[b] Otañez, M.; Glantz, S.A. Social responsibility in tobacco production? Tobacco companies' use of green supply chains to obscure the real costs of tobacco farming. *Tob. Contro*. **2011**, doi:10.1136/tc.2010.039537.

[c] International Service for the Acquisition of Agri-Biotech Applications. ISAAA Brief 42-2010: Executive Summary. http://www.isaaa.org/resources/publications/briefs/42/executivesummary/default.asp

# Chapter 3

# Expression of the *nos* Operon Proteins from *Pseudomonas stutzeri* in Transgenic Plants to Assemble Nitrous Oxide Reductase

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(*Transgenic Research*, In Press, DOI: 10.1007/s11248-011-9555-1)

## 3.1 Author contributions

Shen Wan, author of the current Ph.D. Thesis was the primary author who wrote and revised the manuscript with the assistance of her supervisor (Dr. Illimar Altosaar). S.W. also designed and directed all of the experimental work, and performed the experimental work with the assistance of Y. Mottiar, a former undergraduate honours project student. K. Goto and A. Johnson, also former undergraduate honours project students, assisted with the methyl viologen-linked enzyme assay.

## 3.2 Abstract

Nitrous oxide ($N_2O$) is a stable greenhouse gas that plays a significant role in the destruction of the ozone layer. Soils are a significant source of atmospheric $N_2O$. It is important to explore some innovative and effective biology-based strategies for $N_2O$ mitigation. The enzyme nitrous oxide reductase ($N_2OR$), naturally found in soil bacteria, is responsible for catalysing the final step of the denitrification pathway, conversion of $N_2O$ to dinitrogen gas ($N_2$). To transfer this catalytic pathway from soil into plants and amplify the abundance of this essential mechanism (to reduce global warming), a mega-cassette of five coding sequences was assembled to produce transgenic plants heterologously expressing the bacterial *nos* operon in plant leaves. Both the single-gene transformants (*nosZ*) and the multi-gene transformants (*nosFLZDY*) produced active recombinant $N_2OR$. Enzymatic activity was detected using the methyl viologen-linked enzyme assay, showing that extracts from both types of transgenic plants exhibited $N_2O$-reducing activity. Remarkably, the single-gene strategy produced higher reductase capability than the whole-operon approach. The data indicate that bacterial $N_2OR$ expressed in plants could convert $N_2O$ into inert $N_2$ without involvement of other Nos proteins. Silencing by homologous signal sequences, or cryptic intracellular targeting are possible explanations for the low activities obtained. Expressing $N_2OR$ from *Pseudomonas stutzeri* in single-gene transgenic plants indicated that such ag-biotech solutions to climate change have the potential to be easily incorporated into existing genetically modified organism seed germplasm.

## 3.3 Introduction

Nitrous oxide ($N_2O$) is the third most abundant greenhouse gas after carbon dioxide ($CO_2$) and methane. $N_2O$ plays a significant role in global warming and the destruction of the ozone layer. The warming potential of $N_2O$ is 310 times higher than that of $CO_2$ and its atmospheric lifetime is 114 years (Ravishankara et al. 2009). Due to the anthropogenic perturbation of the global nitrogen cycle, atmospheric concentrations of $N_2O$ have increased from 270 ppb in 1750 to 319 ppb in 2005 and levels have been increasing linearly (about 0.26% $yr^{-1}$) for the past few decades (Forster et al. 2007; Wolf et al. 2010).

The increases in arable land area and the use of synthetic fertilizers have been shown to be the primary drivers for the increase in tropospheric $N_2O$ (Del Grosso 2010). Half of anthropogenic $N_2O$ comes from synthetic fertilizers applied to agricultural soils (Reay et al. 2007; Sutton et al. 2011). In the year 2000, agriculture contributed 13.5% of total global $CO_2$ equivalents, 6% of which were in the form of $N_2O$ derived from agricultural soils. The consumption of synthetic nitrogen has dramatically escalated in recent years, placing current $N_2O$ emissions from this soil–plant–microbial system at 2.6 million metric tons annually (Davidson 2009). $N_2O$ emissions are predicted to continue to increase in the coming decades due to the projected increased use of fertilizer and the aggregate growth of agriculture (Foresight 2011). Furthermore, the ever-increasing world population will swell the demand for food crops even further in the future and a corresponding increase in agricultural greenhouse gas emissions is anticipated.

In soil, there are several enzymatic and microbial routes that produce $N_2O$. Biological denitrification, the reduction of $NO_3^-$ to dinitrogen gas ($N_2$) via four steps, is the major source of $N_2O$ emissions. A fraction of $N_2O$ emissions is a by-product of $NH_3$

oxidation (Wrage et al. 2001). It was shown that $NO_3^-$ assimilated by plants is also lost as $N_2O$ emissions during the process of $NO_3^-$ assimilation in leaves (Smart and Bloom 2001). $N_2O$ emissions from bacterial processes arise largely from a failure of the final step in denitrification, the reduction of $N_2O$ to $N_2$. Therefore, enhancing the conversion of $N_2O$ to $N_2$ could alleviate global warming because $N_2$ is inert and poses no risk to the environment.

In bacteria, $N_2O$ is reduced to inert $N_2$ in the final step of denitrification by nitrous oxide reductase ($N_2OR$), which is the only known enzyme capable of catalysing the reduction of $N_2O$ to $N_2$ (Pomowski et al. 2010; Richardson et al. 2009). Since its initial isolation from *Pseudomonas stutzeri*, $N_2OR$ has been identified in several other microorganisms, including heterotrophic, phototrophic, and chemolithotrophic Gram-negative denitrifying bacteria and Gram-positive denitrifying bacteria (Suharti and de Vries 2005; Zumft1997; Zumft and Matsubara 1982). Structural analysis has revealed that $N_2OR$ is a homodimeric protein of approximately 130 kDa with each subunit containing two different multi-copper centres. The binuclear copper centre ($Cu_A$) is an electron transfer site similar to the cytochrome *c* oxidase electron transfer site. The tetranuclear copper centre ($Cu_Z$) is the catalytic site for the two-electron reduction of $N_2O$ to $N_2$. With a unique structural feature in biological systems, the mixed-valence copper centre ($Cu_4S$) bridges a distorted tetrahedron of copper atoms with a sulfide ion. This novel $Cu_4S$ cluster is liganded by seven conserved histidine residues and a water-derived oxygen atom which is proposed to bridge two of the copper atoms where the substrate, $N_2O$, binds to the enzyme (Dell'Acqua et al. 2010). $N_2OR$ is encoded within the *nos* gene cluster, which contains sequentially encoded components, *nosRZDFYL*. The role of the proteins encoded by the *nos* operon in *P. stutzeri* has been determined. *NosZ* is the structural gene of $N_2OR$ and it is transcribed with the upstream transcriptional regulator NosR. *NosDFY* putatively encode an ATP-binding ABC transporter

system for the biogenesis of the catalytic centers, while NosL functions as a copper chaperone (Zumft and Kroneck 2007). Preliminary studies showed that the functional assembly of $Cu_Z$ is lacking when only *nosZ* is expressed in the non-denitrifiers *Escherichia coli* and *Pseudomonas putida* (Wunsch et al. 2003). Co-expression of *nosZ* with *nosD, nosF* and *nosY* is, however, sufficient for the production of the catalytically active holoenzyme in *P. putida* (Wunsch et al. 2003). Recently, Liu et al. (2008) have expressed the *nosZ* gene alone from *Geobacillus thermodenitrificans* in *Escherichia coli*, producing active, albeit copper-deficient, recombinant $N_2OR$.

In order to reduce $N_2O$ in the atmosphere, we have generated transgenic *Nicotiana tabacum* expressing bacterial $N_2OR$. Tobacco plants were engineered to express the *nosZ* gene, either alone or co-expressed with the other *nos* genes. In this study we demonstrated that extracts from transgenic plants expressing bacterial $N_2OR$ catalyse the conversion of $N_2O$ to $N_2$, presenting a system with the potential to significantly reduce $N_2O$ release into the environment.

## 3.4 Materials and Methods

### *3.4.1 Isolation of genomic DNA from* P. stutzeri

Single colonies of *P. stutzeri ZoBell* (ATCC 14405) were obtained 48 h after plating on LB-agar medium at 30ºC. A 5 ml culture of liquid LB medium was inoculated and incubated overnight with shaking at 30ºC and was then scaled up to 100 ml and incubated for another 3 h. Genomic DNA was obtained using a modified version of the protocol of Neumann et al. (1992) for Gram-negative bacteria. Briefly, the cells were first pelleted by

centrifugation at 3000 g and 4°C for 15 min, washed in PBS buffer and then resuspended in 5 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH7.5). Upon addition of lysozyme to a concentration of 1 mg $ml^{-1}$, the suspension was incubated at 37ºC for 30 min with occasional gentle mixing by inversion. After lysis, 0.5 ml of 10% SDS and 0.5 ml of proteinase K (1 mg $ml^{-1}$) was added and a 2-h incubation at 55ºC with occasional inversion followed. To salt out proteins, 2.5 ml of 6 M NaCl was added and the tube was gently mixed. Phase separation was achieved by the addition of 10 ml of chloroform followed by gentle mixing for 1 h and centrifugation at 3000 g for 10 min. The aqueous upper phase was transferred to a fresh tube using a blunt-ended pipette tip and the DNA was precipitated by the addition of 20 ml of isopropanol followed by gentle inversion and 15 min incubation at -20ºC. The DNA was spooled and transferred to a 1.5 ml tube, rinsed twice with 70% ethanol, dried lightly in a centrifugal evaporator and finally dissolved in 500 µl of deionised water.

### *3.4.2 Anaerobic purification of nitrous oxide reductase from* P. stutzeri

A single colony of *P. stutzeri* was used to inoculate 200 ml of synthetic medium (Matsubara et al. 1982), which was incubated for 24 h at 30ºC with gentle shaking before being used to inoculate 1.8 L of the same medium. After 6 h of growth, $NaNO_3$ was added to a concentration of 1g $L^{-1}$ to induce $N_2$OR expression. After an additional 24 h, a total of 12 L of culture was pelleted by centrifugation and washed with 50 mM $MgCl_2$ in 25 mM Tris buffer (pH7.5) resulting in a 30 g pellet which was purged with argon prior to storing at -80ºC. The pellet was resuspended in 25 mM Tris (pH7.5) buffer, disrupted by sonication and the supernatant was then used for ion-exchange chromatography on a DEAE Sepharose column. Protein elution was performed at a flowrate of 2 ml $min^{-1}$ using 25 mM Tris pH7.5

(buffer A) and 25 mM Tris pH7.5, 0.3 M NaCl (buffer B) at 4ºC over five column volumes with a linear gradient of 0 to 100% for buffer B. Fractions of 5 ml were purged with argon and stored at 4ºC. SDS-PAGE was performed to identify fractions containing $N_2OR$ and nitrite reductase which were pooled and dialysed using a 14 kDa cutoff membrane. Hydroxyapatite chromatography was performed on the pooled fractions using 10 mM and 400 mM $NaH_2PO_4$ buffers, pH 7.2, with a 0-80% linear gradient of the buffer B with 10 column volumes. Active $N_2OR$ protein eluted as a single peak and was then flash frozen and stored at -80ºC. SDS-PAGE was used to assess purity and the Bradford assay was used to evaluate protein concentration (Bradford 1976). All chromatography reagents were degassed and all manipulations were performed under argon.

### *3.4.3 Construction of plant transformation vectors*

Binary expression vectors carrying *nosZ* or *nosFLZDY* were constructed using standard molecular cloning techniques. The *nosZ* gene and other *nos* genes were PCR-amplified from the genomic DNA of *P. stutzeri* using gene-specific primers (Supplementary Table 3.1). Primers used for PCR amplification were designed to introduce restriction enzyme sites allowing for the fusion of segments. The d35S promoter was amplified for each *nos* expression cassette from pCAMBIA1305.1 (CAMBIA, Canberra, Australia). The *extensin* signal sequence (*ex*) was amplified for *nosZ* and *nosD* expression from the pHBV-CO plasmid using forward primer, ExSigZF/ExSigDF, and reverse primer, ExSigZR/ExSigDR (Alli et al. 2002). The alpha amylase inhibitor-1 signal sequence from *Phaseolus vulgaris* (*Pv*) was amplified to regulate *nosY* and *nosL* expression using forward primer, PvSigF, and reverse primer, PvSigR. The nopaline synthase polyadenylation

sequence (NOSter) was amplified for use in each *nos* expression cassette. PCR products were purified using the QIAEX II Gel Extraction Kit (Qiagen, Mississauga, ON, Canada) and cloned using the pPCR-Script Amp SK (+) cloning vector system (Stratagene, La Jolla, CA, USA). Following ligation of the NOSter**,** *nosZ*, *ex* and d35S segments, the resulting sequence was digested with *Kpn*I and *Bam*HI and cloned into the binary vector pRD400 (Datla et al. 1992) (Supplementary Figure 3.1), which carries the neomycin phosphotransferase II (*nptII*) gene for kanamycin resistance as the plant selectable marker. The resulting plasmid pd35S-*nosZ* was verified by DNA sequence analysis (StemCore Laboratories, Ottawa, Canada). Additionally, plant expression constructs were generated for the remaining *nos genes (nosF, nosD, nosY and nosL)* which were used to construct a megacassette pd35S-*nosFLZDY* (Supplementary Figure 3.2).

### *3.4.4 Plant transformation and selection*

The pd35S-*nosZ* and pd35S-*nosFLZDY* plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 strain used to transform tobacco (*Nicotiana tabacum* cv. Xanthi). Mature seeds of *N. tabacum* were first sterilised by submersion in 70% ethanol for 1 min and subsequently in 10% bleach with 2 drops of Tween 20 for 10 min. After rinsing 3 times with sterile deionised water, the seeds were placed in culture vessels containing germination medium (1/2 Murashige and Skoog medium (MS), 3% sucrose, 0.8% agar, pH5.8). Six-week old tobacco leaves were used for *Agrobacterium*-mediated transformation following the method of Horsch et al. (1985) with a slight modification (Blais and Altosaar 2006; Cheng et al. 1998). The young leaf sections were infected with *A. tumefaciens* harbouring either pd35S-*nosZ* or pd35S-*nosFLZDY* plasmid on co-cultivation

medium (MS, 3% sucrose, 0.8% agar, 1.0 mg $L^{-1}$ 6- benzyladenine, 0.l mg $L^{-1}$ α-naphthalene acetic acid, pH5.8). After 2 days, the leaf discs were aseptically transferred to regeneration medium (MS, 3% sucrose, 0.8% agar, 1.0 mg $L^{-1}$ 6- benzyladenine, 0.l mg $L^{-1}$ α-naphthalene acetic acid, pH 5.8, 200 mg $L^{-1}$ of ticarcillin and 300 mg $L^{-1}$ of kanamycin). After 2-3 weeks, the calli from the transformed tobacco cells began to appear on the explants. After roughly 4 weeks, shoots grew from the edge of explants. When the shoots were 1cm in height, the shoots were cut off and transferred to root generating medium (MS medium, 2% sucrose, 0.8% agar, pH 5.8, 200 mg $L^{-1}$ of ticarcillin, 300 mg $L^{-1}$ of kanamycin). Once rooted, the transgenic plantlets were subsequently transferred to potting soil and grown in a greenhouse with 16 h daylight at 400W/$m^2$, 25ºC and 8 h dark, 21ºC and maintained through seed set for further analysis.

### *3.4.5 Detection of the* nosZ *gene in transgenic plants*

PCR analysis was performed to screen for the presence of the transgene. Genomic DNA was isolated from 100 mg young tobacco leaves using the Plant DNeasy Mini kit according to the manufacturer's instructions (Qiagen, Mississauga, ON, Canada). A non-transformed (NT) plant was used as negative control, while the plasmids pd35S-*nosZ* and pd35S-*nosFLZDY* were used as positive controls. For pd35S-*nosZ* transgenic plants, the primers used to detect the *nosZ* gene were as follows: forward primer (ExSigZF) and reverse primer (NosZR). For pd35S-*nosFLZDY* transgenic plants, the primers used to detect the *nosZ* gene were as follows: forward primer (NosZF) and reverse primer (NosZR). PCR was performed in a 25 μl reaction mixture containing 50 ng of genomic DNA, 25 pmol of each

primer, 2.5 µl of 10× PCR buffer (with 1.5 mM $MgCl_2$), 250 µM of each dNTP, 0.5 unit Taq DNA polymerase, with the following cycling condition: initial denaturation at 94°C for 4 min; 30 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 1min, and elongation at 72°C for 2 min; followed by a final elongation at 72°C for 10 min.

### *3.4.6 Detection of the* nosZ *mRNA in transgenic plants*

RT-PCR analysis was performed to analyze the *nosZ* expression in transgenic tobacco plants. Total RNA was extracted from 100 mg of young leaf tissue using the RNeasy Plant Mini kit according to the manufacturer's instructions (Qiagen, Mississauga, ON, Canada). RNA samples were treated with RNase-free DNase (Promega, Madison, WI, USA) to remove contaminating DNA prior to RT-PCR. RT-PCR was performed using the SuperScript II Reverse Transcriptase system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). During the first-strand cDNA synthesis, 250 ng of total RNA was reverse-transcribed at 70°C for 10 min with an oligo (dT) primer in 10 µl reaction mixtures. The synthesis of cDNA was performed for 10 min at 25°C, 60 min at 37°C, and 5 min at 95°C. From the first-strand cDNA reaction, aliquots of 2 µl cDNA were used as template for each PCR mix using *nosZ*-specific primers. For pd35S-*nosZ* transgenic plants, the primers used to detect the *nosZ* gene transcript were as follows: forward primer (ExSigZF) and reverse primer (NosZR). For pd35S-*nosFLZDY* transgenic plants, the primers used to detect the *nosZ* gene were: forward primer (NosZF) and reverse primer (NosZR). PCR was also conducted with total RNA not treated with reverse transcriptase to verify that there was no DNA contamination. The other *nos* gene transcript (*nosF*, *nosL*, *nosD*, *nosY*) were also detected by gel analysis.

### *3.4.7 Protein expression analysis*

To detect the expression of the *nosZ* gene in transgenic plants, Western blot analysis was performed using our previously published protocol (Panahi et al. 2004). Soluble protein was extracted from fresh young leaves of transgenic tobacco using the Plant Total Protein Extraction kit (Sigma, St. Louis, MO, USA). Total protein was quantified using the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA). Protein extracts (40 μg) were heated at 95°C for 10 min in 2x protein sample buffer (0.1 M TrisCl pH6.8, 1 mM EDTA, 6% SDS, 20% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol) and then loaded onto a 10 % acrylamide gel with a 5% stacking gel for subsequent immunoblot analysis**.** The purified $N_2$OR isolated from *P. stutzeri* was used as a positive control. Proteins were transferred onto a nitrocellulose membrane using the Trans-Blot transfer cell (Bio-Rad, CA, USA). After blocking with TBST including 5% skim milk, the membrane was incubated with rabbit anti-$N_2$OR serum (1:5000, provided by W. Zumft), followed by anti-rabbit biotin horseradish peroxidase linked antibody at a concentration of 1:1000 in TBST. Proteins were detected using the ECL western blotting chemiluminescent reagents (Amersham Biosciences, Baie d'Urfé, QC). Image analysis of the Western blots was conducted using a Kodak Imaging Station 2000R (Kodak, Rochester, NY, USA).

### *3.4.8 Reductase activity assay with methyl viologen*

Enzyme activity was measured using a modified version of the methyl viologen-linked assay (Kristjansson and Hollocher 1980). The activity of $N_2$OR was determined

spectrophotometrically by monitoring the oxidation of the chemical electron donor, reduced methyl viologen. All reagents were degassed by vacuum and flushed extensively with high-purity argon. The manipulations were performed inside an anaerobic chamber (37ºC, 10% $H_2$, 5% $CO_2$ and 85% $N_2$). To measure the activity of $N_2$OR in transgenic leaf samples, 50 µl of leaf extract was added to the reaction mixture containing 1.5 ml of 10 mM $KH_2PO_4$, pH 7.1, 0.2 ml of 10 mM methyl viologen, and 0.2 ml of 5 mM sodium dithionite in a stoppered 3.5 ml cuvette (light-path length, 10 mm). For the purified $N_2$OR which served as a positive control, 5 µl of protein (0.956 µg/µl) was added to the reaction mixture containing 2 ml of 10 mM $KH_2PO_4$, 0.05 ml of 10 mM methyl viologen, and 0.05 ml of 5 mM sodium dithionite in a 3.5 ml stoppered cuvette. Upon addition of purified enzyme or leaf extract, the cuvette contents were quickly mixed before addition of substrates. The absorbance at 600 nm was followed for 1 min to establish the small background oxidation rate before initiating the reaction by injecting 25 µl of $N_2O$-saturated water. The enzymatic activity was determined by monitoring the decrease in the absorbance at 600 nm at 10 sec intervals for 5 min. The specific activity of $N_2$OR was expressed as µmol of $N_2O$ reduced per min per mg of $N_2$OR protein.

## 3.5 Results

### *3.5.1 Development of transgenic tobacco plants expressing* nosZ

To facilitate improved processing and extracellular secretion of the $N_2$OR protein *in planta*, the 150-nucleotide endogenous signal sequence for *nosZ*, which directs peptide transport to the bacterial periplasm in *P. stutzeri*, was substituted with the leader sequence of

the *Daucus carota* (carrot) *extensin* gene, which directs the gene product to the plant's extracellular matrix (Chen and Varner 1985)**.** In constructing the pd35S-*nosFLZDY* synthetic genes, the 150-nucleotide endogenous signal sequence for *nosZ* and the 81 bp endogenous signal sequence for *nosD* were replaced with the *extensin* leader sequence. Similarly, 69 bp and 102 bp endogenous signal sequences of *nosL* and *nosY* were substituted with the 63-bp *Phaseolus vulgaris* alpha amylase inhibitor-1 signal sequence (Prescott et al. 2005). The binary vectors pd35S-*nosZ* and pd35S-*nosFLZDY* were engineered to express the *nos* construct under the control of the constitutive cauliflower mosaic virus d35S promoter (Kay et al. 1987) and the NOS polyadenylation terminator. Both binary vectors were constructed using the vector pRD400, which carries the neomycin phosphotransferase II gene for kanamycin resistance as the plant selectable marker. Following transformation of tobacco with the pd35S-*nosZ* and pd35S-*nosFLZDY* constructs, 14 putative pd35S-*nosZ* transformed tobacco lines and 24 putative pd35S-*nosFLZDY* transformed lines regenerated on kanamycin selection medium were recovered, successfully rooted and transferred to soil. The kanamycin-resistant transgenic tobacco lines were designated d35S-*nosZ*1w to d35S-*nosZ*14w, and d35S-*nosFLZDY*1 to d35S-*nosFLZDY*24. The overall growth and developmental traits, such as plant height, leaf broadness, flower size and colour of transgenic plants were similar to those of non-transformed plants.

### *3.5.2 Screening for transgenic plants by PCR*

To determine the presence of the *nosZ* gene, transgenic tobacco plants regenerated on kanamycin selection medium were screened by PCR using sequence-specific primers. Non-transformed plants were used as negative controls, while the plasmids pd35S-*nosZ* and

pd35S-*nosFLZDY* were used as positive controls. PCR analysis showed the presence of an 1869-bp band for the putative pd35S-*nosZ* transformed lines, corresponding to the length of the ex-*nosZ* coding sequence (Figure 3.1, a). For putative pd35S-*nosFLZDY* transformed lines, PCR analysis was performed to amplify a *nosZ* fragment (1767 bp) (Figure 3.1, b) and fragments for *nosL*, *nosF*, *nosD* and *nosY*, respectively (data not shown). PCR analysis results confirmed the presence of *nosZ* in all transgenic lines, whereas no amplification was detected in non-transformed lines.

### *3.5.3 Expression of the* nosZ *transgene in transgenic plants*

To confirm the transcription of the *nosZ* transgene under the control of the d35S promoter, RT-PCR was conducted to amplify the *ex-nosZ* fragment for pd35S-*nosZ* transgenic plants or *nosZ* fragment for the pd35S-*nosFLZDY* transgenic plants with gene-specific primer sets. RT-PCR products with the expected size of 1869 bp for *nosZ*-transformed plants and 1767 bp for *nosFLZDY*-transformed plants were observed in all transgenic lines (Figure 3.2). Similarly, correctly-sized RT-PCR fragments for *nosL*, *nosF*, *nosD* and *nosY*, were also produced from the pd35S-*nosFLZDY* transgenic lines (data not shown). NT plants did not exhibit any bands corresponding to the *nos*Z transcripts. All transgenic lines revealed the presence of the *nos*Z transcript. No notable differences in transcript abundance were detected among the *nosZ*-transgenic lines, while clear differences were observed among *nosFLZDY*-transgenic lines. Furthermore, the *nosZ* transcript abundance was much higher in *nosZ*-transgenic plants than in *nosFLZDY*-transgenic plants.

**Figure 3.1. Identification of d35S-*nosZ* and d35S-*nosFLZDY* transgenic tobacco by PCR screening**. **a** PCR amplification of a 1869 bp fragment of the *ex-nosZ* coding sequence in d35S-*nosZ* transgenic tobacco plants. **b** PCR amplification of a 1767 bp fragment of the *nosZ* coding sequence in d35S-*nosFLZDY* transgenic tobacco plants. 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.

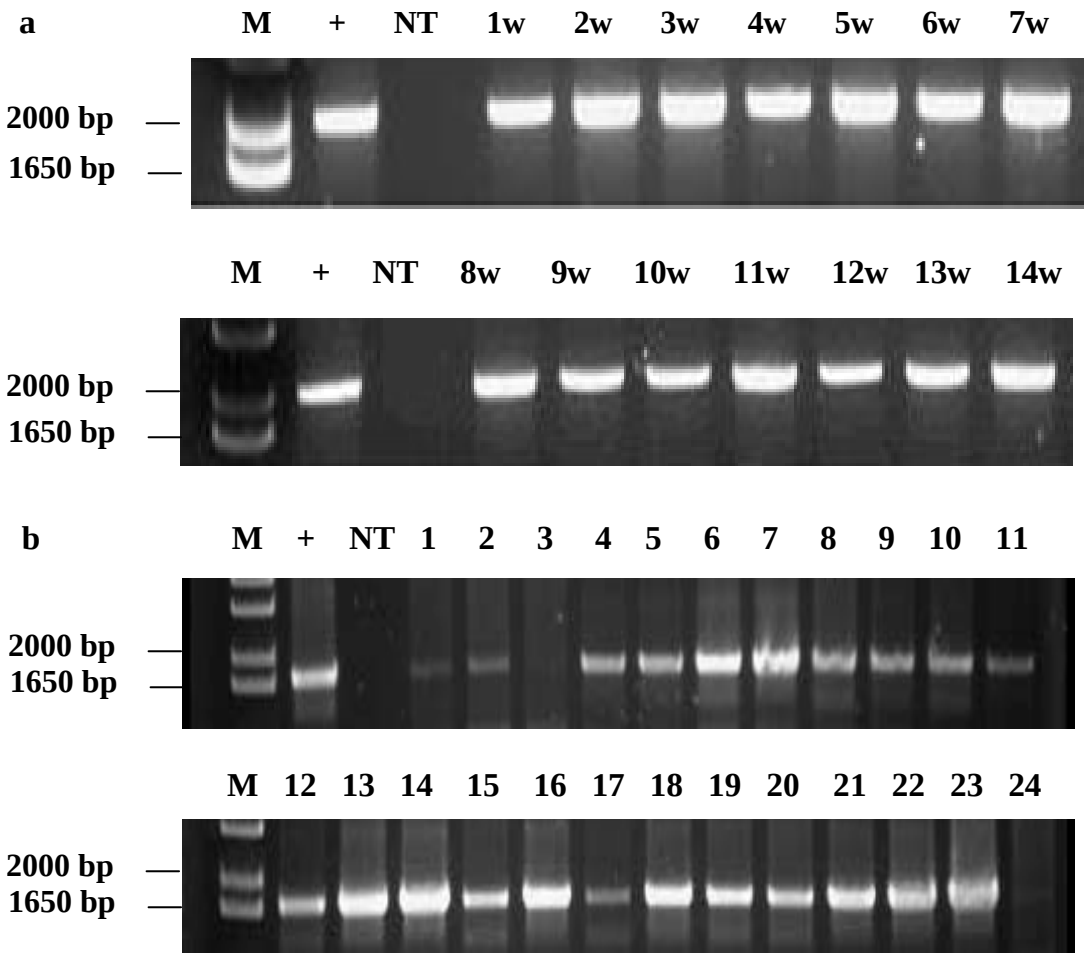
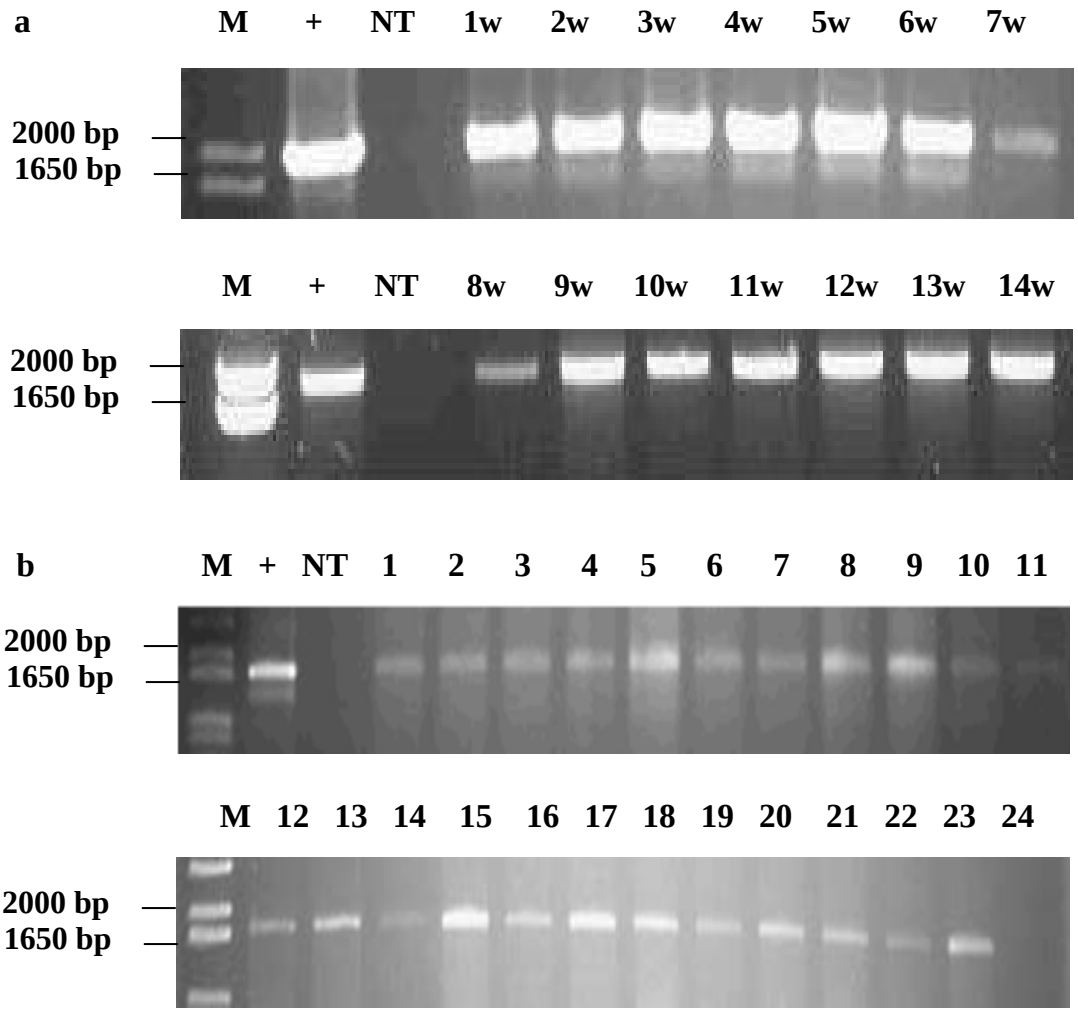

a
M + NT 1w 2w 3w 4w 5w 6w 7w
2000 bp
1650 bp
M + NT 8w 9w 10w 11w 12w 13w 14w
2000 bp
1650 bp
b
M + NT 1 2 3 4 5 6 7 8 9 10 11
2000 bp
1650 bp
M 12 13 14 15 16 17 18 19 20 21 22 23 24
2000 bp
1650 bp

**Figure 3.2. Expression of *nosZ* in d35S-*nosZ* and d35S-*nosFLZDY* transgenic tobacco plants. a** RT-PCR detection of *ex-nosZ* transcripts in leaves of d35S-*nosZ* transgenic tobacco plants. **b** RT-PCR detection of *nosZ* transcripts in leaves of d35S-*nosFLZDY* transgenic tobacco plants. 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
M + NT 1w 2w 3w 4w 5w 6w 7w
2000 bp
1650 bp
M + NT 8w 9w 10w 11w 12w 13w 14w
2000 bp
1650 bp
b
M + NT 1 2 3 4 5 6 7 8 9 10 11
2000 bp
1650 bp
M 12 13 14 15 16 17 18 19 20 21 22 23 24
2000 bp
1650 bp

### *3.5.4 Anaerobic purification of nitrous oxide reductase from* P. stutzeri

Nitrous oxide reductase was purified from *P. stutzeri* cells grown anaerobically with nitrate as an $N_2OR$ expression inducer. A soluble extract from these cells was resolved by anion-exchange chromatography. The 72 kDa $N_2OR$ and 61 kDa nitrite reductase coeluted together at 54.4% to 71.1% buffer B (Figure 3.3). The brownish-green fractions were purified by hydroxyapatite chromatography and $N_2OR$ eluted as a single protein peak between 30-45% 400 mM $NaH_2PO_4$, pH7.2 buffer B. The protein concentration of this purple fraction containing $N_2OR$ was determined to be 1 mg $ml^{-1}$ by the Bradford method.

### *3.5.5 The expression of $N_2OR$ in transgenic plants*

To demonstrate the expression of $N_2OR$ in leaves from transgenic plants, an immunoblot assay of total soluble protein extracts was performed, using an antibody raised against the $N_2OR$ antigen (provided by W. Zumft). No signals for the presence of $N_2OR$ were observed in non-transformed plant extracts, while transgenic lines showed a single band of the expected molecular mass of 72 kDa (Figure 3.4). The size of the protein coincided with the size of the purified $N_2OR$ obtained from *P. stutzeri*. The images were scanned using ImageJ software (http://rsbweb.nih.gov/ij/) to quantify the expression of $N_2OR$. The expression of $N_2OR$ in d35S-*nosZ* plants was estimated to be 0.073-0.138% of total soluble leaf proteins. The expression of $N_2OR$ in d35S-*nosFLZDY* plants was estimated to be 0.005-0.016% of total soluble leaf proteins.

**Figure 3.3. 10% SDS-PAGE analysis of $N_2OR$ protein from different purification steps**. Lane 1: crude clarified *P. stutzeri* lysate (1:50 dilution). Lane 2: pooled fractions from preparative anion exchange chromatography (1:50 dilution). Lane 3: pooled fractions from hydroxyapatite chromatography from half of the sample (1:25 dilution). Lane 4: marker proteins (Fermentas). The molecular weight of $N_2OR$ monomer from *P. stutzeri* is 72 kDa, and the molecular weight of nitrite reductase from *P. stutzeri* is 61 kDa.

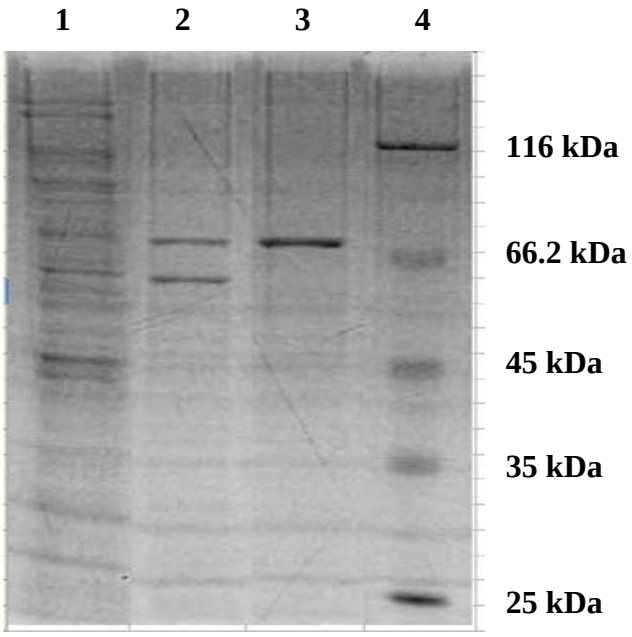

1
2
3
4
116 kDa
66.2 kDa
45 kDa
35 kDa
25 kDa

**Figure 3.4. Western immunoblot analysis of $N_2OR$ in plant protein samples extracted from transgenic plant leaf tissues**. **a** Western immunoblot analysis of $N_2OR$ of d35S-*nosZ* transgenic tobacco plants. **b** Western immunoblot analysis of $N_2OR$ of d35S-*nosFLZDY* transgenic tobacco plants. +: positive control, $N_2OR$ protein purified from *P. stutzeri*, expected size 72 kDa; NT: negative control, protein extracted from a non-transformed plant; transformed plants are identified by number.

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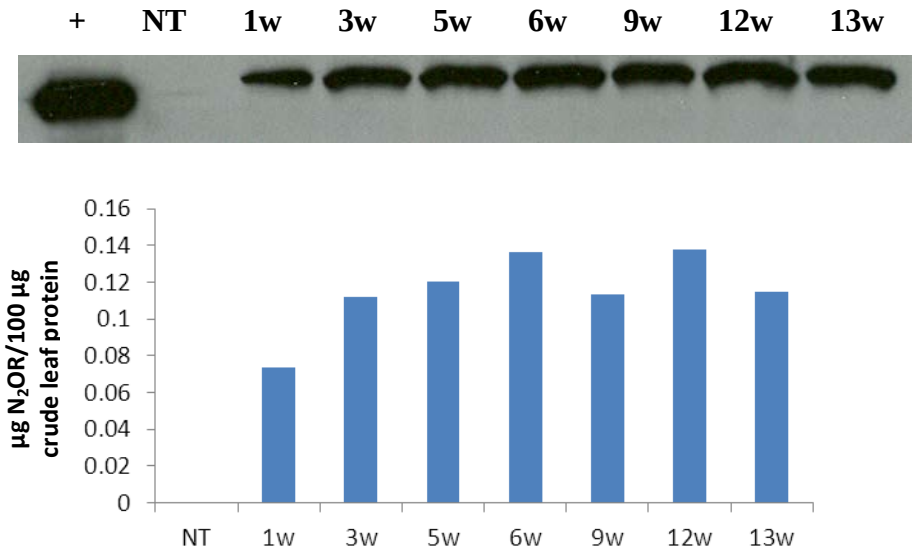
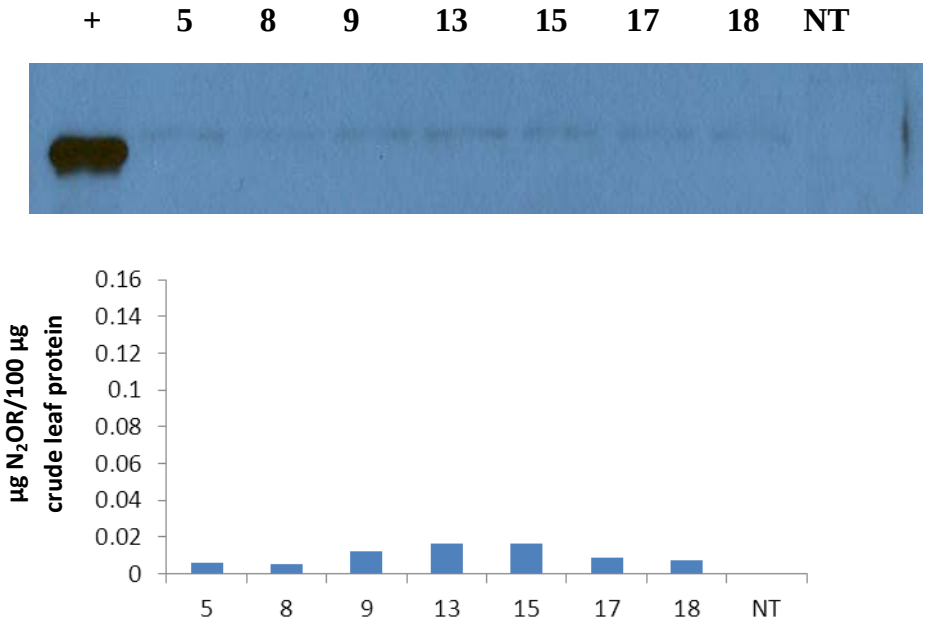
a


+
NT
1w
3w
5w
6w
9w
12w
13w
µg N2OR/100 µg crude leaf protein
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
NT
1w
3w
5w
6w
9w
12w
13w

b


+
5
8
9
13
15
17
18
NT
µg N2OR/100 µg crude leaf protein
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
5
8
9
13
15
17
18
NT

### *3.5.6 The activity of $N_2OR$ expressed in transgenic tobacco plants*

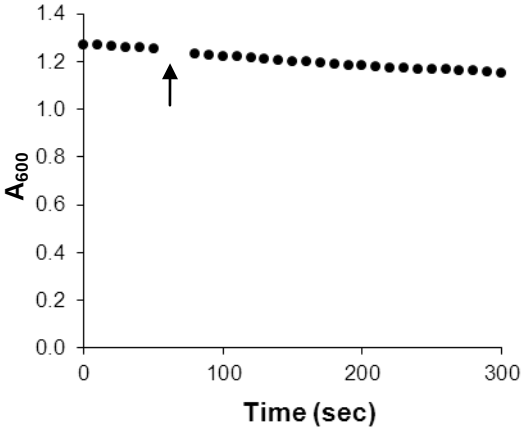
To test the $N_2O$-reducing activity of $N_2OR$ expressed in transgenic tobacco plants, crude protein extracted from transgenic tobacco leaves was analyzed using a methyl viologen-linked enzyme assay. Three of the highest expressors of *nosZ* (d35S-*nosZ*5w, d35S-*nosZ* 6w and d35S-*nosZ*12w) and the highest expressor of *nosFLZDY* (d35S-*nosFLZDY*13), were selected for analysis of $N_2O$-reducing activity. Non-transformed tobacco served as the negative control and purified $N_2OR$ from *P. stutzeri* was used as a positive control. Following the spectrophotometric assay, $A_{600}$ was plotted as a function of time (Figure 3.5). The change in the slope after the addition of $N_2O$ reflects the oxidation of methyl viologen and was used as an indirect indicator for the reduction of $N_2O$ to $N_2$ by $N_2OR$.

All the transgenic plants showed $N_2O$-reducing patterns similar to that of the positive control. The experimental value of specific activity for purified $N_2OR$ was 453.8 μmol $N_2O$ reduced $min^{-1}$ $mg^{-1}$ protein. Of all transgenic plants, d35S-*nosZ*12w showed the highest $N_2OR$ activity of 32.2 μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ leaf protein, with $N_2O$ reduction rates more than 40-fold higher than in the non-transformed plant. The d35S-*nosZ*6w had an $N_2O$-reducing capability of 25 μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$, which was more than 32-fold greater than in the non-transformed plant. Similarly, d35S-*nosZ*5w showed an $N_2O$ reducing rate of 21.47 μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$, more than 27-fold greater than the non-transformed plant. Other plants, such as d35S-*nosFLZDY*13, actually showed lower activity than the non-transformed control (3.5 μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$).

**Figure 3.5. $N_2OR$ activity in transgenic tobacco as measured by the methyl viologen-linked assay**. Trace plots monitoring 600 nm absorbance as a function of time. The arrows depict when $N_2O$-saturated water was added as enzyme substrate. The specific activity of $N_2OR$ in transgenic tobacco plant is shown above each curve. Transformed plants are identified by number.

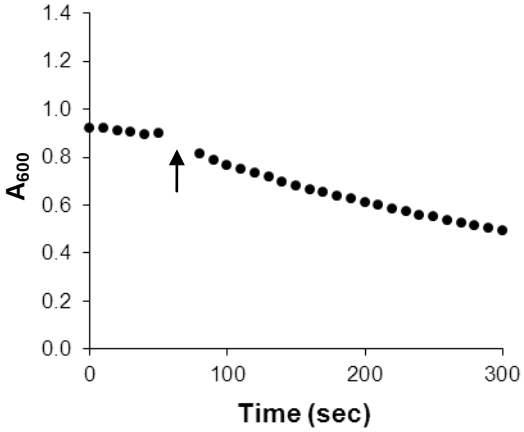
**Non-transformed**

**0.79 µmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ leaf protein**



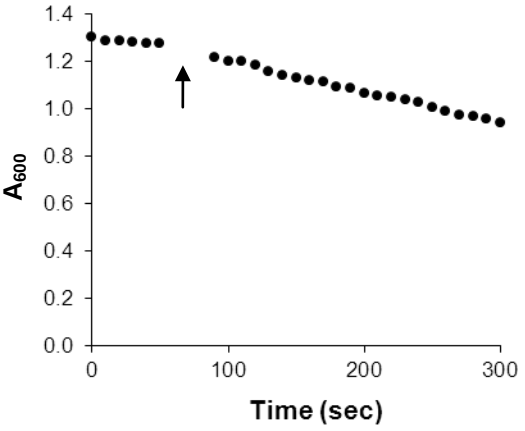

**$N_2OR$ from *P. stutzeri***

**453.8 µmol $N_2O$ reduced $min^{-1}$ $mg^{-1}$ protein**



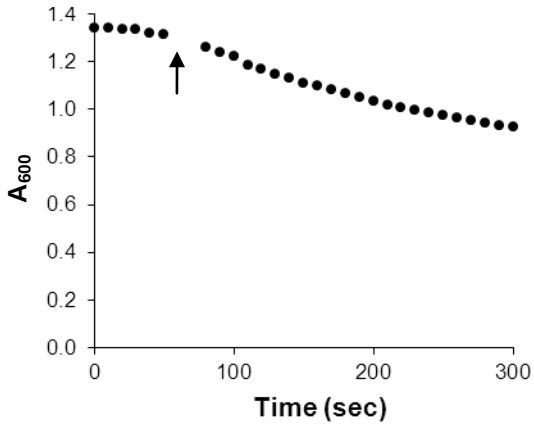

**d35S-*nosZ*5w**

**21.47 µmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ leaf protein**



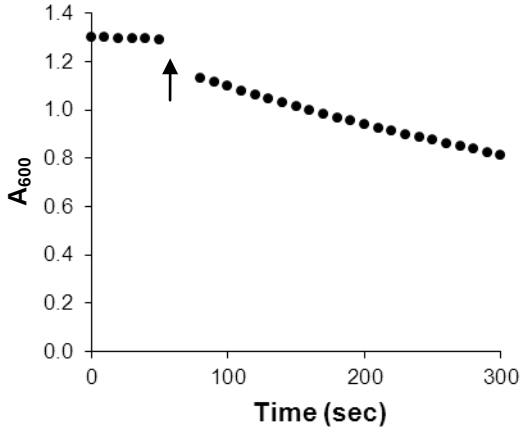

**d35S-*nosZ*6w**

**25.00 µmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ leaf protein**



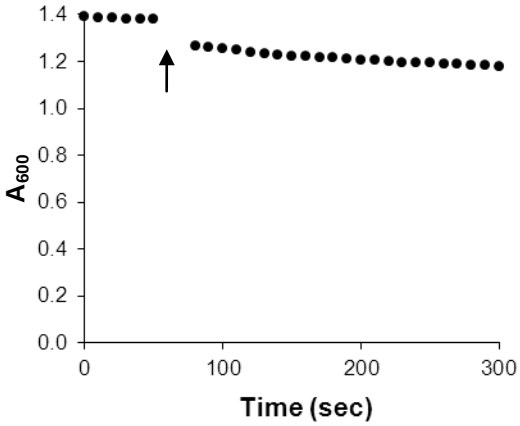

**d35S-*nosZ*12w**

**32.23 µmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ leaf protein**




**d35S-*nosFLZDY*13**

**3.50 µmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ leaf protein**



## 3.6 Discussion

In *P. stutzeri, nosZ* is the structural gene for $N_2OR$, which is located within the *nos* cluster, consisting of six sequentially encoded components, *nosRZDFYL*. Zumft and Kroneck (2007) indicated that the biosynthesis and assembly of $N_2OR$ is a complex process requiring a putative ABC transporter system for the biogenesis of the catalytic centre encoded by *nosDFY*, the copper-chaperone protein NosL for biogenesis of the metal centre, and the transcriptional regulator NosR. To help promote the functional assembly of $Cu_Z$, for production of active $N_2OR$, we co-expressed *nosZ* with *nosD*, *nosF*, *nosY* and *nosL* in transgenic plants. In *P. stutzeri*, the maturation of the NosZ peptide occurs in the periplasm (Wunsch et al. 2003). Due to the absence of a periplasmic space in plant cells and because bacterial signal peptides have no relevance for plant expression, periplasmic signals have to be replaced with extracellular or membrane signal sequences. In plant-based expression, the endogenous signal peptide of NosZ was replaced with a plant signal for extra-cellular targeting - the signal sequence of the carrot *extensin* gene. Furthermore, production of active $N_2OR$ in *P. stutzeri* requires the ABC-type transporter complex comprised of NosD, NosF, and NosY as well as the periplasmic Cu chaperone NosL (Zumft 2005). The endogenous signal sequences of *nosL* and *nosY* were replaced with the alpha amylase inhibitor-1 signal sequence from *Phaseolus vulgaris* which directs peptides to the plasma membrane. Again for *in planta* expression, the endogenous signal sequence of *nosD* was replaced with the extensin signal sequence. Recently, it was found that iron-sulfur flavoprotein NosR is necessary for sustained whole-cell NosZ function in *P. stutzeri* (Zumft 2005). This transcription factor was irrelevant for plant expression of the other *nos* genes since a constitutive promoter was used.

The functions of 'accessory' gene products (NosDFY and NosL) that are highly conserved in the *nos* gene cluster have been elucidated in *P. stutzeri* and other denitrifying bacteria. However, nothing is known about the roles of these gene products in the assembly of the copper cofactors of $N_2OR$ *in planta*. To evaluate the necessity of the ABC transporter and Cu chaperone NosL in the functional assembly of $Cu_Z$, we produced transgenic tobacco carrying either *nosZ* or *nosFLZDY*. Our results indicated that $N_2OR$ was expressed in both *nosZ*-transgenic plants and *nosFLZDY*-transgenic plants. The expression level of $N_2OR$ in *nosZ*-transgenic plants was, unexpectedly, about eight times higher than that in *nosFLZDY* transgenic plants. *NosZ*-transgenic plants showed $N_2O$ reduction rates more than nine-fold higher than those in the *nosFLZDY*-transformed plant. Silencing by homologous promoter sequences, or cryptic intracellular targeting are possible explanations of the low activities obtained. Results showed that the products of the other *nos* genes, such as the putative transporter encoded by *nosDFY,* and the Cu chaperone NosL, did not affect the activity of $N_2OR$ expressed in transgenic plants. It can therefore be inferred that the products of other *nos* genes did not show obvious roles in the biosynthesis and assembly of $N_2OR$ in plants.

Previous studies have shown that in some specific circumstances 'accessory' gene products may play a role in the biosynthesis and assembly of the copper cofactors of $N_2OR$. We suspected that these gene products (NosF, NosL, NosD, NosY) may not be necessary for the expression and assembly of the enzyme *in planta*. The more abundant proteome of an eukaryotic plant cell could fulfil the need for an ABC-type transporter. The analysis of the model plant *Arabidopsis thaliana* and other plant genomes revealed that they encode two to three times more ABC transporters than the genomes of animals and bacteria (Wanke and Kolukisaoglu 2010). Our results demonstrated that these transgenic leaf extracts have $N_2O$-reducing capabilities without the expression of the 'accessory' gene(s). Although the

mechanism assembling the functional $Cu_Z$ site is obscure (Gorelsky et al. 2006), we can conclude that properly synthesized $N_2OR$ apoprotein is amenable to copper atom insertion from the cytoplasm or apoplast of plant cells either spontaneously or through a plant-mediated process. Correctly assembling the $Cu_Z$ site is still a challenge even in heterologous bacterial expression. Although Viebrock and Zumft (1988) showed that heterologous expression of the *nosZ* from *P. stutzeri* cannot yield functional $N_2OR$ holoenzyme because of ineffective copper insertion, Liu *et al*. (2008) evidenced that *nosZ* from *Geobacillus thermodenitrificans* heterologously expressed in *Escherichia coli* could produce active, albeit copper-deficient (only 2.5 Cu/monomer rather than the required 6), recombinant $N_2OR$. Production of active $N_2OR$ was attributed to the ATP-binding cassette proteins present in *E. coli*. Our study confirms the recent evidence of $N_2OR$ activation from *Geobacillus thermodenitrificans* when expressed heterologously without the involvement of other Nos proteins. The existing endosymbiont membrane protein complement may also have assisted in copper-activation of the cytoplasmic $N_2OR$ in these tobacco plants (Awai et al. 2006). Furthermore, there are a variety of cytochrome-based electron donors in the more elaborate and bioenergetically sophisticated plant cells. The endogenous cytochrome(s), such as cytochrome *c* oxidase, could complement the $N_2OR$ holoenzyme in plant cells by providing the electrons needed to drive the redox chemistry.

Our data have demonstrated that extracts from transgenic plants expressing $N_2OR$ are capable of converting $N_2O$ into inert $N_2$. The heterologous expression of the microbial $N_2OR$ offers an innovative and promising approach to the bioremediation of $N_2O$. Biofuel crops are the ideal candidates for $N_2O$ phytoremediation trials. On average, agricultural soils emit 40 gram $N_2O$ $ha^{-1}$ $day^{-1}$ (Stuart Strand, University of Washington, personal communication). If $N_2OR$ was expressed in maize, the conversion of $N_2O$ could be as high as

$1.83\times10^7$ gram $ha^{-1}$ $day^{-1}$ (Supplementary Methods), significantly greater than $N_2O$ emissions from agricultural soil. Using this technology, the emission of $N_2O$ from soils could be prevented, and atmospheric $N_2O$ concentrations could be greatly reduced.

## 3.7 Acknowledgements

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## 3.9 Supplementary Information

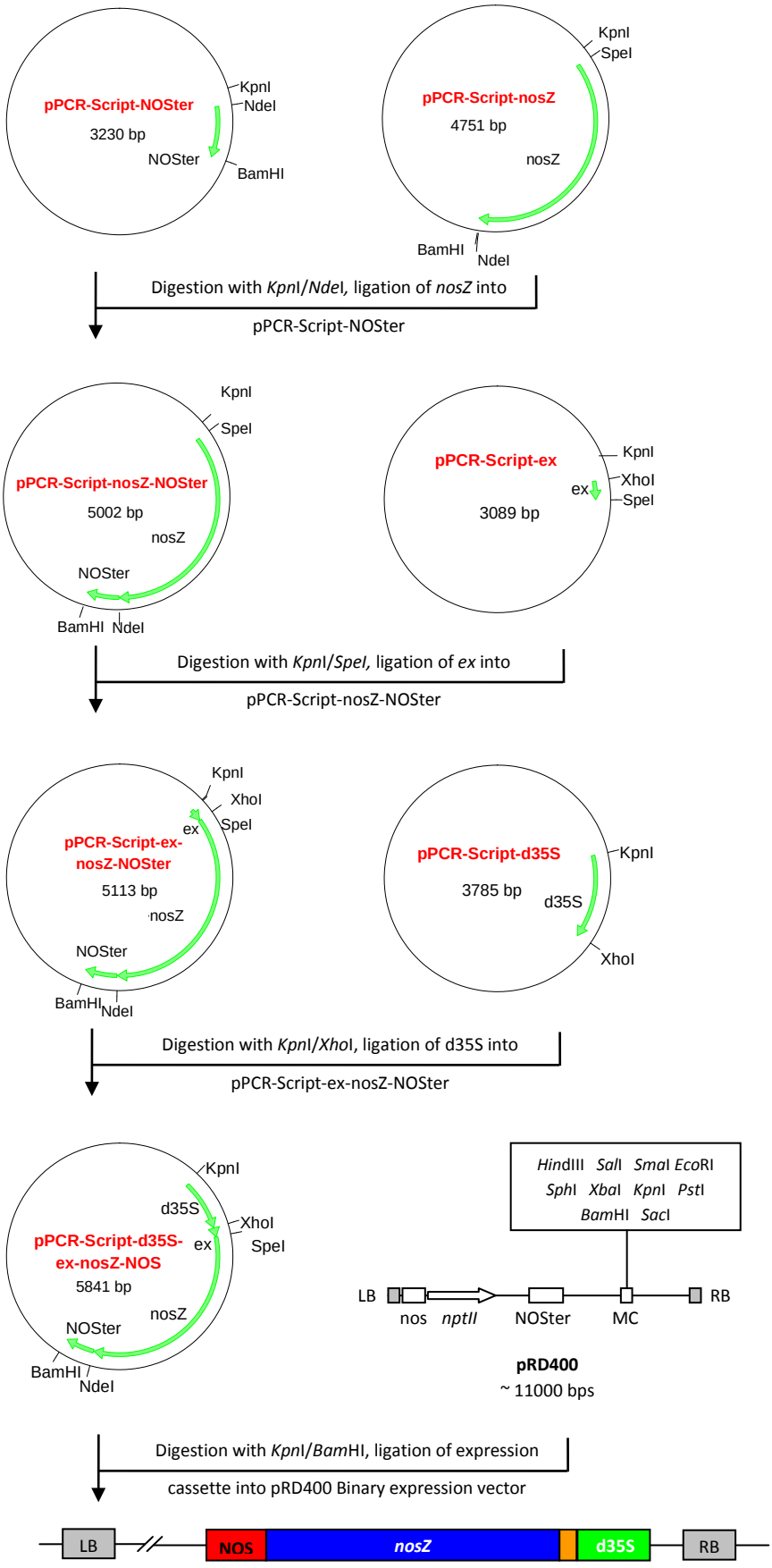
**Supplementary Table 3.1. Optimized PCR conditions for amplification of expression construct components.** Primers and thermal cycler details used for amplification of the d35S promoter sequence, signal sequence, *nos* coding sequence, and NOS terminator sequence.

| Vector | Target | Primer | Sequence | PCR Program Steps |
|---|---|---|---|---|
| **nosZ Unit** | d35S | d35SZF | 5'-GCGGTACCAGAGATAGATTTGTAGAGAGAGACTGGTG-3' | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 58ºC for 35 sec 4: 72ºC for 55 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Kpn*I | |
| | | d35SR2 | 5'-GACTCGAGGCGTATTGGCTAGAGCAGCTTGCC-3' | |
| | | | *Xho*I | |
| | *extensin* Signal sequence | ExSigZF | 5'-TAGGTACCTACTCGAGATGGGAAGAATTGCTAGAGG-3' | 1: 95ºC for 4 min 2: 95ºC for 30 sec<br>3: 50ºC for 30 sec 4: 72ºC for 30 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Kpn*I *Xho*I | |
| | | ExSigZR | 5'-GAGGATCCATACTAGTGGCTGTGGTTTCGGAAG-3' | |
| | | | *Bam*HI *Spe*I | |
| | *nosZ* | NosZF | 5'-GACTAGTCAGGCCGTCAAGGAGTCCAAG-3' | 1: 95ºC for 5 min 2: 95ºC for 1 min<br>3: 58ºC for 1 min 4: 72ºC for 2 min<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Spe*I | |
| | | NosZR | 5'-TAGGATCCAACATATGTTAGGCCGGCTCGACCATC-3' | |
| | | | *Bam*HI *Nde*I | |
| | NOSter | NOSF | 5'-CGCATATGCGTTCAAACATTTGGCAATAAAG-3' | 1: 95ºC for 4 min 2: 95ºC for 30 sec<br>3: 52ºC for 40 sec 4: 72ºC for 40 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Nde*I | |
| | | NOSZR | 5'-TAGGATCCCCCGATCTAGTAACATAGATG-3' | |
| | | | *Bam*HI | |
| **nosF Unit** | d35S | d35SFF | 5'-GAATTCAGAGATAGATTTGTAGAGAGAGACTGGTG-3' | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 58ºC for 35 sec 4: 72ºC for 55 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Eco*RI | |
| | | d35SR1 | 5'-GTAGATCTGCGTATTGGCTAGAGCAGCTTGCC-3' | |
| | | | *Bgl*II | |
| | *nosF* | NosFF | 5'-AGATCTATGAACGCCGTCGAGATC-3' | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 58ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 7 min<br>7: Hold at 4ºC |
| | | | *Bgl*II | |
| | | NosFR | 5'-CATATGTCATAGACGGCCCTCCTG-3' | |
| | | | *Nde*I | |
| | NOSter | NOSFF | 5'-CATATGCGTTCAAACATTTGGCAATAAAG-3' | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 54ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Nde*I | |
| | | NOSFR | 5'-GAGCTCCCCGATCTAGTAACATAGATG-3' | |
| | | | *Sac*I | |
| | d35S | d35SLF | 5'-GTGAGCTCAGAGATAGATTTGTAGAGAGAGACTGGTG-3' | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 58ºC for 35 sec 4: 72ºC for 55 sec |

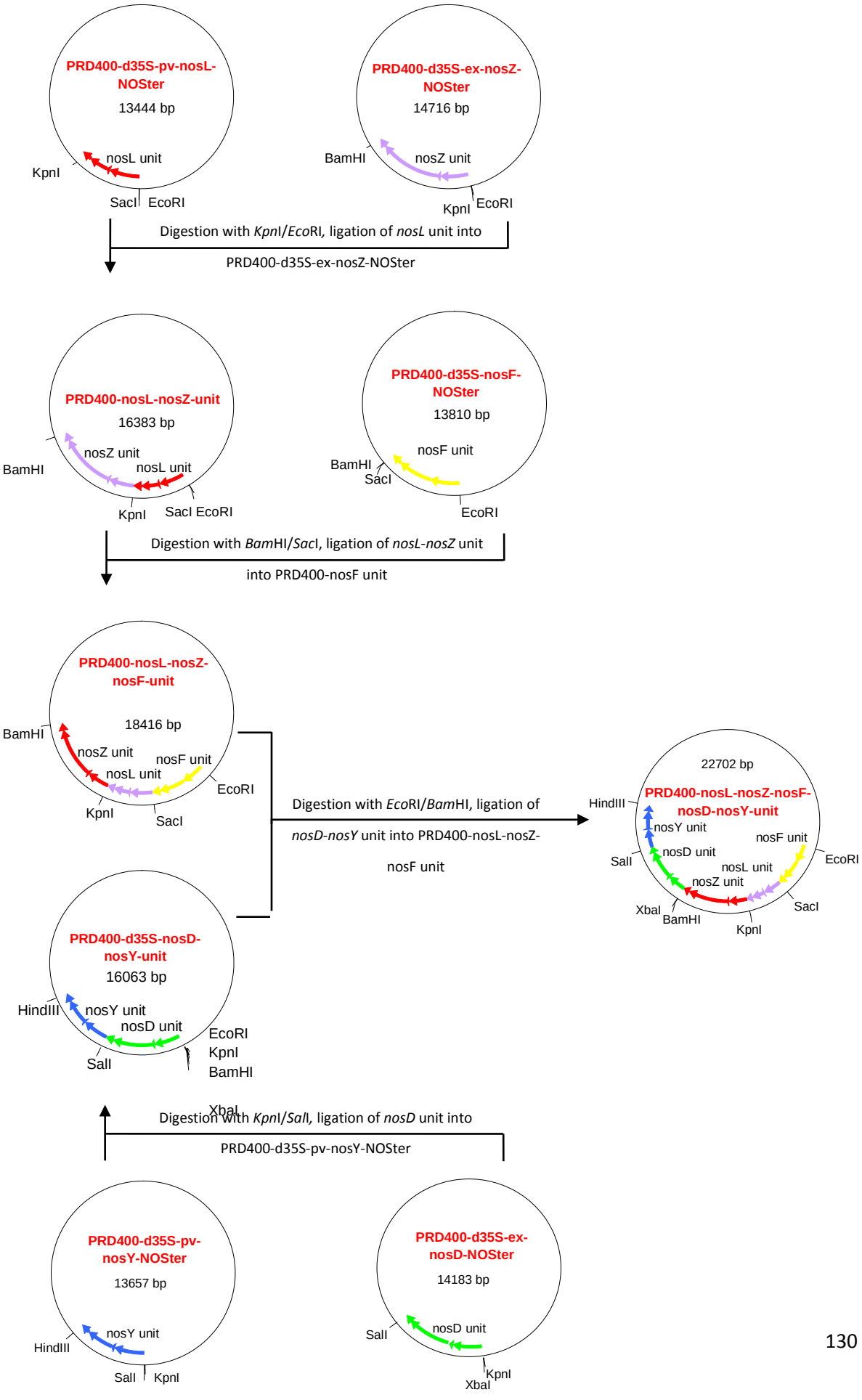
| | | | | |
|---|---|---|---|---|
| **nosL Unit** | | | *Sac*I | 5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | d35SR1 | 5'-GTAGATCTGCGTATTGGCTAGAGCAGCTTGCC-3'<br>*Bgl*II | |
| | *alpha amylase* inhibitor-1 signal sequence | PvSigF | 5'-CAGATCTATGGCTTCCTCCAACTTAC-3'<br>*Bgl*II | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 54ºC for 30 sec 4: 72ºC for 30 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | PvSigR | 5'-GTACTAGTGTTGAAGGAGGTTTCGGTG-3'<br>*Spe*I | |
| | *nosL* | NosLF | 5'-CTACTAGTTGCGGGGAGAAGGAGGAGGTTC-3'<br>*Spe*I | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 60ºC for 30 sec 4: 72ºC for 50 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | NosLR | 5'-GACATATGTCAGTGGCCTGCGTGTGCG-3'<br>*Nde*I | |
| | NOSter | NOSF | 5'-CGCATATGCGTTCAAACATTTGGCAATAAAG-3'<br>*Nde*I | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 54ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | NOSLR | 5'-TAGGTACCCCCGATCTAGTAACATAGATG-3'<br>*Kpn*I | |
| **nosD Unit** | d35S | d35SDF | 5'-CGTCTAGAAGAGATAGATTTGTAGAGAGAGACTGGTG-3'<br>*Xba*I | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 58ºC for 35 sec 4: 72ºC for 55 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | d35SR1 | 5'-GTAGATCTGCGTATTGGCTAGAGCAGCTTGCC-3'<br>*Bgl*II | |
| | *extensin* signal sequence | ExSigDF | 5'-AGATCTATGGGAAGAATTGCTAGAG-3'<br>*Bgl*II | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 57ºC for 30 sec 4: 72ºC for 30 sec<br>5: Repeat 30 times from 2 6: 72ºC for 7 min<br>7: Hold at 4ºC |
| | | ExSigDR | 5'-ACTAGTCGCTGTGGTTTCGGAAG-3'<br>*Spe*I | |
| | *nosD* | NosDF | 5'-ACTAGTGCACCGCAATCGATTACCAC-3'<br>*Spe*I | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 58ºC for 30 sec 4: 72ºC for 85 sec<br>5: Repeat 30 times from 2 6: 72ºC for 7 min<br>7: Hold at 4ºC |
| | | NosDR | 5'-CATATGTGACGTTGGTTCCTGCTTTTC-3'<br>*Nde*I | |
| | NOSter | NOSF | 5'-CGCATATGCGTTCAAACATTTGGCAATAAAG-3'<br>*Nde*I | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 54ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | NOSDR | 5'-TAGTCGACCCCGATCTAGTAACATAGATG-3'<br>*Sal*I | |

| | | | | |
|---|---|---|---|---|
| **nosY Unit** | d35S | d35SYF | 5'-GCGTCGACAGAGATAGATTTGTAGAGAGAGACTGGTG-3'<br>*Sal*I | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 58ºC for 35 sec 4: 72ºC for 55 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | d35SR1 | 5'-GTAGATCTGCGTATTGGCTAGAGCAGCTTGCC-3'<br>*Bgl*II | |
| | *alpha amylase* inhibitor-1 signal sequence | PvSigF | 5'-CAGATCTATGGCTTCCTCCAACTTAC-3'<br>*Bgl*II | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 54ºC for 30 sec 4: 72ºC for 30 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | PvSigR | 5'-GTACTAGTGTTGAAGGAGGTTTCGGTG-3'<br>*Spe*I | |
| | *nosY* | NosYF | 5'-TAACTAGTATCGCCTGGCTCGGCGCTG-3'<br>*Spe*I | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 55ºC for 30 sec 4: 72ºC for 55 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | NosYR | 5'-TACATATGTCAGGTCAAGCGCCGGCG-3'<br>*Nde*I | |
| | NOSter | NOSF | 5'-CGCATATGCGTTCAAACATTTGGCAATAAAG-3'<br>*Nde*I | 1: 94ºC for 4 min 2: 94ºC for 1min<br>3: 54ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | NOSYR | 5'-GCAAGCTTCCCGATCTAGTAACATAGATG-3'<br>*Hind*III | |

**Supplementary Figure 3.1. Construction of the d35S-*nosZ* expression cassette**.


pPCR-Script-NOSter
3230 bp
NOSter
KpnI
NdeI
BamHI
pPCR-Script-nosZ
4751 bp
nosZ
KpnI
SpeI
BamHI
NdeI
Digestion with KpnI/NdeI, ligation of nosZ into
pPCR-Script-NOSter
pPCR-Script-nosZ-NOSter
5002 bp
nosZ
NOSter
KpnI
SpeI
BamHI
NdeI
pPCR-Script-ex
3089 bp
ex
KpnI
XhoI
SpeI
Digestion with KpnI/SpeI, ligation of ex into
pPCR-Script-nosZ-NOSter
pPCR-Script-ex-nosZ-NOSter
5113 bp
nosZ
NOSter
ex
KpnI
XhoI
SpeI
BamHI
NdeI
pPCR-Script-d35S
3785 bp
d35S
KpnI
XhoI
Digestion with KpnI/XhoI, ligation of d35S into
pPCR-Script-ex-nosZ-NOSter
pPCR-Script-d35S-ex-nosZ-NOS
5841 bp
nosZ
NOSter
d35S
ex
KpnI
XhoI
SpeI
BamHI
NdeI
HindIII SalI SmaI EcoRI
SphI XbaI KpnI PstI
BamHI SacI
LB
nos
nptII
NOSter
MC
RB
pRD400
~ 11000 bps
Digestion with KpnI/BamHI, ligation of expression
cassette into pRD400 Binary expression vector
LB
NOS
nosZ
d35S
RB

**Supplementary Figure 3.2. Construction of d35S-*nosFLZDY* expression megacassette.**


PRD400-d35S-pv-nosL-NOSter
13444 bp
nosL unit
KpnI
SacI
EcoRI
PRD400-d35S-ex-nosZ-NOSter
14716 bp
BamHI
nosZ unit
KpnI
EcoRI
Digestion with KpnI/EcoRI, ligation of nosL unit into
PRD400-d35S-ex-nosZ-NOSter
PRD400-nosL-nosZ-unit
16383 bp
nosZ unit
nosL unit
BamHI
KpnI
SacI EcoRI
PRD400-d35S-nosF-NOSter
13810 bp
nosF unit
BamHI
SacI
EcoRI
Digestion with BamHI/SacI, ligation of nosL-nosZ unit
into PRD400-nosF unit
PRD400-nosL-nosZ-nosF-unit
18416 bp
BamHI
nosZ unit
nosF unit
nosL unit
EcoRI
KpnI
SacI
PRD400-d35S-nosD-nosY-unit
16063 bp
HindIII
nosY unit
nosD unit
SalI
EcoRI
KpnI
BamHI
XbaI
Digestion with EcoRI/BamHI, ligation of
nosD-nosY unit into PRD400-nosL-nosZ-
nosF unit
22702 bp
PRD400-nosL-nosZ-nosF-nosD-nosY-unit
HindIII
nosY unit
nosF unit
SalI
nosD unit
EcoRI
nosL unit
nosZ unit
XbaI
BamHI
SacI
KpnI
Digestion with KpnI/SalI, ligation of nosD unit into
PRD400-d35S-pv-nosY-NOSter
PRD400-d35S-pv-nosY-NOSter
13657 bp
nosY unit
HindIII
SalI
KpnI
PRD400-d35S-ex-nosD-NOSter
14183 bp
SalI
nosD unit
KpnI
XbaI

**Supplementary Methods**

**Calculation of mitigation potential of $N_2OR$ biotech crops.** Annual conversion of $N_2O$ was estimated assuming that the $N_2OR$ trait was to be expressed in maize leaves.

Using the $N_2OR$ activity of 32.23 μmol $N_2O$ reduced $min^{-1}$ $gram^{-1}$ leaf protein (Figure 5), and given that shoot mass in maize is about 9 Mg $ha^{-1}$ ,

maize already fluxes $CO_2$ and $O_2$ gases, then the amount of $N_2O$ could be:

= leaf weight × the activity of $N_2OR$ in the leaf × growth period

= 9 Mg $ha^{-1}$ × 32.23 μmol $N_2O$ reduced $min^{-1}$ $gram^{-1}$ leaf protein × 24 hour $day^{-1}$

= $9x10^6$ gram/ha × 32.23 $x10^{-6}$ mol $N_2O$ reduced $min^{-1}$ $gram^{-1}$ leaf protein × 44 gram/mol × 24 hour/day × 60 min/hour

= $1.83×10^7$ gram $ha^{-1}$ $day^{-1}$

# Chapter 4

## Expression of Nitrous Oxide Reductase from *Pseudomonas stutzeri* in Transgenic Tobacco Roots using the Root-specific *rolD* Promoter from *Agrobacterium rhizogenes*

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### 4.1 Author contributions

S. Wan designed, directed and performed all of the experimental work. S. Wan was the primary author who wrote and revised the manuscript with the assistance of A. Johnson, a former undergraduate honours project student, and I. Altosaar, the Ph.D. Thesis supervisor.

## 4.2 Abstract

The nitrous oxide ($N_2O$) reduction pathway from a soil bacterium, *Pseudomonas stutzeri,* was engineered in plants to reduce $N_2O$ emissions. As a proof of principle, transgenic plants expressing nitrous oxide reductase **(**$N_2OR$) from *P. stutzeri,* encoded by the *nosZ* gene, and other transgenic plants expressing $N_2OR$ along with the more complete operon from *P. stutzeri,* encoded by *nosFLZDY,* were generated. Gene constructs were engineered under the control of a root-specific promoter and with a secretion signal peptide. Expression and rhizosecretion of the transgene protein were achieved, and $N_2OR$ from transgenic *Nicotiana tabacum* proved functional using the methyl viologen assay. Transgenic plant line 1.10 showed the highest specific activity of 16.7 umol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root protein. Another event, plant line 1.9, also demonstrated high specific activity of $N_2OR$, 13.2umol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root protein. The availability now of these transgenic seed stocks may enable canopy studies in field test plots to monitor whole rhizosphere N flux. By incorporating one bacterial gene into genetically modified organism (GMO) crops (e.g. cotton, corn, and soybean) in this way, it may be possible to reduce the atmospheric concentration of $N_2O$ that has continued to increase linearly (about 0.26% $yr^{-1}$) over the past half-century.

## 4.3 Introduction

Of all the greenhouse gases, nitrous oxide ($N_2O$) is the most damaging to the environment. With a global warming potential 310 times that of carbon dioxide, $N_2O$ contributes to the rising of atmospheric temperature (Ravishankara *et al.*, 2009). $N_2O$ also

causes acid rain as it reacts with the sun's ultraviolet rays and then ozone to form nitrate (Madigan *et al.*, 2002).

Atmospheric levels of $N_2O$ have increased about 20% since pre-industrial times (Richardson *et al*., 2009), and $N_2O$ continues to accumulate at a rate of 0.26% each year (Forster *et al*., 2007). The leading source of $N_2O$ is agricultural soil (Chapuis-Lardy *et al*., 2007). The amount of $N_2O$ produced is proportional to the amount of nitrogen that enters the soil (Crutzen *et al*., 2008). Nitrogen fertilizer consumption has increased from 31 million metric tonnes in pre-industrial times to 165 million metric tonnes in 2005 (Burney *et al*., 2010) as a result of agricultural intensification. Thus, the increase in atmospheric $N_2O$ since pre-industrial times is largely due to the application of nitrogen-based synthetic fertilizers to agricultural soils.

Nitrogen in the soil is metabolized by denitrifying bacteria such as *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Bradyrhizobium japonicum* and *Wolinella succinogenes* (Wunsch and Zumft, 2005). A closer look into the denitrification process reveals five intermediates between fixable nitrogen and the inert atmospheric $N_2$. The final step in denitrification is the enzymatic conversion of $N_2O$ to $N_2$ by nitrous oxide reductase ($N_2OR$). This reaction often does not occur if ideal metabolic conditions are not met, and results in emission of $N_2O$ (Zumft, 1997). Furthermore, approximately a third of the denitrifying bacteria that have had their genomes sequenced have a truncated denitrification pathway, lacking the *nosZ* gene encoding the $N_2OR$ (Philippot *et al*., 2011). This last step of denitrification could become a core strategy for mitigating $N_2O$ emissions if crops could be improved with this agronomic trait.

The microbial $N_2OR$ is the only known biological catalyst that can catalyze the conversion of $N_2O$ to $N_2$. The $N_2OR$ holoenzyme contains two identical subunits of 65.8-

kDa, each containing six copper atoms. It catalyzes the copper-dependent two-electron reduction of $N_2O$ to water and dinitrogen gas, which takes place in the bacterial periplasm. In *P. stutzeri*, $N_2OR$ is encoded by the gene *nosZ* (Zumft, 1997). The complete *nos* operon contains five additional *nos* genes, *nosR*, *nosD*, *nosF*, *nosY*, and *nosL*, each of which encode proteins that are thought to assist in the assembly of the enzyme in *Pseudomonas stutzeri*. *NosR* encodes a transcriptional regulator, *nosD*, *nosF* and *nosY* encode an ABC-type transporter, and *nosL* encodes a copper chaperone (Honisch and Zumft, 2003).

Here we present a means of mimicking bacterial denitrification in plants by endowing them with the recombinant $N_2OR$ enzyme. This is a novel method of phytoremediation since, to our knowledge, no one has used plants as a means of mitigating this particular greenhouse gas at its source in the soil.

Plant roots are in direct contact with the microbial community in the rhizosphere. They secrete a number of chemicals into the rhizosphere, having a large impact on soil chemistry (Philippot *et al*., 2009). Tobacco plant roots have been used as a recombinant protein production system using root-specific promoters for the gene of interest (Drake *et al*., 2003). Promoting complete denitrification in the rhizosphere in this way may eliminate $N_2O$ emissions at the source. The substrate, $N_2O$, produced by denitrifiers in the rhizosphere could potentially bind to the catalytic enzyme, $N_2OR$, secreted by transgenic tobacco plant roots. $N_2O$ reduction would occur, resulting in the release of $N_2$ gas into the soil air pockets and ultimately into the atmosphere. To test this hypothesis, in an attempt to achieve $N_2OR$ expression *in planta*, *Nicotiana tabacum* cv. Xanthi nc. plants were transformed with the single *nosZ* gene. A second set of transgenic plants were also transformed with the more complete operon, *nosFLZDY*. Transgenic plants were analyzed to confirm transgene incorporation, transgene expression, protein expression, and protein activity. Surprisingly,

extracts from these tobacco plants, isolated from their root tissue and from the medium surrounding their roots, when analyzed by the methyl viologen assay provided evidence of $N_2O$ reduction capacity.

## 4.4 Materials and Methods

### *4.4.1 Genomic DNA Isolation from* P. stutzeri

*Pseudomonas stutzeri* Zobell (ATCC 14405) cells were plated on LB-agar medium and grown at 30ºC for 48 h. A single colony was used to inoculate 5 mL of liquid LB medium, and mixture was incubated at 30ºC overnight with shaking. The bacterial culture was added to 100 mL liquid LB medium and incubated for 3 h. Methods of Neumann *et al*. (1992) were followed for extraction of genomic DNA. 100 mL bacterial culture was centrifuged at 3000 *g* for 15 min at 4ºC, the pellet was washed in PBS buffer, and resuspended in 5 mL SET (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5). Lysozyme was added to a concentration of 1 mg $mL^{-1}$, and the resulting suspension incubated at 37ºC for 30 min, mixing occasionally by inversion. To inactivate DNases, 0.5 mL of proteinase K (1 mg $mL^{-1}$) was added along with 0.5 mL 10% SDS, and mixture was incubated at 55ºC for 2 h with occasional inversion.

To precipitate proteins out of solution, 2.5 mL of 5 M NaCl was added and gently mixed in. Adding 10 mL chloroform, the tube was mixed at low speed for 1 h, and then centrifuged at 3000 *g* for 10 min. The aqueous upper phase was transferred to a new tube. Introduction of 20 mL isopropanol, mixing by inversion, and incubation at -20ºC for 15 min were sufficient to precipitate DNA. The DNA was wound onto a pipette tip, washed twice

with 70% ethanol in a new 1.5 mL tube, placed in a centrifugal evaporator to dry, and resuspended in 500 μL deionized water.

### *4.4.2 Construction of Plant Expression Constructs*

*Pseudomonas stutzeri* genomic DNA was used as a template for PCR amplification of the *nosZ* gene or *nosFLZDY* genes. Primers were gene-specific, and were designed to introduce restriction sites so the resulting segments could be fused (Supplementary Table 4.1). The root-specific promoter of the *rolD* gene from *Agrobacterium rhizogenes* was chosen to direct root-specific expression of $N_2$OR. The promoter sequence 426D (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. The nopaline synthase polyadenylation sequence was used as terminator (NOSter). The leader sequence of the *Daucus carota* (carrot) *extensin* (ex) gene was amplified from the pHBV-CO plasmid using forward primer, ExSigZF/ExSigDF, and reverse primer, ExSigZR/ExSigDR (Alli *et al*., 2002; Chen and Varner, 1985). The *Phaseolus vulgaris* (*Pv*) alpha amylase inhibitor-1 signal sequence was amplified using forward and reverse primers PvSigF and PvSigR, respectively (Prescott *et al*., 2005). Purified PCR products were cloned into the pPCR-Script Amp SK (+) cloning vector system (Stratagene, La Jolla, CA, USA). For assembly of expression vector prolD-*nosZ*, segments of NOSter, nosZ, ex and rolD were ligated together and cloned into the binary vector pRD400 (Datla *et al*. 1992) (Supplementary Figure 4.1) following digestion with *Kpn*I and *Bam*HI. Plasmid pRD400 carries the neomycin phosphotransferase II (nptII) gene, which confers resistance to the antibiotic kanamycin. The resulting prolD-*nosZ* plasmid was sequenced at StemCore Laboratories (Ottawa, Canada) to verify accuracy of the

DNA sequence. The megacassette prolD-*nosFLZDY* was constructed in a similar fashion, ligating individual expression constructs for *nosF*, *nosD*, *nosY* and *nosL* and cloning the resulting sequence into pRD400 (Supplementary Figure 4.2).

### *4.4.3 Plant Transformation and Selection*

*Agrobacterium tumefaciens* strain LBA4404 was introduced with plasmids prolD-*nosZ* and prolD-*nosFLZDY*. Tobacco (*Nicotiana tabacum* cv. Xanthi) seeds were sterilized with a 1-min wash in 70% ethanol, 10-min wash in 10% bleach containing 1-2 drops Tween-20, and three rinses in sterile distilled water. Sterilized seeds were grown on germination medium (1/2 MS, 3% sucrose, 0.8% agar, pH 5.8) in petri dishes. Tobacco leaf transformation followed the general method of Horsch *et al*. (1985). Leaves were collected from 5-6 week-old plants and cut into sections under sterile conditions. *A. tumefaciens* harbouring either prolD-*nosZ* or prolD-*nosFLZDY* plasmid was used to infect leaf sections for 48 h on co-cultivation medium (MS, 3% sucrose, 0.8% agar, 1.0 mg $L^{-1}$ 6-benzyladenine, 0.l mg $L^{-1}$ α-naphthalene acetic acid, pH 5.8).

Transformed leaf cells were grown on regeneration medium (MS, 3% sucrose, 0.8% agar, 1.0 mg $L^{-1}$ 6-benzyladenine, 0.l mg $L^{-1}$ α-naphthalene acetic acid, pH 5.8, 200 mg $L^{-1}$ of ticarcillin and 300 mg $L^{-1}$ of kanamycin). When they had reached a height of 6 cm, transgenic plants were transferred to the greenhouse where they were maintained through seed set with 16 h daylight (400 $W/m^2$) at 25ºC and 8 h dark at 21ºC, with watering as necessary. Seeds were collected once seed pods had matured and planted in hydroponic medium to perform the rhizosecreted protein experiments described below.

### *4.4.4 Polymerase Chain Reaction*

A portion of the root tissue (100 mg) was collected from each six-week old plant and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). PCR was performed in a 25 µl reaction mixture containing 50 ng of tobacco genomic DNA, 25 pmol of each primer, 2.5 µl of 10× PCR buffer (with 1.5 mM $MgCl_2$), 250 µM of each dNTP, 0.5 unit Taq DNA polymerase, with the following cycling condition: initial denaturation at 94°C for 4 min; 30 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and elongation at 72°C for 2 min; followed by a final elongation at 72°C for 10 min. DNA from non-transgenic (NT) plant roots was used as negative control, while either the prolD-*nosZ* or prolD-*nosFLZDY* plasmid was used as positive control. The following primers were used to detect the *nosZ* gene in genomic DNA from prolD-*nosZ* transgenic plants: forward primer (ExSigZF) 5′-TAGGTACCTACTCGAGATGGGAAGAATTGCTAGAGG-3′ and reverse primer (NosZR) 5′-TAGGATCCAACATATGTTAGGCCGGCTCGACCATC-3′. For prolD-*nosFLZDY* transgenic plants, the specific primers used to detect the *nosZ* gene were: forward primer (NosZF) 5′-GACTAGTCAGGCCGTCAAGGAGTCCAAG-3′ and reverse primer (NosZR) 5′-TAGGATCCAACATATGTTAGGCCGGCTCGACCATC-3′.

### *4.4.5 Reverse Transcription Polymerase Chain Reaction*

Root tissue (100 mg) was collected from each six-week old plant and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). After treatment with RNase-free DNase (Promega, Madison, USA), RT-PCR was performed according to the

SuperScript II Reverse Transcriptase system protocol (Invitrogen, Carlsbad, USA). In a 10 µl reaction mixture, 250 ng of total RNA was reverse-transcribed at 70°C for 10 min with an oligo (dT) primer. The conditions for cDNA synthesis were 10 min at 25°C, 60 min at 37°C, and 5 min at 95°C. Each PCR reaction used 2 µl cDNA as a template and *nosZ*-specific primers. DNA from NT plant roots was used as negative control, while either the prolD-*nosZ* or prolD-*nosFLZDY* plasmid was used as positive control. For prolD-*nosZ* transgenic plants, the following primers were used to detect the *nosZ* gene: forward primer (ExSigZF) 5′-TAGGTACCTACTCGAGATGGGAAGAATTGCTAGAGG-3′ and reverse primer (NosZR) 5′-TAGGATCCAACATATGTTAGGCCGGCTCGACCATC-3′. For prolD-*nosFLZDY* transgenic plants, the following primers were used to detect the *nosZ* gene: forward primer (NosZF) 5′-GACTAGTCAGGCCGTCAAGGAGTCCAAG-3′ and reverse primer (NosZR) 5′-TAGGATCCAACATATGTTAGGCCGGCTCGACCATC-3′. A control PCR, with total RNA not treated with reverse transcriptase, was performed to ensure that no DNA was present. Expression of other genes in the *nos* cassette (*nosF*, *nosL*, *nosD*, and *nosY*) was also analyzed using RT-PCR in the same way, with gene-specific primers.

### *4.4.6 Growth of Transgenic Plants in Hydroponic Medium*

Hydroponic cultures were established for the harvest of recombinant protein from root tissue and the rhizosphere according to the method of Drake *et al*. (2003). Transgenic tobacco seeds containing either the rolD-*nosZ* or the rolD-*FLZDY* expression cassette were surface-sterilized using 20% v/v bleach, washed in sterile distilled water, and sown onto a 9 cm petri dish containing 0.7% w/v agar-solidified MS medium (Murashige and Skoog, 1962). When seedlings reached 1 cm in height, they were transferred to liquid MS medium

by placing the shoot through a perforation in a plastic platform in a 50 ml container containing 25 ml liquid MS medium. The roots of the seedling were immersed in the liquid medium and the shoots were supported above the platform. Seedlings were maintained at 25°C with 16 h daylight and 8 h dark, and were grown to a height of 6 cm before further analysis.

### *4.4.7 Crude Protein Extraction from Roots and from Hydroponic Medium*

Root tissue was harvested, washed three times in dd$H_2O$, immediately frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. The soluble protein was extracted from root tissue of transgenic tobacco using Plant Total Protein Extraction Kit (Sigma, St. Louis, USA). Total protein extract was quantified using the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, USA).

Rhizosecreted protein from each plant culture was collected over a period of 25 days. Aliquots of MS medium (500 µL) from both rolD-transgenic and NT plant cultures, were collected at regular intervals. Samples were concentrated to a volume of 20 µl by means of YM50 Microcon filters (Millipore, Billerica, USA) and stored at -20°C. Protein samples were pooled together prior to western blotting and $N_2$OR activity analysis.

### *4.4.8 Anaerobic Purification of Nitrous Oxide Reductase from* P. stutzeri

*Pseudomonas stutzeri* Zobell (ATCC 14405) cells were plated on LB-agar medium and grown at 30ºC for 48 h. A single colony was used to inoculate 200 mL of synthetic medium (Matsubara *et al*., 1982), and culture was incubated at 30ºC for 24 h with low-speed

shaking. This culture was added to 1.8 L of synthetic medium and grown for 6 h. To induce $N_2OR$ expression, sodium nitrate was added to a concentration of 1 g $L^{-1}$. The culture was scaled up to 12 L and incubated at 30ºC for 24 hours. After centrifugation, the resulting pellet was washed with 50 mM $MgCl_2$ in 25 mM Tris buffer (pH 7.5), purged with argon, and stored at -70ºC. After dissolving the pellet in 25 mM Tris buffer (pH 7.5), cells were disrupted by sonication.

The supernatant was applied to a DEAE Sepharose ion-exchange chromatography column and eluted at 2 mL $min^{-1}$ using 25 mM Tris (pH 7.5) for buffer A, and 25 mM Tris (pH 7.5), 0.3 M NaCl for buffer B. Protein was eluted at 4ºC over five column volumes with a linear gradient of 0 to 100% buffer B. Collected 5 mL fractions were stored at 4ºC after purging with argon. Those containing $N_2OR$ and nitrite reductase were identified using SDS-PAGE, pooled and then dialysed using a membrane with a 14 kDa cutoff. Fractions were subjected to hydroxapatite chromatography using 10 mM $NaH_2PO_4$ (pH 7.2) for buffer A and 400 mM $NaH_2PO_4$ (pH 7.2) for buffer B. Protein was eluted over 10 column volumes with a linear gradient of 0 to 80% buffer B. Collected 2 mL fractions were purged with argon and stored at 4ºC prior to SDS-PAGE analysis (Supplementary Figure 4.3). Chromatography was performed under anaerobic conditions, with all reagents degassed by vacuum and procedures carried out under 100% argon. Concentration of purified nitrous oxide reductase was determined using the Bradford method (1976) with bovine serum albumin as the standard.

### *4.4.9 Western Blotting*

Samples were prepared by heating 40 μg protein extract for 10 min at 95°C 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). The positive control was $N_2OR$ purified from *Pseudomonas stutzeri*, and the negative control was protein extract from a non-transformed plant. Samples were run on a 10% acrylamide gel with a 5% stacking gel, then transferred onto a nitrocellulose membrane using the Trans-Blot transfer cell (Bio-Rad, Hercules, USA). To prevent non-specific binding, membrane was blocked with TBST plus 5% skim milk before incubation with rabbit anti-$N_2OR$ serum (1:5000, provided by W. Zumft) and subsequently anti-rabbit biotin horseradish peroxidase linked antibody (1:1000 in TBST) (Cell Signaling Technology, Danvers, USA). Immunoblotted $N_2OR$ was visualized using ECL western blotting chemiluminescent reagents (Amersham Biosciences, Baie d'Urfé, QC).

### *4.4.10 Methyl Viologen-linked Reductase Activity Assay*

*In vitro* $N_2OR$ activity was determined using a modified protocol from Kristjansson and Hollocher (1980). This assay uses reduced methyl viologen as the chemical electron donor, allowing the reduction of $N_2O$ to be monitored spectrophotometrically. Assays were carried out in an anaerobic chamber (10% $H_2$, 5% $CO_2$ and 85% $N_2$; Model 1025, Thermo Fisher Scientific, Waltham, USA) at 37ºC, and all reagents were degassed by vacuum and purged with 100% argon gas. For the positive control, the reaction mixture comprised 5 µl purified $N_2OR$ (0.956 µg/µl), 0.05 ml of 10 mM methyl viologen and 0.05 ml of 5 mM sodium dithionite in 2 ml of 10 mM $KH_2PO_4$ (pH 7.1). For transgenic root samples, the mixture comprised 50 µl root extract, 0.2 ml of 10 mM methyl viologen and 0.2 ml of 5 mM sodium dithionite in 1.5 ml of 10 mM $KH_2PO_4$ (pH 7.1). For all samples, reactions took place in a stoppered 3.5 ml cuvette (light-path length 1cm).

Absorbance at 600 nm was monitored for 1 min to quantify the background oxidation rate. The substrate, $N_2O$, was added in one 25-µl injection of $N_2O$-saturated $ddH_2O$. By monitoring the change in absorbance at 600 nm of the reaction mixture, the specific activity of the $N_2OR$ therein was determined (for specific activity calculations, see Supplementary Method 1). The specific activity was expressed as micromoles of $N_2O$ reduced per min per milligram of $N_2OR$ protein.

## 4.5 Results

### *4.5.1 Engineering and Growth of nosZ-expressing Tobacco Plants*

We generated transgenic *Nicotiana tabacum* cv. Xanthi nc. plants expressing the *nosZ* gene under the transcriptional control of the root-specific *Agrobacterium rhizogenes* rolD promoter (Slightom *et al*., 1986). Fourteen kanamycin-resistant $T_0$ *nosZ*-transgenic tobacco lines and 15 kanamycin-resistant $T_0$ *nosFLZDY*-transgenic tobacco lines were generated by leaf-disk Agrobacterium-mediated transformation. The transgenic tobacco lines were designated rolD:*nosZ*-1.1 to rolD:*nosZ*-1.14, and rolD:*nosFLZDY*-5.1 to rolD:*nosFLZDY*-5.15. First generation transformed ($T_1$) plant lines were grown from the seeds of the $T_0$ generation, with no apparent phenotypic differences between transgenic and non-transgenic plants.

### *4.5.2 Detection of nosZ DNA in Transgenic Plants*

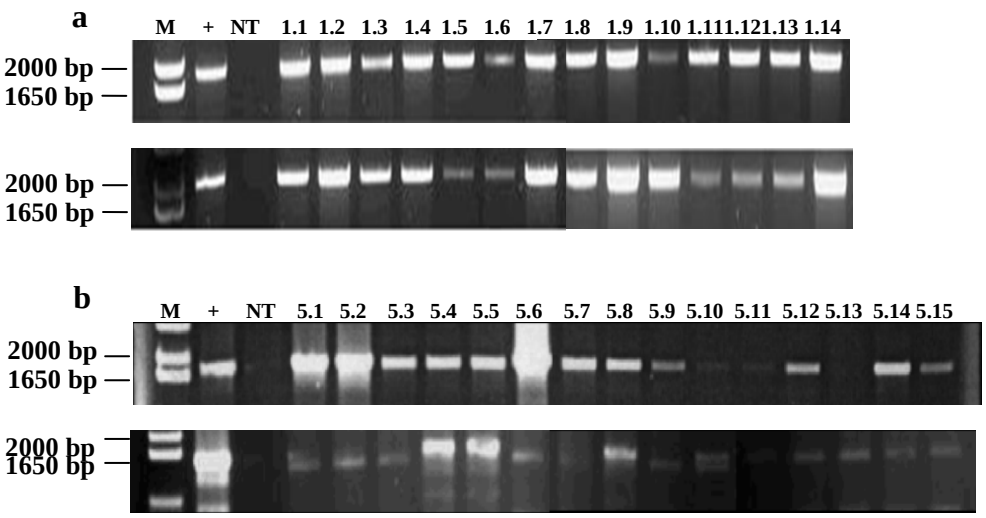
To confirm the presence of the *nosZ* transgene under the control of the rolD promoter, transgenic plants were screened by PCR using sequence-specific primers. Non-

transgenic plants were used as negative controls, while the plasmid prolD-*nosZ* or prolD-*nosFLZDY* was used as a positive control. PCR analysis of the putative prolD-*nosZ* transformed lines showed the presence of an 1869 bp band (Figure 4.1, a, top row) corresponding to the length of the *ex-nosZ* coding sequence. For putative prolD-*nosFLZDY* transformed lines, PCR analysis was performed to amplify the 1767 bp *nosZ* coding sequence (Figure 4.1, b, top row), as well as fragments from *nosF*, *nosL*, *nosD*, and *nosY* (data not shown). PCR analysis confirmed the presence of *nosZ* in all transgenic lines, whereas no amplification was observed in non-transgenic lines.

### *4.5.3 Detection of nosZ mRNA in Transgenic Plants*

To confirm the transcription of the *nosZ* transgene under the control of the *rolD* promoter, RT-PCR was conducted to amplify the *nosZ* fragment of the transgene with gene-specific primer sets. RT-PCR products with the expected size of 1869 bp for *nosZ* mRNA were observed in all *nosZ*-transformed and 1767 bp for *nosZ* mRNA were observed in *nosFLZDY*-transformed transgenic lines (Figure 4.1, a and b, bottom row). In addition, RT-PCR fragments of the expected size for *nosF*, *nosL*, *nosD*, and *nosY* were produced from the rolD-*nosFLZDY* transgenic line (data not shown). Non-transgenic controls did not exhibit any bands corresponding to *nosZ* transcripts. There were notable differences in transcript abundance among the *nosZ* and *nosFLZDY* transgenic lines. Transcript abundance was much higher in *nosZ*-transgenic plants than in *nosFLZDY*-transgenic plants. PCR was also

**Figure 4.1. Identification of rolD-*nosZ* and rolD-*nosFLZDY* transgenic tobacco by PCR and RT-PCR screening.** **a** 1869 bp *ex-nosZ* coding sequence in rolD-*nosZ* transgenic tobacco plants was detected by PCR (top row) and RT-PCR (bottom row). **b** 1767 bp *nosZ* coding sequence in rolD-*nosFLZDY* transgenic tobacco plants was detected by PCR (top row) and RT-PCR (bottom row). M, 1 kb Plus DNA ladder (Invitrogen); +, positive control, recombinant plasmid prolD-*nosZ*/prolD-*nosFLZDY* isolated from *E. coli*; NT, negative control, was genomic DNA from a non-transformed plant. Transformed plants are identified by number, with 1-series plants representing those expressing rolD-*nosZ* and 5-series plants representing those expressing rolD-*nosFLZDY*.


a
M + NT 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 1.10 1.11 1.12 1.13 1.14
2000 bp
1650 bp
2000 bp
1650 bp
b
M + NT 5.1 5.2 5.3 5.4 5.5 5.6 5.7 5.8 5.9 5.10 5.11 5.12 5.13 5.14 5.15
2000 bp
1650 bp
2000 bp
1650 bp

conducted with total RNA not treated with reverse transcriptase to verify that there was no DNA contamination (data not shown).

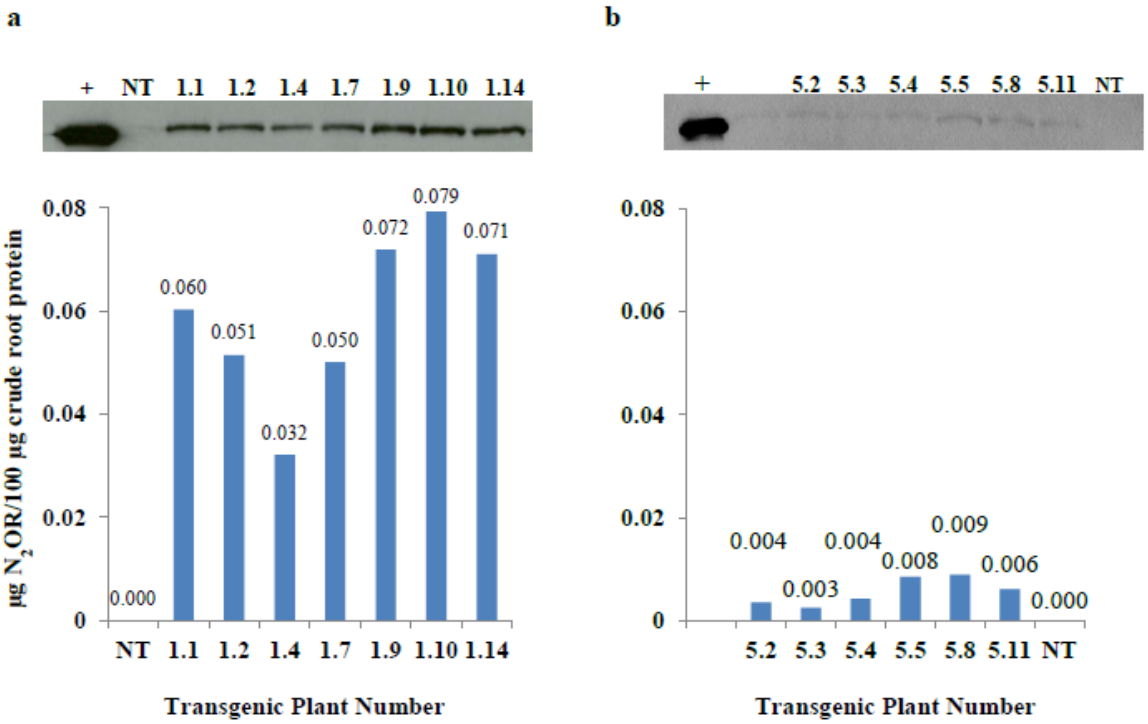
### *4.5.4 Protein Expression Analysis of Root Tissue by Western Immunoblot*

To demonstrate the expression of the $N_2OR$ in tobacco root from $T_0$ transgenic plants, an immunoblot assay of total soluble protein extracts was performed using a polyclonal antibody against the $N_2OR$ antigen. Figure 4.2 displays the immunoblots for rolD-*nosZ* and rolD-*nosFLZDY* samples, showing a single band of the expected molecular mass of 72 kDa. The positive control, $N_2OR$ obtained from *P. stutzeri*, also yielded a signal band at 72 kDa. There was no signal for the presence of $N_2OR$ in non-transformed plant extracts. The images were scanned using ImageJ software (http://rsbweb.nih.gov/ij/) to semi-quantify the expression of $N_2OR$. The corresponding histogram shows yield of recombinant $N_2OR$ in µg $N_2OR$/100 µg crude root protein. Recombinant protein yield varied among rolD-*nosZ* samples, with the lowest *nosZ* yield of 0.032 µg $N_2OR$/100µg crude root protein and the highest 0.079 µg $N_2OR$/100 µg crude root protein. On average, the rolD-*nosZ* plants expressed $N_2OR$ at levels 10-fold higher than rolD-*nosFLZDY* plants, which varied from 0.003 µg $N_2OR$/100 µg crude root protein to 0.009 µg $N_2OR$/100 µg crude root protein.

### *4.5.5 Activity of $N_2OR$ in Root Tissue*

The specific activity of $N_2OR$ in four transgenic tobacco lines was assessed using a spectrophotometric methyl viologen-linked assay. $A_{600}$ was plotted against time for the

**Figure 4.2. Western immunoblot analysis detecting $N_2$OR in total soluble protein extracted from transgenic plant root tissue. a** Histogram showing the yield of recombinant $N_2$OR in *nosZ*-expressing transgenic plants, with the corresponding Western immunoblot. **b** Histogram showing the yield of recombinant $N_2$OR in *nosFLZDY*-expressing transgenic plants, with the corresponding Western immunoblot. +: positive control, $N_2$OR protein purified from *P. stutzeri*; NT: negative control, protein extracted from a non-transformed plant; transformed plants are identified by number.


a
+ NT 1.1 1.2 1.4 1.7 1.9 1.10 1.14
µg N2OR/100 µg crude root protein
0.08
0.06
0.04
0.02
0
0.000
0.060
0.051
0.032
0.050
0.072
0.079
0.071
NT 1.1 1.2 1.4 1.7 1.9 1.10 1.14
Transgenic Plant Number
b
+ 5.2 5.3 5.4 5.5 5.8 5.11 NT
0.08
0.06
0.04
0.02
0
0.004
0.003
0.004
0.008
0.009
0.006
0.000
5.2 5.3 5.4 5.5 5.8 5.11 NT
Transgenic Plant Number

slope-based activity calculations, and the calculated specific activity of the recombinant enzyme (expressed as µmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root protein) was presented in Figure 4.3. The non-transformed control showed minimal activity (0.60 µmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root protein). The positive control, $N_2OR$ from *P. stutzeri*, gave a specific activity of 454 µmol $N_2O$ reduced $min^{-1}$ $mg^{-1}$ protein. $N_2OR$ from the four samples assayed showed considerable variability in their specific activity. Activity was assayed for one individual plant per transgenic line, for the same lines assayed in Figure 4.1 and Figure 4.2.

### *4.5.6 Protein Expression Analysis of Rhizosecreted $N_2OR$*

A Western immunoblot was performed to test for the expression and subsequent excretion of recombinant $N_2OR$ into the rhizosphere. Fractions of hydroponic medium collected over a 25-day period from individual plants were pooled, and the crude protein extracted. The resulting immunoblot is shown in Figure 4.4. A 72-kDa band coinciding with the size of the protein from *P. stutzeri* is present in the exudates from all transgenic lines. The non-transformed control did not exhibit a signal for the presence of $N_2OR$. Yield of recombinant $N_2OR$ was determined based on the chemiluminescent signal and ranged from 0.016 µg $N_2OR$/100 µg crude root secreted protein (rolD:*nosZ*-5.8) to 0.044 µg $N_2OR$/100 µg crude root secreted protein (rolD:*nosZ*-1.7).

### *4.5.7 Activity of $N_2OR$ in Rhizosphere*

The crude protein extracted from the hydroponic culture medium was assayed for

**Figure 4.3. The specific activity of $N_2OR$ extracted from transgenic root tissue measured by the methyl viologen-linked assay**. Trace plots monitor 600 nm absorbance as a function of time. The arrows depict when $N_2O$-saturated water was added as enzyme substrate. The immediate sharp but minor drop in absorbance is due to dilution of the reaction mixture in the cuvette. The change in slope after $N_2O$ addition was used to calculate the specific activities shown above each curve.

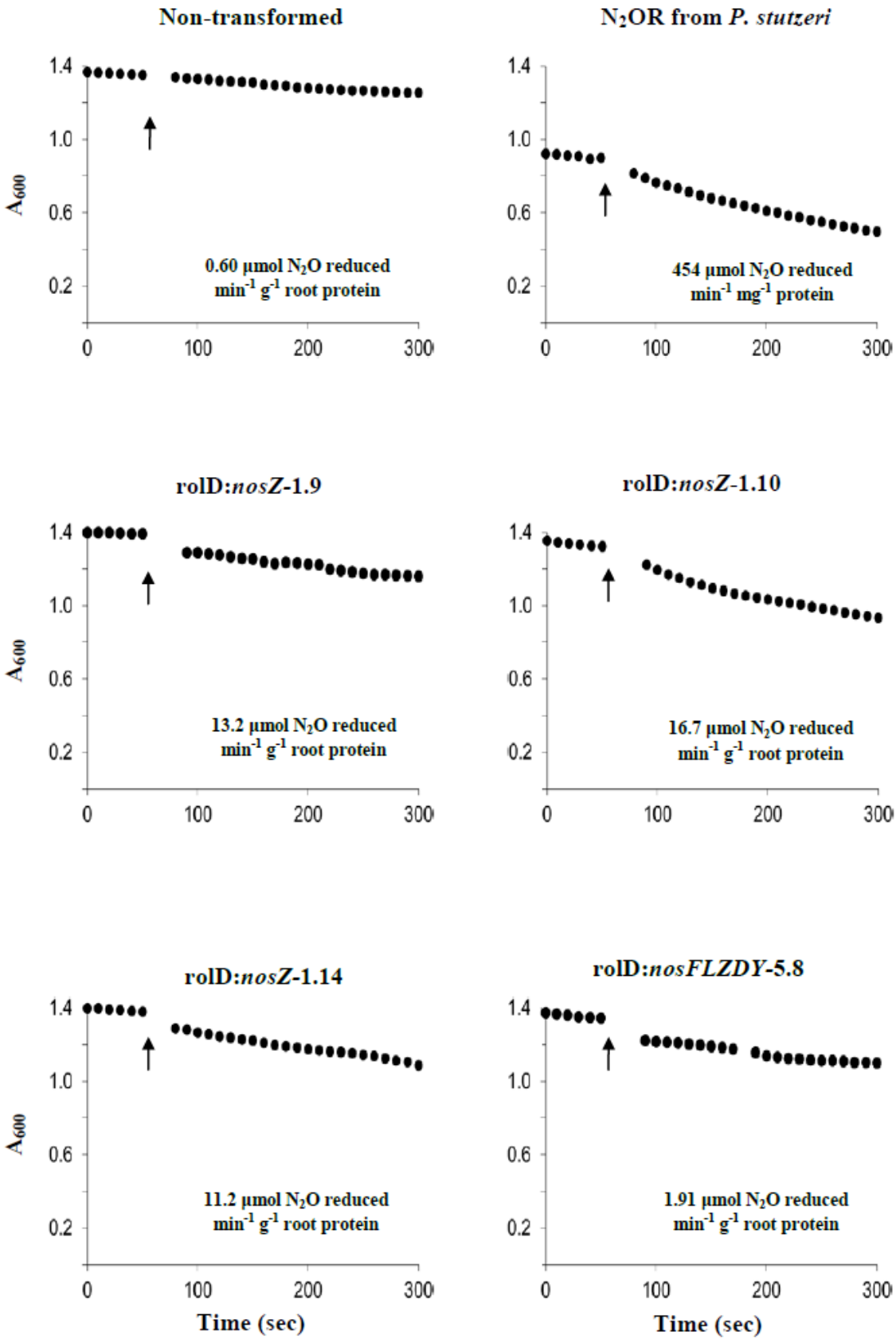
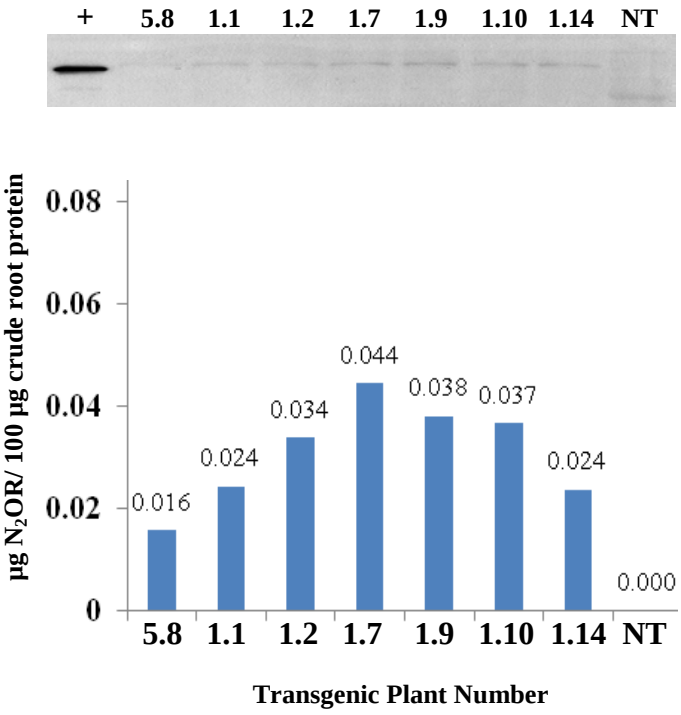

Non-transformed
N2OR from P. stutzeri
rolD:nosZ-1.9
rolD:nosZ-1.10
rolD:nosZ-1.14
rolD:nosFLZDY-5.8
A600
1.4
1.0
0.6
0.2
0
100
200
300
0.60 µmol N2O reduced min-1 g-1 root protein
454 µmol N2O reduced min-1 mg-1 protein
13.2 µmol N2O reduced min-1 g-1 root protein
16.7 µmol N2O reduced min-1 g-1 root protein
11.2 µmol N2O reduced min-1 g-1 root protein
1.91 µmol N2O reduced min-1 g-1 root protein
Time (sec)

**Figure 4.4. Western immunoblot analysis detecting $N_2OR$ in the rhizosphere secreted from transgenic plant root tissue**. Histogram showing the yield of recombinant $N_2OR$ in *nosZ*-expressing and *nosFLZDY*-expressing transgenic plants, with the corresponding Western immunoblot. +: positive control, $N_2OR$ protein purified from *P. stutzeri*; NT: negative control, protein extracted from a non-transformed plant; transformed plants are identified by number.

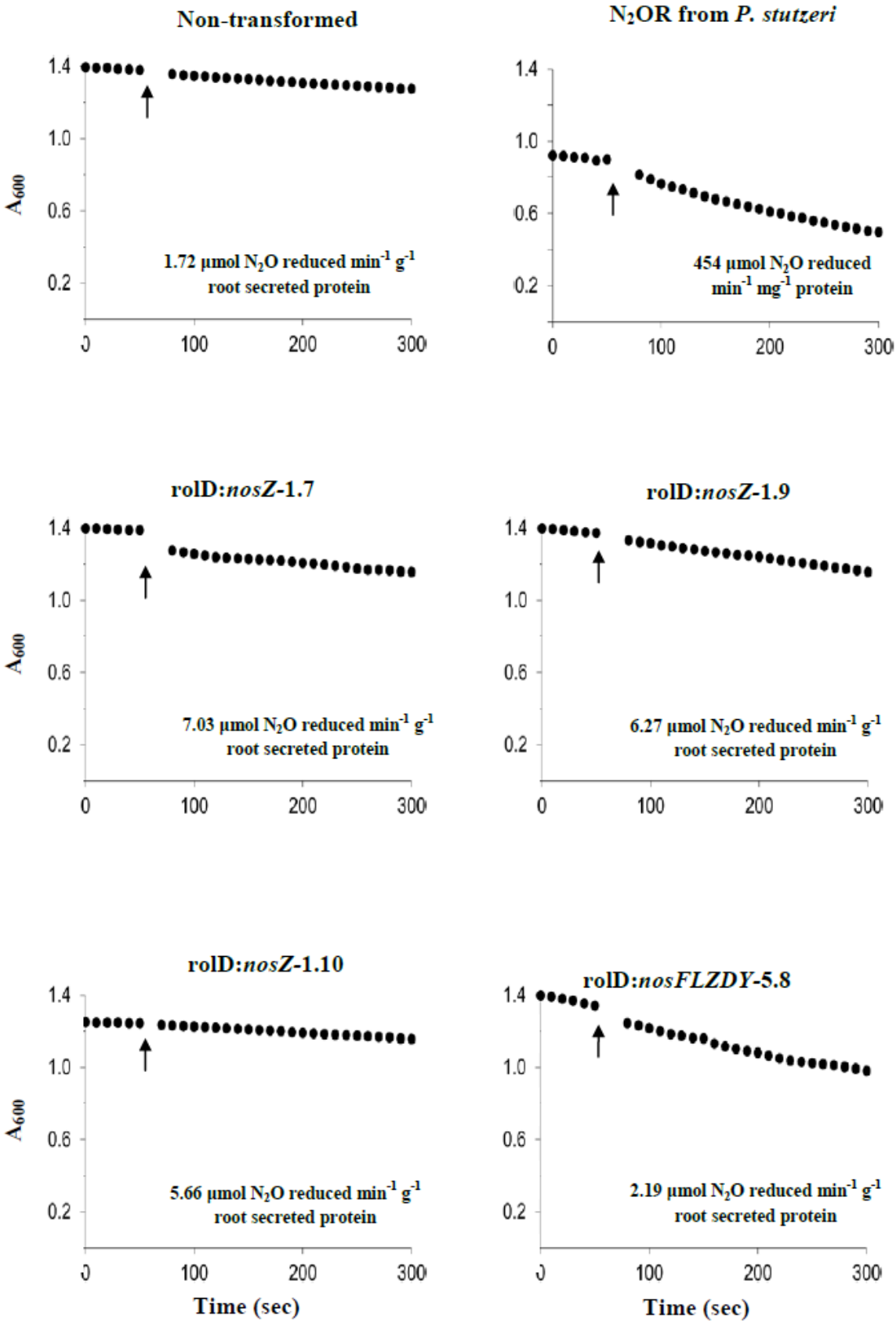

+
5.8
1.1
1.2
1.7
1.9
1.10
1.14
NT
µg N2OR/ 100 µg crude root protein
0.08
0.06
0.04
0.02
0
0.016
0.024
0.034
0.044
0.038
0.037
0.024
0.000
5.8
1.1
1.2
1.7
1.9
1.10
1.14
NT
Transgenic Plant Number

specific activity using the methyl viologen-linked assay. Figure 4.5 reports the calculated specific activity of the recombinant enzyme, expressed as μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root secreted protein. The non-transformed control showed a background specific activity (1.72 μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root secreted protein). The purified enzyme itself serving as a positive control, $N_2OR$ from *P. stutzeri*, gave a specific activity of 454 μmol $N_2O$ reduced $min^{-1}$ $mg^{-1}$ protein. $N_2OR$ from the rolD:*nosZ*-1.7, rolD:*nosZ*-1.9 and rolD:*nosZ*-1.10 transgenic lines showed the highest specific activities of 7.03, 6.27 and 5.66 μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root secreted protein respectively. This is less than half the specific activities of $N_2OR$ located in the root tissue of the same transgenic lines (see Figure 4.3). In contrast, the specific activity of $N_2OR$ extracted from the hydroponic media of rolD:*nosFLZDY*-5.8 plants was slightly higher than $N_2OR$ compartmentalized in the roots (compare 2.19 μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root secreted protein versus 1.91 μmol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root protein**)**.

## 4.6 Discussion

Human-induced climate change has become one of the defining issues of our time given the immense environmental, social and economic consequences of our continued production and release of greenhouse gases (Callendar, 1938). $N_2O$ emission rates have been exacerbated with the increasing use of synthetic nitrogen-based fertilizers whereby annual $N_2O$ emissions from global soils now exceed 2.6 Tg (1 Tg = 1 million tons) of $N_2O$-N (Davidson, 2009).

**Figure 4.5. The specific activity of $N_2OR$ in the rhizosphere, excreted from transgenic plant root tissue by the methyl viologen-linked assay**. Trace plots monitor 600 nm absorbance as a function of time. The arrows depict when $N_2O$-saturated water was added as enzyme substrate. The immediate sharp but minor drop in absorbance is due to dilution of the reaction mixture in the cuvette. The change in slope after $N_2O$ addition was used to calculate the specific activities shown above each curve.


Non-transformed
1.72 µmol N2O reduced min-1 g-1 root secreted protein
N2OR from P. stutzeri
454 µmol N2O reduced min-1 mg-1 protein
rolD:nosZ-1.7
7.03 µmol N2O reduced min-1 g-1 root secreted protein
rolD:nosZ-1.9
6.27 µmol N2O reduced min-1 g-1 root secreted protein
rolD:nosZ-1.10
5.66 µmol N2O reduced min-1 g-1 root secreted protein
rolD:nosFLZDY-5.8
2.19 µmol N2O reduced min-1 g-1 root secreted protein
A600
Time (sec)

This study presents a proof-of-concept for the use of $N_2$OR-expressing tobacco plants for the phytoremediation of the environmental pollutant $N_2O$. We produced expression cassettes for both the *nosZ* gene and *nosFLZDY* genes. The root-specific promoter, rolD, provided sufficient expression in tobacco roots. Our findings suggest that *nosZ* gene expression cassettes, both the single cassette prolD:*nosZ* and the megacassette prolD:*nosFLZDY,* were correctly integrated into the tobacco genome and expressed (Figure 4.1). Transgenic lines differentially expressed rolD:*FLZDY* (Figure 4.1, b). This is presumably because the transgene is inserted into the plant genome at random, and expression varies depending on the sequences surrounding the integration site (Clark *et al*., 1993).

In calculating recombinant protein yield from these transgenic plant roots, two patterns became evident. First, the yield is positively correlated to rolD:*nosZ* expression levels determined by RT-PCR. For example, plant line 1.10 has very strong transcription despite the apparent low content of the transgene (Figure 4.1, a). This 1.10 mRNA is correspondingly translated into the most abundant level of protein detected by anti-$N_2$OR antibodies by western hybridization (Figure 4.2, a). Plant line 1.10 extracts correspondingly contained the highest amount of protein, 0.079 µg $N_2$OR/100 µg root protein. And finally, it turned out that this plant line 1.10 also had the highest specific activity for this novel enzyme, 16.7 umol $N_2O$ reduced $min^{-1}$ $g^{-1}$ root protein (Figure 4.3). As for the second pattern, plants expressing rolD:*nosZ* yielded ten-fold more $N_2$OR than those expressing rolD:*nosFLZDY*. A possible explanation for the lower-expressing rolD:*nosFLZDY* plants is gene silencing. Since gene silencing increases with increasing transcript size (Johnston *et al*., 2011; Melnyk *et al*., 2011), the larger rolD:*nosFLZDY* construct may have been subject to gene silencing in the tobacco plant, whereas the rolD:*nosZ* construct was not.

$N_2OR$ from rolD:*nosZ* and rolD:*nosFLZDY* plant root tissue was shown to be correctly assembled. Western blot analysis showed, by electrophoretic mobility, that $N_2OR$ purified from *P. stutzeri* and $N_2OR$ in crude protein extract isolated from plant root tissue were the same size (Figure 4.2). Likewise, in hydroponic culture experiments we detected $N_2OR$ in transgenic root exudates, but in a lower quantity than in the root tissue (Figure 4.4). These results contrast those from previous experiments, which revealed more recombinant protein in the hydroponic medium than in the tobacco root tissue (Borisjuk *et al*., 1999). This is due to inadequate transport of the 140-kDa recombinant $N_2OR$ dimer from root cells across the epidermis and into the rhizosphere. Increasing the permeability of the root cell wall by supplying the plant with a plant growth regulator such as auxin may facilitate better secretion of $N_2OR$ (Drake *et al*., 2009).

The present results demonstrate that functional $N_2OR$ was expressed in the roots and was secreted into the hydroponic rhizosphere in a form able to catalyze the conversion of $N_2O$ to $N_2$ (Figure 4.3, 4.5). The specific activity of rhizosecreted $N_2OR$ was half that of $N_2OR$ isolated from root tissue. We infer that this loss in activity is due to the aerobic nature of the hydroponic culture, as oxygen can cause the inactivation of $N_2OR$ (Clays-Josserand *et al*., 1995).

In both plant roots and root exudates, the specific activity of $N_2OR$ was less than that of the native enzyme in *P. stutzeri* (Figure 4.3, 4.5). The lower activity of recombinant protein might be due to oxygen inactivation or low partial pressure of $N_2O$. The extent to which denitrification occurs is highly dependent upon $O_2$ partial pressure and nitrogen availability (Philippot *et al.*, 2009). Since, in these experiments, transgenic plants were grown in the presence of oxygen and with normal levels of nitrogen, optimal $N_2OR$ production was not fully realized. Just as bacteria grown in anaerobic conditions with $N_2O$

most highly express $N_2OR$ (Madigan *et al.*, 2002), the engineered plants also may express more $N_2OR$ in anaerobic soil types such as those that are heavy-textured or which contain a high percentage of organic material (Inglett *et al*., 2005). An additional factor is exposure to $N_2O$. Experiments on *P. stutzeri* showed that the micro-organisms are induced to express $N_2OR$ when grown in the presence of $N_2O$ (Matsubara *et al*., 1982). Thus, increasing the concentration of $N_2O$ in the greenhouse might increase $N_2OR$ transcription. If the plants were grown in the presence of denitrifying bacteria (e.g. in a field), roots might be exposed to sufficient levels of $N_2O$ to induce higher production of recombinant $N_2OR$ (Richardson *et al*., 2009).

The present results indicate that transgenic tobacco plants stably expressing the *nosZ* transgene are capable of producing functional recombinant $N_2OR$. Further analysis will include examination of the ability of these plants to reduce $N_2O$ in field trials using various soil types and environments together with single plant canopy enclosures to measure nitrogen flux. Once the reduction of $N_2O$ by the transgenic plant lines has been proven effective in test plots, studies to improve recombinant enzyme yield, as well as stability and activity of the enzyme will be of interest.

Since “arable lands” produce two-thirds of the world’s $N_2O$ (Otanez and Glantz, 2011), field crops are logical candidates for $N_2O$ remediation trials. Crops can be used to amplify the limited capacity of tilled and fertilized soils to reduce $N_2O$ emissions. About 30 countries have adopted biotech crops like maize, soybean, canola and cotton (ISAAA) that are engineered to protect soil and groundwater by reducing the spraying of insecticides and herbicides. Such gene-pyramiding is growing rapidly in genetic engineering, delivering many input and output advantages to producers and consumers, e.g. enhanced water-use efficiency, enhanced cold tolerance, and increased yield. Extending the remit of biotech crops to include

soil gas flux, namely, reduction of fertilizer-induced $N_2O$ emission, should encounter positive regulatory approval given the potential contribution to purge those same soils of this deleterious gas (Ghimire *et al.,* 2011). The $N_2O$ emissions from agricultural soil are 40 grams $N_2O$ $ha^{-1}$ $d^{-1}$ (Stuart Strand, University of Washington, personal communication). If $N_2OR$ was expressed in maize, the conversion of $N_2O$ by the roots of maize could be as high as $2.11\times10^3$ grams $N_2O$ $ha^{-1}$ $day^{-1}$ (Supplementary Method 2), more than 50 times greater than $N_2O$ emissions from agricultural soil. By accumulating sufficient levels of $N_2OR$, soils growing these transgenic crops could capture and alleviate $N_2O$ pollution.

## 4.7 Acknowledgements

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## 4.9 Supporting information

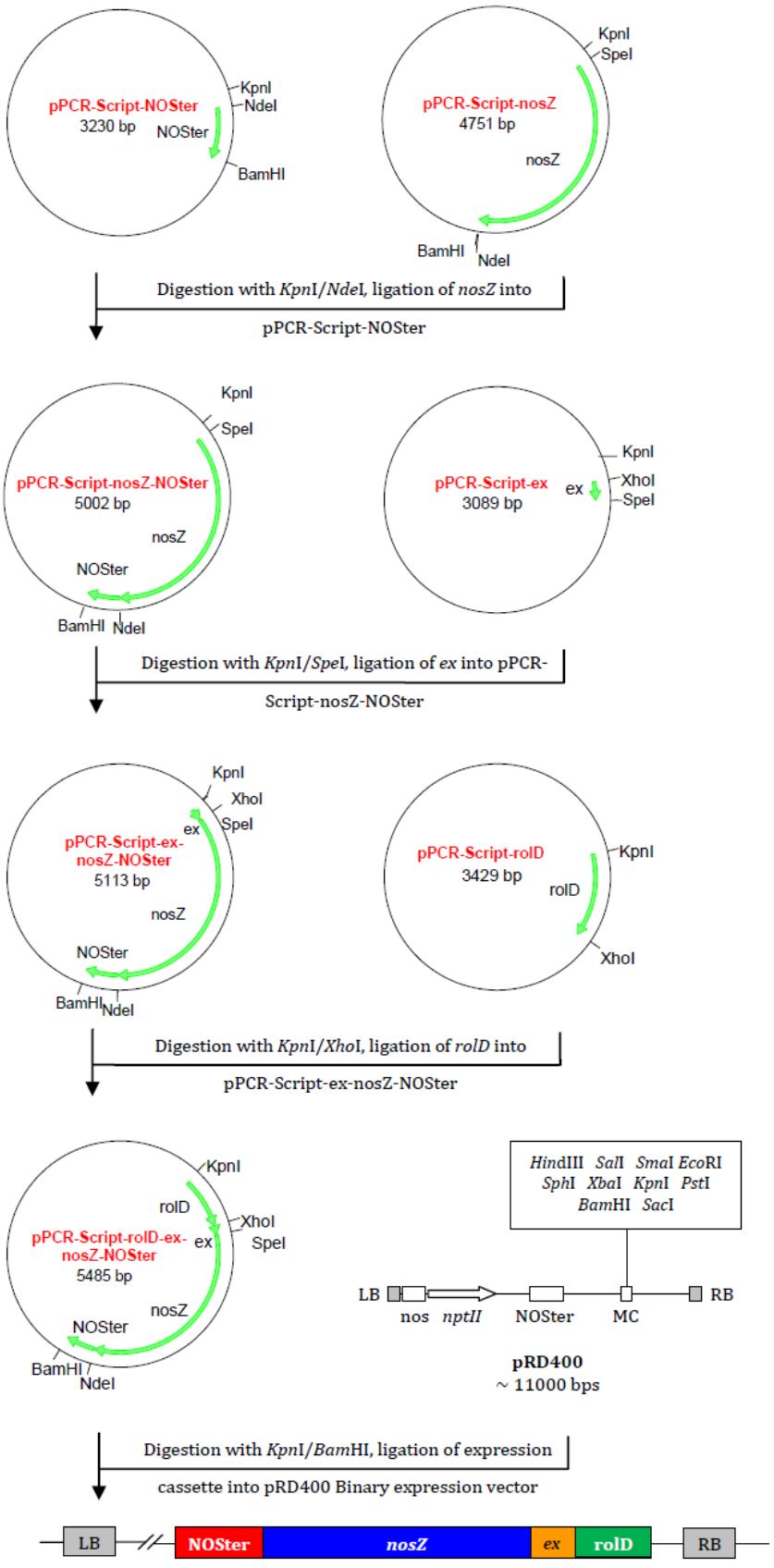
**Supplementary Table 4.1. PCR reaction conditions for amplification of expression construct components**. Primers, reaction conditions and PCR cycling details used for amplification of the *rolD* promoter sequences, signal sequences, *nos* DNA coding sequence, and *Noster* sequences.

| Vector | Target | Primer | Sequence | PCR Program |
|---|---|---|---|---|
| **nosZ Unit** | rolD | RolDZF | 5'-GCGGTACCAGTTGTATCCGAATACTCATA-3'<br>*Kpn*I | 1: 95ºC for 4 min 2: 95ºC for 30 sec<br>3: 50ºC for 45 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | RolDR2 | 5'-ATCTCGAGCTGCCTTGTAGGTGCGA-3'<br>*Xho*I | |
| | *extensin* signal sequence | ExSigZF | 5'-TAGGTACCTACTCGAGATGGGAAGAATTGCTAGAGG-3'<br>*Kpn*I *Xho*I | 1: 95ºC for 4 min 2: 95ºC for 30 sec<br>3: 50ºC for 30 sec 4: 72ºC for 30 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | ExSigZR | 5'-GAGGATCCATACTAGTGGCTGTGGTTTCGGAAG-3'<br>*Bam*HI *Spe*I | |
| | *nosZ* | NosZF | 5'-GACTAGTCAGGCCGTCAAGGAGTCCAAG-3'<br>*Spe*I | 1: 95ºC for 5 min 2: 95ºC for 1 min<br>3: 58ºC for 1 min 4: 72ºC for 2 min<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | NosZR | 5'-TAGGATCCAACATATGTTAGGCCGGCTCGACCATC-3'<br>*Bam*HI *Nde*I | |
| | NOSter | NOSF | 5'-CGCATATGCGTTCAAACATTTGGCAATAAAG-3'<br>NdeI | 1: 95ºC for 4 min 2: 95ºC for 30 sec<br>3: 52ºC for 40 sec 4: 72ºC for 40 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | NOSZR | 5'-TAGGATCCCCCGATCTAGTAACATAGATG-3'<br>*Bam*HI | |
| **nosF Unit** | rolD | RolDFF | 5'-GAATTCAGTTGTATCCGAATACTCATATATG-3'<br>*Eco*RI | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 53ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 7 min<br>7: Hold at 4ºC |
| | | RolDR1 | 5'-AGATCTCTGCCTTGTAGGTGCGAATTTTC-3'<br>*Bgl*II | |
| | *nosF* | NosFF | 5'-AGATCTATGAACGCCGTCGAGATC-3'<br>*Bgl*II | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 58ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 7 min<br>7: Hold at 4ºC |
| | | NosFR | 5'-CATATGTCATAGACGGCCCTCCTG-3'<br>*Nde*I | |
| | NOSter | NOSFF | 5'-CATATGCGTTCAAACATTTGGCAATAAAG-3'<br>*Nde*I | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 54ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 7 min<br>7: Hold at 4ºC |
| | | NOSFR | 5'-GAGCTCCCCGATCTAGTAACATAGATG-3'<br>*Sac*I | |

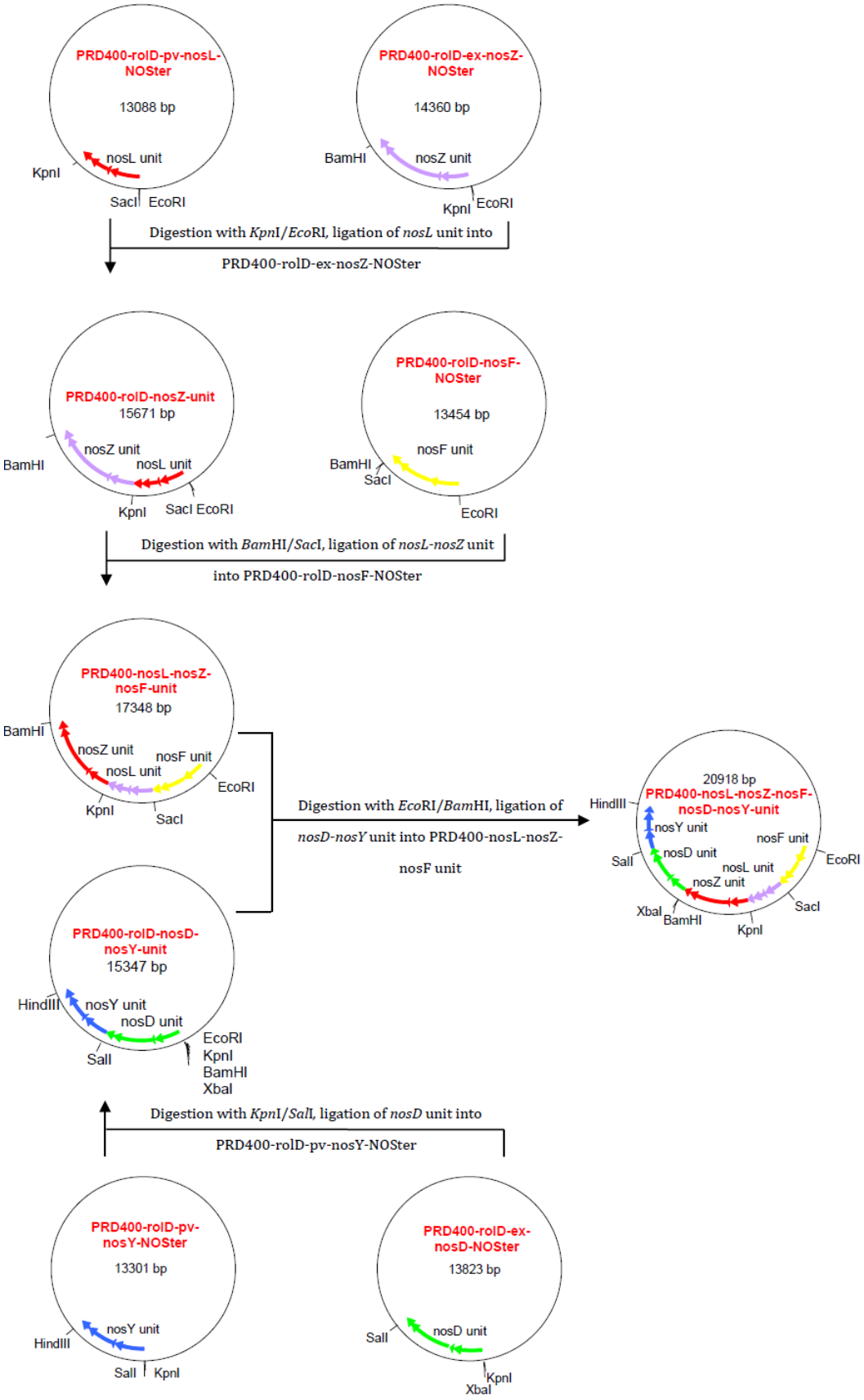
| | | | | | |
|---|---|---|---|---|---|
| **nosL Unit** | rolD | RolDLF | 5'-GTGAGCTCAGTTGTATCCGAATACTCATA-3' *Sac*I | 1: 94ºC for 4 min<br>3: 53ºC for 30 sec<br>5: Repeat 30 times from 2<br>7: Hold at 4ºC | 2: 94ºC for 1 min<br>4: 72ºC for 45 sec<br>6: 72ºC for 10 min |
| | | RolDR1 | 5'-AGATCTCTGCCTTGTAGGTGCGAATTTTC-3' *Bgl*II | | |
| | *Phaseolus vulgaris* alpha amylase inhibitor-1 signal sequence | Pv SigF | 5'-CAGATCTATGGCTTCCTCCAACTTAC-3' *Bgl*II | 1: 94ºC for 4 min<br>3: 54ºC for 30 sec<br>5: Repeat 30 times from 2<br>7: Hold at 4ºC | 2: 94ºC for 1 min<br>4: 72ºC for 30 sec<br>6: 72ºC for 10 min |
| | | PvSigR | 5'-GTACTAGTGTTGAAGGAGGTTTCGGTG-3' *Spe*I | | |
| | *nosL* | NosLF | 5'-CTACTAGTTGCGGGGAGAAGGAGGAGGTTC-3' *Spe*I | 1: 94ºC for 4 min<br>3: 60ºC for 30 sec<br>5: Repeat 30 times from 2<br>7: Hold at 4ºC | 2: 94ºC for 1 min<br>4: 72ºC for 50 sec<br>6: 72ºC for 10 min |
| | | NosLR | 5'-GACATATGTCAGTGGCCTGCGTGTGCG-3' *Nde*I | | |
| | NOSter | NOSF | 5'-CGCATATGCGTTCAAACATTTGGCAATAAAG-3' *Nde*I | 1: 94ºC for 4 min<br>3: 54ºC for 30 sec<br>5: Repeat 30 times from 2<br>7: Hold at 4ºC | 2: 94ºC for 1 min<br>4: 72ºC for 45 sec<br>6: 72ºC for 10 min |
| | | NOSLR | 5'-TAGGTACCCCCGATCTAGTAACATAGATG-3' *Kpn*I | | |
| **nosD Unit** | rolD | RolDDF | 5'-CGTCTAGAAGTTGTATCCGAATACTCATA-3' *Xba*I | 1: 94ºC for 4 min<br>3: 53ºC for 30 sec<br>5: Repeat 30 times from 2<br>7: Hold at 4ºC | 2: 94ºC for 1 min<br>4: 72ºC for 45 sec<br>6: 72ºC for 7 min |
| | | RolDR1 | 5'-AGATCTCTGCCTTGTAGGTGCGAATTTTC-3' *Bgl*II | | |
| | *extensin* signal sequence | ExSigDF | 5'-AGATCTATGGGAAGAATTGCTAGAG-3' *Bgl*II | 1: 94ºC for 4 min<br>3: 57ºC for 30 sec<br>5: Repeat 30 times from 2<br>7: Hold at 4ºC | 2: 94ºC for 1 min<br>4: 72ºC for 30 sec<br>6: 72ºC for 7 min |
| | | ExSigDR | 5'-ACTAGTCGCTGTGGTTTCGGAAG-3' *Spe*I | | |
| | *nosD* | NosDF | 5'-ACTAGTGCACCGCAATCGATTACCAC-3' *Spe*I | 1: 94ºC for 4 min<br>3: 58ºC for 30 sec<br>5: Repeat 30 times from 2<br>7: Hold at 4ºC | 2: 94ºC for 1 min<br>4: 72ºC for 85 sec<br>6: 72ºC for 7 min |
| | | NosDR | 5'-CATATGTGACGTTGGTTCCTGCTTTTC-3' *Nde*I | | |
| | NOSter | NOSF | 5'-CGCATATGCGTTCAAACATTTGGCAATAAAG-3' | 1: 94ºC for 4 min | 2: 94ºC for 1 min |

| | | | | |
|---|---|---|---|---|
| | | | *Nde*I | |
| | | NOSDR | 5’-TAGTCGACCCCGATCTAGTAACATAGATG-3’ | 3: 54ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 7 min<br>7: Hold at 4ºC |
| | | | *Sal*I | |
| | rolD | RolDYF | 5’-GCGTCGACAGTTGTATCCGAATACTCATA-3’ | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 53ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Sal*I | |
| | | RolDR1 | 5’-AGATCTCTGCCTTGTAGGTGCGAATTTTC-3’ | |
| | | | *Bgl*II | |
| | *Phaseolus vulgaris* alpha amylase inhibitor-1 signal sequence | PvSigF | 5’-CAGATCTATGGCTTCCTCCAACTTAC-3’ | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 54ºC for 30 sec 4: 72ºC for 30 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Bgl*II | |
| | | PvSigR | 5’-GTACTAGTGTTGAAGGAGGTTTCGGTG-3’ | |
| | | | *Spe*I | |
| **nosY Unit** | *nosY* | NosYF | 5’-TAACTAGTATCGCCTGGCTCGGCGCTG-3’ | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 55ºC for 30 sec 4: 72ºC for 55 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Spe*I | |
| | | NosYR | 5’-TACATATGTCAGGTCAAGCGCCGGCG-3’ | |
| | | | *Nde*I | |
| | NOSter | NOSF | 5’-CGCATATGCGTTCAAACATTTGGCAATAAAG-3’ | 1: 94ºC for 4 min 2: 94ºC for 1 min<br>3: 54ºC for 30 sec 4: 72ºC for 45 sec<br>5: Repeat 30 times from 2 6: 72ºC for 10 min<br>7: Hold at 4ºC |
| | | | *Nde*I | |
| | | NOSYR | 5’-GCAAGCTTCCCGATCTAGTAACATAGATG-3’ | |
| | | | *Hind*III | |

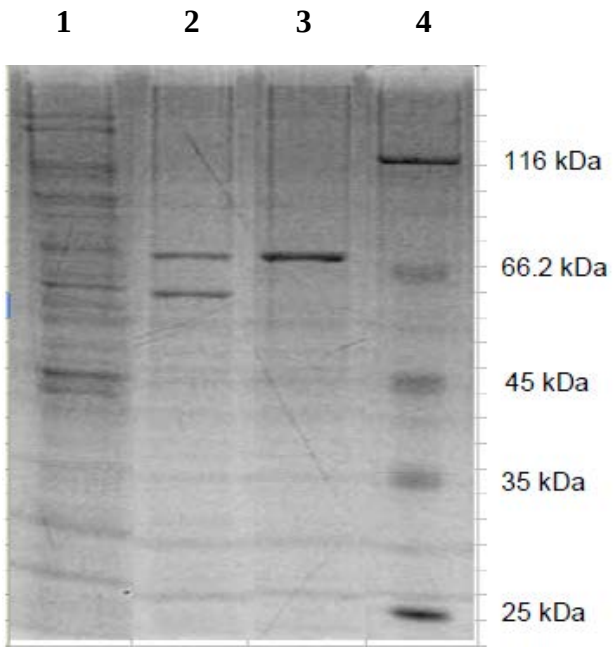
**Supplementary Figure 4.1. Construction of the rolD-*nosZ* expression cassette**. The nosZ expression construct is comprised of rolD promoter, *extensin* signal sequence, *nosZ* from *P. stutzeri*, and NOSter polyadenylation signal.


pPCR-Script-NOSter
3230 bp
NOSter
KpnI
NdeI
BamHI
pPCR-Script-nosZ
4751 bp
nosZ
KpnI
SpeI
BamHI
NdeI
Digestion with KpnI/NdeI, ligation of nosZ into
pPCR-Script-NOSter
pPCR-Script-nosZ-NOSter
5002 bp
nosZ
NOSter
KpnI
SpeI
BamHI
NdeI
pPCR-Script-ex
3089 bp
ex
KpnI
XhoI
SpeI
Digestion with KpnI/SpeI, ligation of ex into pPCR-
Script-nosZ-NOSter
pPCR-Script-ex-
nosZ-NOSter
5113 bp
ex
nosZ
NOSter
KpnI
XhoI
SpeI
BamHI
NdeI
pPCR-Script-rolD
3429 bp
rolD
KpnI
XhoI
Digestion with KpnI/XhoI, ligation of rolD into
pPCR-Script-ex-nosZ-NOSter
pPCR-Script-rolD-ex-
nosZ-NOSter
5485 bp
rolD
ex
nosZ
NOSter
KpnI
XhoI
SpeI
BamHI
NdeI
HindIII SalI SmaI EcoRI
SphI XbaI KpnI PstI
BamHI SacI
LB
nos
nptII
NOSter
MC
RB
pRD400
~ 11000 bps
Digestion with KpnI/BamHI, ligation of expression
cassette into pRD400 Binary expression vector
LB
NOSter
nosZ
ex
rolD
RB

**Supplementary Figure 4.2. Construction of the rolD-*nosFLZDY* expression megacassette**. The nosFLZDY expression megacassette is comprised of 5 units, *nosF*, *nosL*, *nosZ*, *nosD*, and *nosY* unit. RolD is the root-specific promoter; Ex is the *extensin* signal sequence; Pv is the *Phaseolus vulgaris* alpha amylase inhibitor-1 signal sequence; *nosF*, *nosL*, *nosZ*, *nosD*, and *nosY* encode their respective genes from *P. stutzeri*; and NOSter is the polyadenylation termination sequence from *Agrobacterium tumefaciens*.


PRD400-rolD-pv-nosL-NOSter
13088 bp
nosL unit
KpnI
SacI
EcoRI
PRD400-rolD-ex-nosZ-NOSter
14360 bp
BamHI
nosZ unit
KpnI
EcoRI
Digestion with KpnI/EcoRI, ligation of nosL unit into
PRD400-rolD-ex-nosZ-NOSter
PRD400-rolD-nosZ-unit
15671 bp
BamHI
nosZ unit
nosL unit
KpnI
SacI EcoRI
PRD400-rolD-nosF-NOSter
13454 bp
nosF unit
BamHI
SacI
EcoRI
Digestion with BamHI/SacI, ligation of nosL-nosZ unit
into PRD400-rolD-nosF-NOSter
PRD400-nosL-nosZ-nosF-unit
17348 bp
BamHI
nosZ unit
nosF unit
nosL unit
EcoRI
KpnI
SacI
PRD400-rolD-nosD-nosY-unit
15347 bp
HindIII
nosY unit
nosD unit
SalI
EcoRI
KpnI
BamHI
XbaI
Digestion with EcoRI/BamHI, ligation of
nosD-nosY unit into PRD400-nosL-nosZ-
nosF unit
20918 bp
PRD400-nosL-nosZ-nosF-nosD-nosY-unit
HindIII
nosY unit
nosF unit
SalI
nosD unit
nosL unit
EcoRI
nosZ unit
XbaI
BamHI
SacI
KpnI
Digestion with KpnI/SalI, ligation of nosD unit into
PRD400-rolD-pv-nosY-NOSter
PRD400-rolD-pv-nosY-NOSter
13301 bp
HindIII
nosY unit
SalI
KpnI
PRD400-rolD-ex-nosD-NOSter
13823 bp
SalI
nosD unit
KpnI
XbaI

**Supplementary Figure 4.3. 10% SDS-PAGE analysis of $N_2OR$ protein from different purification steps**. Lane 1: crude clarified *P. stutzeri* lysate (1:50 dilution). Lane 2: pooled fractions from preparative anion exchange chromatography (1:50 dilution). Lane 3: pooled fractions from hydroxyapatite chromatography from half of the sample (1:25 dilution). Lane 4: marker proteins (Fermentas). Molecular weight of $N_2OR$ monomer from *P. stutzeri* is 72 kDa, and molecular weight of nitrite reductase from *P. stutzeri* is 61 kDa.


1
2
3
4
116 kDa
66.2 kDa
45 kDa
35 kDa
25 kDa

**Supplementary Method 1**

**$N_2$OR activity calculation.** The activity of $N_2$OR was evaluated using the Beer-Lambert law. The molar absorptivity (ε) of methyl viologen is 11400 $M^{-1}$ $cm^{-1}$ and the path length (ℓ) was 1 cm. A: absorbance; C: concentration; n is moles; and V is volume. By substitution and simplification, the following expression can be obtained for the rate of dye oxidised as a function of the rate of absorbance change.

$$A = \varepsilon L C$$

$$\frac{dA}{dt} = \varepsilon L \frac{dC}{dt}$$

$$\frac{dC}{dt} = \left(\frac{1}{\varepsilon L}\right)\frac{dA}{dt}$$

$$\frac{d\left(n/V\right)}{dt} = \left(\frac{1}{\left(11400\ M^{-1}cm^{-1}\right)\left(1\ cm\right)}\right)\frac{dA}{dt}$$

$$\frac{dn}{dt} = \left(\frac{mol}{11400}\right)\frac{dA}{dt}$$

$$\frac{dn}{dt} = \left(\frac{\mu mol}{0.0114}\right)\frac{dA}{dt}$$

The value used for the rate of absorbance change should be corrected for background. And since the stoichiometric ratio between methyl viologen and $N_2O$ reduced is 2:1, the activity per minute per gram of sample can be evaluated as follows where m is the mass of leaf extract or purified protein added:

$$Activity = \frac{\left(\frac{1\ \mu mol\ N_2O}{2\ \mu mol\ dye}\right)\left(\frac{\mu mol\ dye}{0.0114}\right)\left(dA/dt\right)}{m}$$

For example, if the background-corrected rate of absorbance change was $\left(dA/dt\right) = 0.0486 \times 10^{-4}\ min^{-1}$ for a purified protein sample of $m = 0.00478\ mg$, then the activity is calculated as follows:

$$Activity = \frac{\left(\frac{\mu mol\ N_2O}{0.0228}\right)\left(0.0486\ min^{-1}\right)}{0.00478\ mg\ protein} = 446\ \frac{\mu mol\ N_2O}{min\ mg\ protein}$$

**Supplementary Method 2.**

**Calculation of mitigation potential of $N_2OR$ biotech crops.** Annual conversion of $N_2O$ was estimated assuming that the $N_2OR$ trait were to be expressed in maize roots.

Using the $N_2OR$ activity of 16.7 μmol $N_2O$ reduced $min^{-1}$ $gram^{-1}$ root protein (Fig.3), and given that root mass in maize agriculture ranges from 2 to 4 Mg roots $ha^{-1}$ (References a,b) and assuming that, root protein content is 1mg $gram^{-1}$, and given that maize already fluxes $CO_2$ and $O_2$ gases, then the amount of $N_2O$ could be:

= root weight × root protein content × the activity of $N_2OR$ in the root × tobacco growth period

= 2 Mg $ha^{-1}$ × 1 mg root protein $gram^{-1}$ root × 16.7 μmol $N_2O$ reduced $min^{-1}$ $gram^{-1}$ root protein × 24 hour $day^{-1}$

= $2x10^6$ gram/ha × 1 $x10^{-3}$ gram root protein/gram root × 16.7 $x10^{-6}$ mol $N_2O$ reduced $min^{-1}$ $gram^{-1}$ root protein × 44 gram/mol × 24 hour/day × 60 min/hour

= $2.11 \times 10^3$ gram $ha^{-1}$ $day^{-1}$

[a] Kemanian AR, Stockle CO, Huggins DR, Viega LM (2007) A simple method to estimate harvest index in grain crops. *Field Crops Research*, **103**, 208-216.

[b] http://www.uoguelph.ca/~mgoss/two/Sr_ratio.html (last viewed 9 August 2011).

# Chapter 5

# General Discussion

## 5.1 Summary of results

The bulk of human-produced $N_2O$ originates from the agricultural sector – most notably from nitrogen-fertilised soils. To our knowledge, this thesis provides the first report of mitigating $N_2O$ emissions from agriculture using transgenic strategies.

To help develop a phytoremediation system whereby genetically modified crops might be able to "scrub" the $N_2O$ emissions from their own soils, bacterial $N_2OR$ was heterologously expressed in transformed tobacco plants as a proof-of-principle in a model plant system. Preliminary studies by others in recombinant bacterial systems showed that the functional assembly of the catalytic centres ($Cu_Z$) of $N_2OR$ was lacking when only *nosZ* was expressed (Viebrock and Zumft, 1988). In transformed bacteria, it was found that the coexpression of *nosZ* with *nosD, nosF* and *nosY* was necessary for production of the catalytically active holoenzyme. To explore the possibility of eukaryotic plant cytoplasm being diverse enough to support reconstitution of this copper-activated enzyme, I generated two series of transformation events: transgenic tobacco plants expressing the *nosZ* gene alone (Chapter 2), as well as a series of tobacco plants in which the other *nos* genes were coexpressed with *nosZ* (Chapters 3 and 4). More than 100 transgenic tobacco lines, expressing *nosZ* and *nosFLZDY* under the control of *rolD* promoter and d35S promoter, were tested. The *nosZ* gene from *Pseudomonas stutzeri* when heterologously expressed in tobacco provided evidence that active recombinant $N_2OR$ was produced, when assayed by methyl viologen (Figure 2.1). By purifying the enzyme from bacteria itself and using it as a positive control, the activity of $N_2OR$ expressed in transgenic plants, analyzed with methyl viologen-

linked enzyme assay, showed detectable $N_2O$ reducing activity in Figure 2.1, Figure 3.5, Figure 4.3 and Figure 4.5. The $N_2O$-reducing patterns were similar to that of the positive control purified bacterial $N_2OR$ (Figure 2.1 lowest right panel).

The data indicated that the heterologous expression of bacterial $N_2OR$ in plants, without the expression of the accessory Nos proteins, could convert $N_2O$ into inert $N_2$. This suggests that atmospheric phytoremediation of $N_2O$ by plants harbouring $N_2OR$ could be invaluable in efforts to slash emissions. The data confirmed the original three hypotheses:

There is a cellular pool of available copper in tobacco plant cells sufficient to activate the nosZ protein *in planta* (Chapter 2);

The proteome of a plant cell is sophisticated enough to complement the need of nosZ assembly for an ABC-type transporter (Chapter 3);

The root-expressed $N_2OR$ is more active than that secreted from roots (Chapter 4).

## 5.2 Outstanding challenges in the heterologous expression of bacterial $N_2OR$ *in planta*

As a theoretical way to mitigate the harmful effects of the greenhouse gas, $N_2O$, enhancing the conversion of $N_2O$ to $N_2$ by heterologously expressing bacterial $N_2OR$ in plants could be used to 'scrub' the emissions (Inatomi, 1999). But at present this is an ambitious and challenging genetic engineering project, especially given the global scale of the challenge and the socio-ethical issues surrounding increased fertilizer use (Richardson et al., 2009). High expression of $N_2OR$ in commercial plants would require intensive work with some risk, but the potential benefits in terms of reduced greenhouse gas levels and greatly reduced radiative forcing may turn out to be significant if implemented in a regulated ramp-

up as was done for the introduction of GMO cotton in Australia and Bt cotton in India and China (ISAAA, 2010).

The expression and assembly of the complex metalloenzyme $N_2OR$ in plants is challenging. For expression in bacteria $N_2OR$ assembly requires at least five proteins other than the basic subunit NosZ. The present study posited whether the highly conserved "accessory" gene products are necessary in the assembly of the catalytic center of $N_2OR$ *in planta*. The results as presented in Chapters 2, 3, and 4 indicated that the copper atoms could have inserted into the $N_2OR$ apoprotein either spontaneously or through a plant-mediated process, without the products of the ABC-type transporter complex comprised of NosD, NosF, and NosY as well as the periplasmic Cu chaperone NosL.

Numerous forms of $N_2OR$ have been isolated from various species of denitrifiers based on different protein isolation procedures, concerned especially with the levels of exposure to oxygen (Rasmussen et al., 2002; Zumft and Kroneck, 2007). These spectroscopically different forms of enzyme vary dramatically in activities towards $N_2O$ reduction, and in the colour of the purified protein due to the changes at the catalytic site ($Cu_Z$). For example, the active form of $N_2OR$ isolated from *P. stutzeri* under oxygen-free conditions is a type I reductase ($N_2OR$ I) known as the purple form, while the completely aerobicly prepared $N_2OR$ form is called 'pink' $N_2OR$ or $N_2OR$ II (Coyle et al., 1985; Riester et al., 1989). When $N_2OR$ is exposed to oxygen, the cofactor $Cu_Z$ becomes a redox-inactive form. The $Cu_Z$ can be altered in two ways, due to the levels of exposure to oxygen. Oxygen incubation can alter the catalytic site from $Cu_Z$ to $Cu_Z$* which may still be capable of catalysis, or such exposure may produce the 'pink' form which has most likely lost the overall $Cu_Z$ (Rasmussen et al., 2002). This behaviour seems to protect its catalytic site from $O_2$ damage or to even allow enzyme turnover in the presence of limited amounts of $O_2$. The

transient exposure to low levels of $O_2$ probably results in the temporarily inactivated $N_2OR$. Once $O_2$ is depleted, the $N_2O$ reduction activity could recover, either by reactivating existing enzymes or *de novo* enzyme synthesis of $N_2OR$ (Morley et al., 2008; Richardson et al., 2009). It was also found that the sensitivity to oxygen may depend on the source of $N_2OR$. $N_2OR$ from *Achromobacter cycloclastes* and *Pseudomonas nautica* are less sensitive to oxygen, showing high activities even when they were isolated under oxygen conditions (Coyle et al., 1985; Hulse and Averill, 1990).

The activity of $N_2OR$ may be damaged within bacterial cells by prolonged exposed to $O_2$. As an outstanding question if $N_2OR$ were to be deployed in transgenic crops, the implications of the presence of oxygen *in planta* ostensibly as a by-product of photosynthesis need to be addressed with regards to such potential functionality of $N_2OR$. The presence of, or lack of oxygen *in planta* is a very valid consideration especially since $N_2OR$ is primarily evolved in bacteria that are in anoxic environments, where access to oxygen is restricted. Would $N_2OR$ be functional if exposed to oxygen when expressed within a plant cell and will it be damaged *in vivo*? In this regard it is important to note that Geigenberger has advanced some very strong arguments to indicate that even when the external oxygen concentration is high, oxygen can fall to low concentrations within plant tissues, especially in the roots, because plants lack efficient oxygen delivery systems (Geigenberger, 2003). Since the movement of oxygen through the plant tissues, driven by a large diffusion gradient, cannot keep pace with the rate of oxygen consumption, plant cells actually experience anoxia/hypoxia. Hypoxia is a widespread phenomenon in plants that has been largely ignored in the past. Some plant tissues, that lack large intercellular air spaces, or are poorly vacuolated, have the propensity to fall into hypoxia. For example, root meristems have high rates of oxygen consumption and few intercellular air spaces (Ober and Sharp, 1996). In this

thesis study, bacterial $N_2OR$ was expressed heterologously in the plant's extracellular matrix and root tissues, two locales that might provide low oxygen tensions. This could allow $N_2OR$, normally classified as an anaerobic enzyme, to function to some even limited degree, well enough to effect a change in environmental levels of harmful nitrous oxide gas. The intracellular micro-environment was not directly studied but the positive enzyme assays obtained from transgenic plant extracts (Figure 2.1, Figure 3.5, Figure 4.3) do confirm the presence of a sufficiently anoxic state *in planta*, one that is now coming more and more to light in the recent literature (van Dongen et al., 2011).

## 5.3 Significance of phytoremediation of nitrous oxide by transgenic plants

Bioremediation is an emerging technology that uses microorganisms or their enzymes to return a contaminated environment to its original condition. Because of its potential for the sustainable mitigation of environmental pollution, there is considerable interest in developing cost-effective, and no or limited negative impact alternatives based on microorganisms or plants (Van Aken et al., 2010). Transgenic plants are a new generation of genetically modified organisms with the capability to treat an environment contaminated with pollutants in an efficient and environment-friendly manner. I hypothesized that genetic engineering technology to express $N_2OR$ constitutively in plants that can be grown to high biomass under cultivation could be used to remove $N_2O$ from the atmosphere at a large rate, to force down their concentrations, reducing radiative forcing. Since total enzymatic activity is the product of expression and biomass, a method of increasing either activity or biomass could be used to increase biodegradation of $N_2O$. Thus, it is urgently needed to develop

some farming practices that completely or mostly eliminate the denitrifier-$N_2O$ emission from agriculture (Richardson et al., 2009).

The engineered environment, where crop yields have been boosted to unprecedented levels, has concomitantly driven excess fertilizer into the soil. With the resultant greenhouse gas, $N_2O$, constantly increasing in the troposphere, the widest possible alert and conscription of innovative remedies must be promoted. The global denitrification cycle could be augmented by genetically modified plants expressing microbial enzyme, $N_2OR$, transferring the soil-borne enzyme machinery to crops. This soil-microbe-crop-atmosphere axis inherently beckons the broadest possible array of environmental scientists to advance remediation technologies. This thesis was a modest attempt to explore the intracellular environment of tobacco leaves and roots to see if this biocatalyst has a future in such mop-up exercises aimed at the atmosphere itself. The positive results in these preliminary proof-of-principle experiments are the first such reports to our knowledge to test the Mitsubishi patent filing of 1999.

Although heterologous expression of $N_2OR$ has been previously reported in prokaryotes, this report marks the first time that this bacterial enzyme has been produced in a eukaryotic host, one that is a significant commercial crop. My results showed that a proteome such as that existing in tobacco can complement the *nosZ* gene product from *Pseudomonas stutzeri* and lead to active $N_2OR$ that endows plants with a functional $N_2O$-cracking mechanism. Crops expressing $N_2OR$ could mitigate emissions directly at the source before $N_2O$ reaches the stratosphere or troposphere.

To my knowledge, the expression of bacterial $N_2OR$ in plant is an innovative approach to remediation of atmospheric $N_2O$. The bacterial $N_2OR$ and the accessory Nos proteins have been heterologously expressed in a model plant, one that is a global

commercial crop for its leaves while at the same time tobacco has been a favourite organism for biochemical and DNA transformation investigations (Vanlarebeke et al., 1975; Wildman et al., 1949). Wheat transformation is another option that could be explored using the construct found to be most effective in tobacco. The bacterial "$N_2O$-cracking system" could be reproduced in plants just as "carbon sequestration", removing $CO_2$ from the atmosphere via photosynthesis and storage in vegetation, is already accepted as a mitigation strategy to offset carbon emissions and abate climate change. The cultivation of genetically engineered energy crops makes it possible to remove $CO_2$, and thermal conversion of these crops can act as a substitute for fossil fuels. In future, this genetic engineering strategy could be used as an option to reduce nitrous oxide emissions from cropping systems.

In some political jurisdictions there has been debate on the pros and cons of GM crops, but more and more consumers have tended to accept GM foods. For example, the latter appear to be gaining more acceptance amongst Chinese and North American consumers. However, some consumers, particularly in Europe, have expressed opposition on this issue. Despite such concerns, the production of GM crops steadily increases in the global market (ISAAA 2010).

Gene-stacking this $N_2OR$ capability in commercial crops is eminently feasible and may even lead to measureable reduction in atmospheric $N_2O$ levels. In 2010, 10% of all 1.5 billion hectares of cropland in the world (148 million hectares) were occupied by biotech crops, and more than 4 billion people (59% of the world's population) live in the 29 countries where approved biotech crops were grown (ISAAA, 2010). Since the adoption rate of current biotech crops in the world is increasing, there is considerable potential for increasing the hectarage of the biotech crops, such as soybean, maize, cotton, and canola. Since 1996, herbicide tolerance, insect resistance or herbicide tolerance/insect resistance are

the dominant traits of such commercial crops. As the first generation biotech crops, the yield and production of crops have been significantly increased by protecting losses from pests, weeds, and diseases. The farmers' benefit from the growing of biotech crops is in increasing their farm-gate incomes.

To meet the multiple needs of consumers (quality traits, such as omega-3, pro-Vitamin A) and address the multiple yield constraints of the world's farmers, stacked crop products appear poised to become the future trend. For the long term, the stacked double, triple traits and more traits are expected to become an important feature of biotech crops. The most important commercial crop of the new biotech crops is Golden Rice that comprises five stacked transgenes (Ronald, 2011). These types of second generation biotech crops offering consumers and farmers with a growing number of input and output traits will become more prevalent. For example, Smartstax$^{TM}$, biotech maize with eight genes encoding three traits including protection against above-ground insects, below-ground insects, and broad herbicide tolerance, was launched in the USA and Canada in 2010 (Reay-Jones and Wiatrak, 2011). On a global basis, stacked traits are a trend that will increasingly affect both farmers and the world.

If the $N_2$OR capability is stacked in commercial biotech crops it may significantly reduce the atmospheric $N_2O$ levels and even help mitigate climate change. Importantly this technology would be inexpensive, probably much cheaper than any other greenhouse gas reduction technology. The global biotech maize seed alone sells for a US$11.2 billion premium in 2010 (ISAAA, 2010). A mandate for the addition of $N_2$OR genes to biotech maize could be supplemented with a subsidy for participating farmers, at say 10% of the biotech maize premium, about $1.12 billion per year. If strong $N_2$OR expression *in planta* were accomplished, annual fluxes to genetically modified maize could be as much as 64kg

$N_2O$ per hectare (Stuart Strand, unpublished data). The calculated annual uptake by the 46 million hectares of biotech maize would be 2.944 million tonnes. So $N_2O$ could be removed for about $380/tonne $N_2O$ destroyed. If the 298 fold warming potential factor is applied, the potential removal would be $1.28/ tonne $CO_2$ equivalent. When compared to the present value of sequestration on carbon markets, $4-10/ tonne $CO_2$ (Den Elzen & De Moor, 2002), then this thesis could help support a regulatory case advocating such remedial transgenesis.

## 5.4 Methyl viologen-linked enzyme assay

The methyl viologen-linked enzyme assay is widely used to measure $N_2OR$ activity. In principle, the oxidation of methyl viologen dye is coupled with the reduction of nitrous oxide by $N_2OR$ (Kristjansson and Hollocher, 1980). The dye colour changes from blue in the reduced form to colourless in the oxidized form, thus allowing indirect monitoring of $N_2O$ reduction by $N_2OR$ using $N_2O$ saturated water.

The major challenge of this assay was the relatively high background oxidation rate. The background oxidation is the oxidation of reduced methyl viologen solution before adding the $N_2O$. To achieve anaerobic conditions in the experiments described herein, all the reagents were degassed and bubbled with argon to displace any dissolved oxygen. Although manipulations were performed in an anaerobic chamber, considerable background rate of autooxidation of the dye was observed (less than 3.3 μmol $O_2$ reduced $min^{-1}$ in Figure 2.1). This is likely due to the presence of small amount of oxygen in the reagents which remained even after all the reagents have been degassed and bubbled. Also, there are always the unavoidable generation of reactive oxygen species such as hydrogen peroxide ($H_2O_2$) and superxoxide anions ($O_2^-$) in plant since they are involved in aerobic metabolism (Moller,

2001). The $O_2^-$ and $H_2O_2$ can be toxic for biological systems, since they can form singlet oxygen which was shown to inactivate some of the important enzymes (Englander et al., 1987).

The $N_2OR$ activity could be assayed using the alternative approaches, such as the direct measurement of $N_2O$ reduction and $N_2$ formation by gas chromatography (Coyle et al., 1985; Shrestha et al., 2001) or detection of $^{15}N_2$ by using $^{15}N$-labelled $N_2O$ using the GC-MS (Chan et al., 2004). The advantage over this method is that oxygen and reactive oxygen species do not interfere with the measurement of $N_2O$ reduction.

Changes in absorbance in the plant tissue samples are subtle when compared with the positive control (Figure 2.1 lowest right panel). One possible reason for this is that the $N_2OR$ was expressed in the plant's extracellular matrix, so the recombinant $N_2OR$ was not properly extracted completely from the leaf /root tissue and thus was not in the solution. Previous work showed that pre-incubating the reduced dye with the enzyme solution could yield higher activity (Prudencio et al., 2000). Although the mechanism has not yet been elucidated, it is thought that the fully reduced enzyme would be more capable than a partially reduced enzyme since the reduced dye participates in a further reduction of $N_2OR$. For future experiments, the pre-incubation time of reduced dye with enzyme could be enhanced longer to 40 min (Ghosh et al., 2003).

## 5.5 Future work

We have demonstrated that extracts from transgenic plants encoding $N_2OR$ are capable of converting $N_2O$ into inert $N_2$. Expressions of the prokaryotic *nosZ* gene in a eukaryotic cellular environment maybe endow plants with "$N_2O$-cracking" capabilities.

Future analyses of the transgenic plants will examine the ability of the plants to reduce $N_2O$ in the field. A wider set of parameters, such as the $N_2$ production against high atmospheric $N_2$ concentration, the biological copper availability at agricultural sites, should be monitored at the gross field study level. Once the transgenic plant lines are proven effective, the following studies will focus on the improvement of recombinant enzyme yield, activity and stability.

Given that the $N_2O$ reduction pathway in fertilised soils is limiting, engineering it in a manner so it could be further enhanced by tissue-specific expression remains a key long-term goal so it can be field-tested in commercial elite lines of maize or wheat in Canada. Transgenic plants expressing $N_2OR$ under the control of a root-specific promoter with a secretion signal could deliver $N_2OR$ into the rhizosphere and surrounding soil to prevent the release of $N_2O$ at the source. This is an even more appealing strategy given new findings that in actual ecosystems where $NO_3^-$ may be relatively scarcer than ammonia, the contribution of nitrifier denitrification is also a major and significant source of $N_2O$ with the potential of being the premier $N_2O$ production pathway in biogeochemistry (Kool et al., 2011). Concomitantly we are also exploring expanding the deployment venues for empowering crops with $N_2OR$ activity. Given that *Pseudomonas* is a prokaryote, and that plant mitochondria are also ancient prokaryotes, we will explore the possibility of transforming plant mitochondria, especially those in roots. We have established discussions and potential collaboration with The Richardson Lab at University of East Anglia in England to try expressing $N_2OR$ in root mitochondria so that the NosZ might interact with the mitochondrial electron transport system in a more optimal manner, facilitating full copper-charging and electron-transfer.

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**Curriculum Vitae**

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*2006- 2011* **University of Ottawa**, Ottawa, Ontario, Canada
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*1993-1997* **Liaoning Normal University**, Dalian, China
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Research Center of Plant Genetic Engineering, Jilin University, China
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*1997-2000* **Lecturer**
Department of Food Science, Jilin Normal Institute of Engineering & Technology, China
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**TEACHING**

*2001-2003* **Teaching Assistant**
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**PUBLICATIONS**

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*2010* Nitrogen 2010 - 1st International Symposium on the Nitrogen Nutrition of Plants. Inuyama, Japan. July 26-30, 2010.

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