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# The Subcellular Targeting and *In Vivo* Metal Binding Characteristics of AgNt84 using Transgenic Tobacco and BY-2 Suspension Cell Cultures

Brook Kay Nelson
*University of Tennessee, Knoxville*

To the Graduate Council:

I am submitting herewith a thesis written by Brook Kay Nelson entitled "The Subcellular Targeting and *In Vivo* Metal Binding Characteristics of AgNt84 using Transgenic Tobacco and BY-2 Suspension Cell Cultures." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

Beth Mullin, Major Professor

We have read this thesis and recommend its acceptance:

Michael Essington, Andreas Nebenfuhr, Albrecht von Arnim

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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# The Subcellular Targeting and *In Vivo* Metal Binding Characteristics of AgNt84 using Transgenic Tobacco and BY-2 Suspension Cell Cultures

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville.

Brook Kay Nelson

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# ABSTRACT

AgNt84 is a nodulin protein expressed in *Alnus glutinosa*, an actinorhizal tree that participates with the actinomycete *Frankia* in symbiotic nitrogen fixation. Expression of AgNt84 mRNA is exclusively found within Zone 2 of the nodules, which is the zone that contains cells in the process of being infected by *Frankia*. A truncated form of the protein was found to bind $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Cd^{2+}$, and $Hg^{2+}$ *in vitro*. AgNt84 was predicted to be targeted extracellularly based on the presence of an N-terminal signal sequence. The purpose of this research was to determine the subcellular targeting and *in vivo* metal binding properties of AgNt84. Transient transformation of onion and tobacco tissue and stable transformation of tobacco and tobacco BY-2 cells were used to accomplish these goals.

Transient expression of AgNt84-GFP fusion protein in onion epidermal cells confirmed that AgNt84 is targeted to the endoplasmic reticulum. The fusion protein was not found in the cytosol. Expression of AgNt84-GFP with an HDEL retention tag resulted in the accumulation of the fusion protein at what appear to be the plasmadesmata as well as its presence in the endoplasmic reticulum. The presence of AgNt84-GFP in the endoplasmic reticulum is consistent with the pathway that a protein targeted to the plasma membrane would follow. However, the possibility that the protein may be targeted to other organelles or remain in the endoplasmic reticulum remains.

To determine the ability of AgNt84 to bind metal *in vivo*, three hydroponically grown tobacco lines transgenic for AgNt84 and wild-type tobacco plants were incubated in 5 mM MES buffer containing 200 μM $Cd(NO_3)_2 \cdot 4H_2O$. The amount of cadmium in the plants at day 1 and day 3 was analyzed by inductively coupled argon plasma spectrometry (ICP). Two of the transgenic tobacco lines tested had significantly more cadmium in the roots than wild-type tobacco at day 1 and day 3. One of these, T10, was a transgenic line expressing AgNt84, and the expression of AgNt84 in the other tobacco lines remains to be confirmed. Histochemical staining of tobacco tissue using dithizone supported ICP measurements of cadmium content.

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# LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| AgNt84-GFP | AgNt84 fusion to GFP |
| $dH_2O$ | deionized water |
| $ddH_2O$ | deionized water that had been deionized a second time; nanopure water |
| MES | 2-[N-morpholino]ethanesulfonic acid |
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| LSD | Least Significant Difference |
| EDTA | ethylene diamine tetraacetate |
| NaOH | sodium hydroxide |
| SDS | sodium dodecylsulfate |
| HCl | hydrochloric acid; hydrochloride |
| $HNO_3$ | nitric acid |
| $Cd(NO_3)_2 \cdot 4H_2O$ | cadmium nitrate tetrahydrate |
| NaCl | sodium chloride |
| $MgCl_2$ | magnesium chloride |
| $KNO_3$ | potassium nitrate |
| $MgSO_4 \cdot 7H_2O$ | magnesium sulfate heptrahydrate |
| $MnSO_4 \cdot H_2O$ | manganous sulfate monohydrate |
| $ZnSO_4 \cdot 7H_2O$ | zinc sulfate heptahydrate |
| $CuSO_4 \cdot 5H_2O$ | cupric sulfate |
| $CaCl_2 \cdot 2H_2O$ | calcium chloride dihydrate |
| KI | potassium iodide |
| $CoCl_2 \cdot 6H_2O$ | cobalt chloride |
| $NaH_2PO_4 \cdot H_2O$ | sodium phosphate monohydrate |
| $H_3BO_3$ | boric acid |
| $NaMoO_4 \cdot 2H_2O$ | sodium molybdate |
| $FeSO_4 \cdot 7H_2O$ | ferrous sulfate |

| | |
|---|---|
| $Na_2EDTA$ | disodium EDTA |
| $Na_3PO_4$ | sodium phosphate |
| DMSO | dimethyl sulfoxide |
| g | gram |
| mg | milligram |
| µg | microgram |
| ng | nanogram |
| L | liter |
| mL | milliliter |
| µL | microliter |
| M | molar |
| mM | millimolar |
| µM | micromolar |
| $Zn^{2+}$ | zinc divalent cation |
| $Ni^{2+}$ | nickel divalent cation |
| $Co^{2+}$ | cobalt divalent cation |
| $Cu^{2+}$ | copper divalent cation |
| $Cd^{2+}$ | cadmium divalent cation |
| $Hg^{2+}$ | mercury divalent cation |
| $Ca^{2+}$ | calcium divalent cation |
| $Mg^{2+}$ | magnesium divalent cation |
| $Mn^{2+}$ | manganese divalent cation |
| RNA | ribonucleic acid |
| RNAase A | ribonuclease A |
| DNA | deoxyribonucleic acid |

# CHAPTER I

# INTRODUCTION

## A. METAL CONTAMINATION & THE ENVIRONMENT

Over the past decades, exposure to high concentrations of metals has become an environmental hazard (Nedelkoska and Doran 2000a). Industrialization, mining, and agriculture are some reasons for the increase in exposure to metals (Nedelkoska and Doran 2000a). Unlike organic chemicals, metals remain in the environment because they cannot be degraded. Some metals are essential for growth of organisms (Nedelkoska and Doran 2000a; Macek et al., 2002). However, all metals are toxic at high levels, regardless of whether or not the element is essential (Nedelkoska and Doran 2000a; Macek et al., 2002). Metals interfere with basic physiological processes by interacting with redox reactions, creating free radicals that damage cells, or by replacing essential metals involved in protein complexes, preventing the proteins from functioning properly (Pilon-Smits and Pilon 2002).

Humans are exposed to metals through several sources. Water contamination, cigarette smoke, food, inhaling particulate matter in the air from burning fossil fuels, and absorption of metals from surgical implants and dental fillings are some of the sources of exposure (Coen et al., 2001; Riley et al., 2003; Report on Carcinogens 2005). Many fertilizers contain lead and cadmium in trace amounts, and as a result produce grown using these fertilizers may contain elevated levels of lead and cadmium (Lee et al., 2003). The metals to which humans are exposed may cause several health effects. Cadmium is a nonessential toxic element that interacts with calcium and causes renal and skeletal problems mainly by loss of minerals through urination and by negatively interacting with calcification of bones, respectively (Goyer 1997; Şişman et al., 2003). In addition, iron deficiency can increase cadmium uptake (Goyer 1997). In an *in vitro* study, the addition of cadmium and nickel to human cell cultures resulted in a variety of delayed

chromosomal defects and cell death long after exposure (Coen et al., 2001). Lead, a teratogen and nonessential element, affects many organs and can lead to behavioral and physical problems, and schizophrenia is a problem that may be attributed to lead (Goyer 1997; Opler et al., 2004). Also, copper, an essential element, causes oxidative stress in the mitochondria at high levels (Thomas et al., 2003). Copper is involved in the neurodegenerative effects of prions, and copper is implicated in the development of several neurological problems, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (White and Cappai 2003). It has also been shown that high concentrations of copper can cause cell death in neurons (White and Cappai 2003).

Tobacco has been widely used in studies on the potential for a protein to bind cadmium or in studies that involve hyperaccumulation of metals (Nedelkoska and Doran, 2000; Dorlhac de Borne et al., 1998; Macek et al., 2002; and Dhankher et al., 2003). The mechanism by which tobacco tolerates cadmium has also been studied (Choi et al., 2001). The interest in tobacco and cadmium may stem from concerns with cadmium contamination of tobacco. Smokers on average contain twice the amount of cadmium than do nonsmokers (Dorlhac de Borne et al., 1998). In fact, tobacco is one of a small number of plants that accumulate most of the cadmium in the shoot tissue (Dorlhac de Borne et al., 1998).

Because of the detrimental effects of high concentrations of metals on the environment and on humans, remediation of sites contaminated with metals has become an important priority over the last several years. All current methods of remediation are expensive (Probst et al., 2001; Raskin and Ensley 2001). Some current methods of cleaning toxic metals involve moving, incinerating, flushing, or washing the soil (Prasad and de Oliveira Freitas 2003). These methods involve physically handling, digging, and/or removing the soil to reduce the contamination of metals. Over 500,000 sites were recognized as being suspected of contamination from 1980 to 1996 (EPA 1997). As of 2004, there are still estimated to be 294,000 sites within the United States that need some form of remediation, and many of these sites are projected to take 10 to 30 years to clean up while several may take longer (EPA 1997). The projected cost for remediation of these sites is estimated to be $209 billion (EPA 2004). A small number of these sites are

considered superfund sites, or sites that are considered to be highly contaminated sites (EPA 1997). As of 1999, the number of superfund sites was 2,053 (Probst et al., 2001). The additional superfund sites added to this list are projected to range from 23 to 49 sites per year (Probst et al., 2003). Between 1990 and 1999, the amount of money spent each year on remediation of superfund sites ranged from $1.3 billion to $1.6 billion (Probst et al., 2001). The estimated cost for funding the remediation of superfund sites from 2000 to 2009 is between $15.6 billion and $18.3 billion (Probst et al., 2001). The use of less expensive solutions, such as phytoremediation, may aid in reducing the cost of remediation.

## B. PHYTOREMEDIATION

### 1. PHYTOREMEDIATION & THE CLEAN UP OF METALS

Phytoremediation is the term used to describe the method of remediation where plants are used to clean up the waste sites (Raskin and Ensley 2000). In 2000, there were 194 projects that used phytoremediation, and 33% of these projects involved phytoremediation of toxic metals (Prasad and de Oliveria Freitas 2003). Metal contamination is problematic because the metals do not leave the environment or break down; rather, the most common methods of dealing with metal contamination through phytoremediation are those that change the metal to a less toxic form or sequester the metal from the environment (Cobbet and Meagher 2002).

There are four common methods of phytoremediation: rhizofiltration, phytovolatilization, phytostabilization, and phytoextraction. Rhizofiltration is the method of using hydroponically grown plants to remove metals from water by concentrating the metals in roots or shoots (Prasad and de Oliveria Freitas 2003). Phytovolatilization is a complementary method to rhizofiltration and uses plants to convert the metal into a gaseous substance (Prasad and de Oliveria Freitas 2003). For example, the phytovolatilization and rhizofiltration of selenium is performed by *Hydrilla verticilla* (Carvalho et al., 2001). Phytostabilization, also known as phytorestoration, is the method by which plants are used to stabilize metals in the soil or near the roots to prevent the movement of metals into ground water (Prasad and de Oliveria Freitas 2003). This

method is used mainly for sites that are not considered superfund sites (Prasad and de Oliveria Freitas 2003). Phytoextraction, or hyperaccumulation, is the method of using plants to extract metals from the soil and to accumulate the metals in certain tissues, such as leaves (Prasad and de Oliveria Freitas 2003).

## 2. Advantages & Disadvantages of Phytoremediation

The use of plants to clean up the environment has several advantages. The main advantage of phytoremediation is the relatively low cost. Phytoremediation is less expensive than traditional methods of remediation because the plant is sown in the field and at a later time parts of the plant containing the accumulated metals are harvested (Raskin and Ensley 2001). Secondly, plants have mechanisms to deal with metals. They can sequester metals in specific tissues or convert some metals into a less toxic form (Pilon-Smits and Pilon 2002; Prasad and de Oliveria Freitas 2003). Hyperaccumulators are plants that can accumulate high levels of metals within their tissue (Prasad and de Oliveria Freitas 2003).

There are several disadvantages to using phytoremediation. Since many of the contaminated sites tend to contain more than one metal, the use of hyperaccumulators may not be possible because hyperaccumulators tend to accumulate a small number of specific metals (Cobbett and Meagher 2002). A second disadvantage of phytoremediation is time (Raskin and Ensley 2001). Because the phytoremediating plants needed to be planted and grown, there may be a long time between planting and harvesting of biomass (Raskin and Ensley 2001). Another disadvantage is that the plant may convert the metal into a more bioavailable form (Stolz and Gregor 2002). A metal in a bioavailable form increases the chance of metal toxicity to other organisms, and there is less control over potentially volatile products (Prasad and de Oliveria Freitas 2003).

## 3. Improving Phytoremediators

Improvements in phytoremediation can involve either the use of hyperaccumulating or nonhyperaccumulating plants. With hyperaccumulating plants, there are two possible characteristics that can be improved. The first is to increase the

amount of accumulated metal within a tissue and/or to increase the size of the tissue so that more metals can accumulate. The second involves genetically modifying the hyperaccumulating plant so that plant would have a greater capacity to deal with a wider range of metals (Lasat 2002). With plants that do not naturally hyperaccumulate metals, the ideal plants to use would be those that have a large biomass so that metals can be efficiently removed (Lasat 2002). The improvements in nonhyperaccumulating plants would involve modifying a plant's ability to sequester, change, or tolerate metals (Lasat 2002).

*Arabidopsis thaliana* is a plant that can be used in the improvement of phytoremediation. Although *Arabidopsis* is a nonhyperaccumulating plant, *Arabidopsis* has several advantages for use in studying phytoremediation in transgenic plants (Cobbett and Meagher 2002; Pilon-Smits and Pilon 2002). It has been proposed that *Arabidopsis* be used as an initial step to rapidly determine in whether a phytoremediation strategy could work prior to using the strategy on a closely related plant (Cobbett and Meagher 2002; Pilon et al. 2003). *Arabidopsis* is a fast-growing plant that is considered a model plant, and Arabidopsis is closely related to other hyperaccumulating plants in the Brassicaceae, such as *Brassica juncea* and *Thlaspi caerulescens* (Cobbett and Meagher 2002).

Two types of plant metal-binding proteins are metallothioneins and phytochelatins (Cobbett and Meagher 2002). Metallothioneins are cysteine-rich, low molecular weight metal-binding proteins containing cysteine residues. There are three classes of metallothioneins. Class I metallothioneins are metallothioneins that have a conserved arrangement of cysteine residues. Class II metallothioneins are other metallothioneins that do not have a conserved sequence, and plant metallothioneins are found in this category. Class III metallothioneins include phytochelatins and other peptides closely related to phytochelatins. A novel class of small molecular weight metal-binding proteins has been recently discovered (Gupta et al., 2002). These proteins, which are called metallohistins, are glycine and histidine-rich rather than cysteine-rich like the metallothioneins, and metallohistins, such as the protein AgNt84, may have potential uses in phytoremediation (Gupta et al., 2002).

## C. AN INTRODUCTION TO AGNT84

### 1. DISCOVERY OF AGNT84

AgNt84 is a NOD-GHRP, or a nodule-specific glycine and histidine-rich protein, from *Alnus glutinosa* (Pawlowski et al., 1997). AgNt84 was isolated by subtractive hybridization of nodule and root cDNA libraries to find nodule-specific clones (Twigg 1993). The cDNA *agNt84* codes for a 10.57-kDa protein consisting of 99 amino acids. Of the 99 amino acids, 26 amino acids are predicted to be an N-terminal signal sequence while 35 amino acids are rich in glycine and histidine, which is thought to be the metal-binding domain (Gupta et al., 2002). Using *A. glutinosa* tissue, northern blot analysis detected *agNt84* expression in nodules but not in other tissues, and Southern blot analysis indicates that AgNt84 belongs to a gene family (Pawlowski et al., 1997). *In situ* hybridization of *Alnus glutinosa* nodules was used to identify the region of the nodule where the mRNA of AgNt84 is being expressed (Pawlowski et al., 1997). AgNt84 mRNA is expressed in Zone 2 of the nodule only in cells where *Frankia* filaments are visible (Pawlowski et al., 1997). The presence of a putative N-terminal signal sequence that is predicted to target AgNt84 to the endoplasmic reticulum (ER), and the lack of ER retention and vacuole targeting sequences leads to a prediction that AgNt84 is targeted extracellularly.

### 2. METAL-BINDING PROPERTIES OF A TRUNCATED FORM OF AGNT84

Expression of AgNt84 in *E. coli* was toxic to the bacteria, so a truncated form of the protein, known as AgNt84-6, was expressed in *E. coli* for further characterization of the protein (Dobritsa and Mullin 1996; Gupta et al., 2002). AgNt84-6 has the first 26 amino acids deleted. AgNt84-6 was tested for metal binding properties using the metals $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ (Gupta et al., 2002). These experiments included metal-chelate affinity chromatography, MALDI-TOF MS, equilibrium dialysis, ICP analysis, and NMR analysis. In metal-chelate affinity chromatography, resin was prepared so that one of the following metals was present on the charged resin: $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$. Resin that had

been charged with $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, or $Cd^{2+}$ was able to bind AgNt84 whereas resin charged with $Ca^{2+}$, $Mg^{2+}$, or $Mn^{2+}$ did not. When AgNt84-6 was eluted from the $Cu^{2+}$-charged resin, the solution had a blue color, indicating that $Cu^{2+}$ remained bound to the protein (Gupta et al., 2002). The eluted protein from each treatment was electrophoresed on 12% SDS-PAGE, and the protein migrated faster in the presence of the following metals than with the protein alone: $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, and $Cd^{2+}$. Because of these results, the eluted proteins from each treatment were further analyzed using MALDI-TOF. The results showed several peaks increasing in mass equivalent to the mass of the protein and metal in addition to a peak equivalent to the mass of the protein alone. Depending on the type of the metal used, the maximum amount of metal bound to the protein monomer ranged from 2 to 6 atoms. Equilibrium dialysis followed by ICP analysis of the solution resulted in similar conclusions, and the conclusions were that the multimeric protein binds 3 to 12 metal atoms. NMR was used to identify the amino acids involved in binding the metal atoms, and proton shifts were detected only in histidine residues (Gupta et al., 2002).

AgNt84 has several possible functions in *Alnus glutinosa* nodules. Two hypothetical functions are based on the metal-binding properties of AgNt84. One is that AgNt84 may be involved in sequestering the metals from *Frankia* to control its growth, which suggests that AgNt84 has antimicrobial properties similar to the small histidine-containing histatins of saliva (Gupta et al., 2002; Brewer and Lajoie 2000). Alternatively, AgNt84 may be involved in providing metals, such as cobalt or zinc, to *Frankia* because the bacteria obtain all of their nutrients from the host plant in symbiotic nitrogen fixation (Gupta et al., 2002). The final possible function is that AgNt84 may be involved in structural support of the cell wall, and AgNt84 may not bind metals *in vivo*.

### 3. Potential for Phytoremediation

AgNt84 may be a possible candidate for use in phytoremediation for several reasons. First of all, preliminary experiments show that AgNt84 is targeted extracellularly. The prediction of extracellular targeting of AgNt84 is based on three observations: (1) the beginning 26 amino acids are predicted to be a signal peptide based

on several protein prediction programs, (2) immunocytochemistry shows that AgNt84 may be bound to the cell wall of the nodule, and (3) AgNt84 can only be extracted from nodule tissue using harsh methods of protein extraction (Pawlowski et al., 1997; Mullin, unpublished results). It would be an advantage to a metal accumulating plant for toxic metals to be sequestered in the extracellular space. Secondly, metallohistins, such as AgNt84, have different characteristics than metallothioneins and phytochelatins. Metallothioneins and phytochelatins, have a large number of cysteine residues (Cobbett and Meagher 2002). The metallohistin AgNt84 contains many histidine residues alternating with glycine residues. The amino acid cysteine would give different properties to a protein than the amino acid histidine. Finally, AgNt84-6 can bind multiple atoms of several metals, namely $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Cd^{2+}$, and $Hg^{2+}$. If AgNt84 is found to bind metals *in vivo* and if its extracellular location can be confirmed, it has the potential to be an excellent candidate for improving the phytoremediating capabilities of plants.

## D. RESEARCH OBJECTIVES

The function of AgNt84 in *Alnus glutinosa* nodules is not known. Furthermore, the metal-binding research that has been done on AgNt84 did not include *in vivo* studies. *In vivo*, the protein may or may not bind metals since the metal-binding experiments were conducted using only truncated, purified protein from *E. coli* expressing AgNt84-6. In addition, the targeting of the protein is predicted based on sequence alignments with other proteins and from protein prediction programs. The protein also may be tightly bound to the cell wall, and AgNt84 may not bind metals as a result. Immunocytochemistry results show that the protein may be present in the middle lamella of nodule cells, but the antibody to AgNt84 cross-reacts with *Frankia* and with cellulose, making these results unreliable. Because of these unresolved questions, the experimental objectives are first to determine the subcellular location of AgNt84 in a transgenic plant and then to determine the *in vivo* metal binding properties of AgNt84.

It is not feasible to use *A. glutinosa* for these experiments. No transformation system has been developed for *A. glutinosa*, and there are no reports of the availability of a stable cell culture or of a regenerating line of *A. glutinosa*. For reasons that will be

discussed later, tobacco was the plant of choice for studying the metal uptake and metal binding properties of AgNt84.

In summary, the overall objectives of this research are:

1. **To determine the subcellular location of AgNt84 in transgenic tobacco.**
2. **To determine the *in vivo* metal-binding properties of AgNt84.**

# CHAPTER II

# EXPERIMENTAL DESIGN AND APPROACH

## A. RESEARCH OBJECTIVES

As stated in the previous chapter, the overall objectives of this research are:

1. **To determine the subcellular location of AgNt84**
2. **To determine the *in vivo* metal-binding properties of AgNt84**

The first step to complete these goals was to decide what plant and bacterial organisms to use in the experiments.

## B. CHOICE OF PLANT SPECIES

*Nicotiana tabacum* (tobacco) was chosen as the plant species to use to discover if AgNt84 could bind metals *in vivo* before taking the time to transform a plant better suitable for phytoremediation. Tobacco was chosen for transgenic expression of AgNt84 for several reasons. Both wild type and transgenic tobacco have previously been used in several experiments studying phytoremediation (Macek et al., 2002; Nedelkoska and Doran 2000a; Nedelkoska and Doran 2000b; Thomas et al., 2003). Hairy root cultures and suspension cell cultures were initially chosen over whole transgenic plants for two reasons. The sample collection and measurements could be more easily standardized, and the hairy root and suspension cell cultures would be faster to regenerate after transformation. However, problems with obtaining transgenic lines of hairy roots made the use of whole transgenic plants an attractive alternative to hairy roots. Finally, tobacco was chosen because it should be fairly easy to transform with *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*. Other plants more suitable for phytoremediation would be more difficult to transform, and these plants would take longer to regenerate transformed tissue than tobacco would.

A second important decision that had to be made was which suspension cell culture line to use. Two plant suspension cell cultures often used are *Arabidopsis thaliana* suspension cell cultures and tobacco BY-2 suspension cell cultures. Several advantages of using BY-2 suspension cell cultures over *Arabidopsis* suspension cell cultures made BY-2 suspension cultures the choice for the proposed experiments. BY-2 cells are uniform in size, and they form small clusters (Planchais et al., 2000). In addition, BY-2 cells are homogeneous, and their growth rate is both fast and uniform (Planchais et al., 2000; Nagata et al., 1992). They also do not have a high level of background autofluorescence, and they can be easily induced by glucocorticoids and heat shock (Bonifacino et al., 2004). *Arabidopsis* suspension cell cultures, on the other hand, are composed of cells that have size variability, and the suspension cells have different growth rates (Planchais et al., 2000).

## C. *AGROBACTERIUM* STRAINS

*Agrobacterium*-mediated transformation was chosen for stable transformation of tobacco. The *Agrobacterium* strains used were *Agrobacterium tumefaciens* strain LBA4404, *Agrobacterium rhizogenes* strain A4RS, *Agrobacterium rhizogenes* strain AR10, and *Agrobacterium rhizogenes* strain 15834. *A. tumefaciens* strain LBA4404 is used to transform both tobacco plants and BY-2 cells (Su et al., 2004; Nebenführ et al., 1999). All three *A. rhizogenes* strains were utilized to induce hairy roots. AR10 was used because the pMDC7-derived plasmids contained the same bacterial antibiotic selection as the selection for the A4RS Ri plasmid making it difficult to select for A4RS that have been transformed with these plasmids. Transgenic and wild-type tobacco were inoculated with *A. rhizogenes* strain 15834 to form hairy roots. The reason for using 15834 is that it is reported to transform tobacco (Medina-Bolívar and Cramer. 2004).

## D. MAJOR OBJECTIVES

Once the organisms were chosen, details of the experiments had to be outlined. To complete both research objectives, five major objectives needed to be carried out in the following order:

- Design binary plasmids for transforming *Agrobacterium tumefaciens* and *rhizogenes* for later use in stable transformation of tobacco.
- Confirm expression of AgNt84-GFP in plant tissue by transient transformation of onion epidermal cells by particle bombardment and of tobacco leaves by agroinfiltration.
- Transform tobacco plants and tobacco BY-2 suspension cell culture via stable *Agrobacterium*-mediated transformation.
- Determine the subcellular localization of AgNt84 in transformed tobacco.
- Determine the short-term cadmium uptake of AgNt84in transgenic tobacco expressing AgNt84.

The experimental design and experimental approach of each of these five major objectives will be discussed briefly. More detail will be given in later chapters.

### 1. Design Binary Plasmids for Transforming *Agrobacterium tumefaciens* and *rhizogenes* for Later Use in Stable Transformation of Tobacco

The main goal in the design of the binary plasmids was to make plasmids so the expression of AgNt84 could be visualized in the cell with a reporter gene, yet the metal-binding properties of AgNt84 would be based with confidence on AgNt84, not a fusion protein. Regardless of the promoter driving the expression of AgNt84, two constructs should be made for each promoter used so both experiments could be conducted.

There were three main decisions that had to be made in the design of the plasmids to be utilized in this study. They were the choice of promoter, the choice of a reporter gene, and the system to be used for combining all of the DNA sequences. Decisions about each of these three are presented below.

#### 1.a. Promoters for Driving the Expression of AgNt84 or AgNt84 fusion protein

The use of several different promoters to drive the expression of AgNt84 would provide results useful in the visualization of the location of AgNt84 and the

quantification of metal bound to AgNt84. The 2X CaMV 35S promoter is a strong constitutive promoter, which should give a high level of AgNt84 expression. Inducible promoters would however allow better control of the expression of AgNt84. In addition, inducible promoters could prevent any potential detrimental effects of expressing AgNt84 in tobacco. If inducible promoters were used, any differences between expressing and not expressing AgNt84 in transgenic tobacco could be studied. Each type of promoter has benefits for completing the goals of the research, so both types of promoters were used in the design of vectors.

### 1. B. Reporter Genes

When trying to discover the localization of a protein within a cell or tissue, direct visualization of the protein is valuable. Reporter genes are frequently used for this visualization. There are two common reporter genes used in making promoter-gene-reporter constructs, and they are the genes for green fluorescent protein (GFP) and ß-glucuronidase (GUS).

Before discussing the reporter gene chosen for use, the concept of fusion proteins needs to be introduced. For a reporter protein to track the protein's location, it has to be attached to the protein, meaning the reporter gene has to be constructed as a transcriptional and translational fusion to the gene of interest, in this case AgNt84. This is done by cloning the two genes so that the stop codon of AgNt84 is replaced with the reporter gene. The stop codon of reporter gene terminates the fusion protein. Thus, when the promoter drives the expression of the gene, the gene will be transcribed and translated so the protein product contains AgNt84 with the reporter gene attached to its C-terminal end.

When deciding which reporter gene to include in the construct, several factors were considered. With GFP, the visualization can be done in live, intact tissue. With GUS, detection is based on the product of an enzymatic reaction and involves preparing the plant tissue in a way that kills the cell. For these reasons, GUS is not the best choice for determining the subcellular localization of proteins. Also, because GUS forms a

tetramer, it might interfere with AgNt84's function and location. The use of GUS as a reporter gene was not chosen for the reasons given above.

The location of AgNt84 is thought to be extracellular in the region of the middle lamella in *Alnus glutinosa* nodules, so the design of the plasmids would have to take this into consideration. As stated later in Chapter IV, a protein targeted extracellularly would appear first in the endoplasmic reticulum. The protein then goes to the Golgi apparatus. The three possible destinations of a protein leaving the Golgi apparatus are transport back to the endoplasmic reticulum, transport to the vacuolar compartments, and transport to the plasma membrane. Targeting to other organelles—such as the plastids, the mitochondria, and the nucleus—does not involve transport to the endoplasmic reticulum. The possibility that GFP may not be visualized extracellularly was anticipated. GFP may be constrained by the cell wall matrix, and the fluorophore may not fluoresce at a detectable level. The extracellular pH may hinder GFP fluorescence since protein fluorescence is related to the pH of the environment. If this is the case the location of GFP can be determined using anti-GFP antibodies.

A second form of GFP, GFP with the C-terminal HDEL endoplasmic reticulum retention tag, can be used to determine if the signal sequence of AgNt84 directs the protein to the ER. This method of GFP visualization was used as an indirect method of supporting the hypothesis that the protein is targeted extracellularly since a newly synthesized protein following the pathway to secretion is first seen in the endoplasmic reticulum. However, this method is indirect because not all the proteins targeted to the endoplasmic reticulum will end in the plasma membrane or will be secreted.

### 1. C. The Gateway System

Since several promoters were to be used, traditional cloning of AgNt84 would be a lengthy process. Therefore, a new and simpler method for cloning was used. The Gateway system made cloning plasmids easier by reducing the number of steps involved. In addition, the Gateway system preventing false positives because *E. coli* would not grow if transformed with an undesirable product (Invitrogen 2000). The Gateway system is discussed in detail in Chapter III.

The Gateway vectors chosen for designing the binary plasmids contained both inducible and constitutive promoters. Plasmids were made so the CaMV 35S promoter, a heat shock promoter, or an estradiol-inducible promoter drove the expression of AgNt84. Three other plasmids were constructed with these promoters driving the expression of AgNt84-GFP. A seventh plasmid was designed so the 35S promoter was driving the expression of AgNt84-GFP-HDEL. The AgNt84 fusion with GFP could be used for localization studies, and the AgNt84 constructs without a reporter fusion could be used in metal uptake experiments. Further explanations of the plasmids used are given in Chapter III.

### 2. Confirm Expression of Plasmids in Plant Tissue by Transient Transformation of Onion Epidermal Cells by Particle Bombardment and of Tobacco Leaves by Agroinfiltration

Particle bombardment and agroinfiltration were two methods of transient expression used to verify that *Agrobacterium* could transform tobacco with T-DNA that would express GFP. Particle bombardment and agroinfiltration save time by avoiding potential problems with stable transformation and expression while giving information about the fusion protein's location.

Particle bombardment was a method for transient gene expression in which a plasmid could be expressed directly in onion cells and provided a way to verify that the plasmid construct was made properly and could lead to expression in plant cells. More information on particle bombardment is given in Chapter IV. Being aware that the expression of the plasmid works ensures that the sequence of the reporter gene is fine.

Agroinfiltration is a method of transient *Agrobacterium*-mediated transformation that can be used to determine if a particular *Agrobacterium* strain is compatible with the species or variety of plant used. This was especially helpful since the proposed *A. rhizogenes* strain AR10 (R24E7) had not been shown previously in literature to transform tobacco.

## 3. Transform Tobacco Plants and Tobacco BY-2 Suspension Cell Culture via Stable *Agrobacterium*-mediated Transformation

Seven binary plasmids were designed, and each of these was used to transform *A. tumefaciens* strain LBA4404, *A. rhizogenes* strain A4RS, and *A. rhizogenes* strain AR10. The method chosen for transformation was electroporation because electroporation is fast, and some of the *Agrobacterium* strains were known to be amenable to electroporation. However, triparental mating was tried on A4RS because electroporation of A4RS did not work. Successful transformation of some of the transformed *Agrobacterium* was confirmed by back transformation into *E. coli.*

The method for transforming tobacco plants initially centered on forming hairy roots. The protocols followed were for leaf disc transformation (from Mentewab Ayalew) and hypocotyl wounding (Chian 1998). The leaf disc method was initially tried because a transformation protocol that worked for *A. tumefaciens* might also work for *A. rhizogenes*. The technique of wounding the hypocotyl with a needle was derived from a method used on other plants in the laboratory with *A. rhizogenes*, so this was thought to work. However, the procedure with the fruitful results is from a procedure in which the midvein of a tobacco leaf is used to produce tobacco hairy roots (Medina-Bolívar and Cramer 2004). Medina-Bolívar and Cramer (2004) mention that selection of hairy roots directly from *A. rhizogenes* transformed tissue is not reliable because untransformed roots could grow among the transformed roots. The plasmid pRedRoot (Limpens et al., 2004), which marks transformed hairy roots with constitutive expression of red fluorescent protein was not available to us at the time that these experiments were done. An alternate strategy was to generate hairy roots from already transformed plants expressing AgNt84, so leaf disc transformation with a transformed tobacco line was attempted. Attempts were also made to transform BY-2 cells using using several different protocols and *A. tumefaciens* LBA4404.

### 4. Determine the Subcellular Localization of AgNt84 in Transformed Tobacco

Plants that had been transformed with binary plasmids containing GFP were checked for GFP fluorescence using a fluorescence microscope, and any information regarding the subcellular localization of GFP was obtained. It was possible that expression of GFP was not visible extracellularly, but not all the possible methods for confirming that GFP was targeted extracellularly were tried because of the lack of stable transformed lines and/or time. The information gained was not a complete understanding of the targeting of AgNt84, but the information gave further insight about what may be happening *in vivo*.

### 5. Determine the Short-term Cadmium Uptake of AgNt84 in Transgenic Tobacco Expressing AgNt84

Transformation of hairy roots or tobacco suspension cells did not yield stable transgenic lines for cadmium uptake experiments. Transgenic tobacco seeds from another source were used for cadmium uptake. However, expression of AgNt84 in the tobacco lines had not yet been confirmed by northern analysis. The approach taken was to check for the presence of AgNt84 RNA by northern analysis of the tobacco lines used for metal uptake experiments to be certain that an expressing line was included in the experiments.

In addition, the best method for setting up the metal uptake experiments had to be determined. The final setup for the cadmium uptake experiments was to use 3-week-old plants and hydroponically grow the plants for two more weeks. The 5-week-old plants were at the stage that they could be placed in MES buffer containing 200 μM $Cd(NO_3)_2 \cdot 4H_2O$. The choice of solution was based Nedelkoska and Doran (2000b), who report that the uptake of metals increases in plant tissue incubated in MES buffer for a short time. They reason that the cadmium interacts with ions in the MS media reducing the uptake of cadmium. In Nedelkoska and Doran (2000b) used tobacco hairy roots for 9 hour short-term uptake experiments. Since tobacco plants were used, uptake of cadmium would also occur in the shoot, so the time period for short-term metal uptake experiments was extended. Samples of plants incubated for one day in cadmium and for three days in

cadmium were analyzed. Inductively coupled argon spectrometry (ICP) was the method used for analysis of the cadmium concentration. Dithizone staining was used to visualize the location and concentration of cadmium within plant tissue. Information about ICP analysis and dithizone staining is presented in Chapter V.

## E. AN OUTLINE OF ALL THE OBJECTIVES

Each major objective contained goals, or minor objectives, to be completed before the major objective could be carried out. Each chapter is divided so related major objectives are discussed together. The outline below contains a summary of all the objectives from each chapter that should be completed to accomplish the two research objectives stated at the beginning of this chapter:

**Chapter III:**

***Major objective:***

- Design binary plasmids for transforming *Agrobacterium tumefaciens* and *rhizogenes* for later use in stable transformation of tobacco.

***Minor objectives:***

- To design seven plasmids for expression of agNt84 *in planta.*
- To construct the seven plasmids.
- To transform *E. coli* with each of the seven plasmids
- To isolate each plasmid from *E. coli*
- To confirm that the correct plasmid was made.

**Chapter IV:**

***Major objectives:***

- Confirm expression of plasmids in plant tissue by transient transformation both of onion epidermal cells by particle bombardment and of tobacco leaves by agroinfiltration.
- Transform tobacco plants and tobacco BY-2 suspension cell culture via stable *Agrobacterium*-mediated transformation.

- Determine the subcellular localization of AgNt84 in transformed tobacco.

***Minor objectives:***

- to transform onion epidermal cells by particle bombardment to verify GFP expression
- to transform *Agrobacterium tumefaciens* strain LBA4404, *Agrobacterium rhizogenes* strain AR10, and/or *Agrobacterium rhizogenes* strain A4RS with the seven binary plasmids
- to transform tobacco leaves by agroinfiltration
- to transform tobacco plants and tobacco BY-2 cells with *Agrobacterium*
- to verify the presence of the correct plasmid in *Agrobacterium* by transforming the plasmids back into *E. coli* and confirming their identity.

**Chapter V:**

***Major objective:***

- Determine the short-term cadmium uptake in transgenic tobacco expressing AgNt84.

# CHAPTER III

# CONSTRUCTION OF BINARY PLASMIDS & *E. COLI* TRANSFORMATION

## A. INTRODUCTION

A major objective of this research was to design plasmids to be used in plant transformation experiments in order to observe the location of AgNt84 and the effect of its expression on the ability of plants to sequester cadmium. Before making any plasmid, decisions needed to be made about how the plasmid should be designed, including which gene or genes should be included in the plasmid, which promoter should drive expression and what selection system should be used. These decisions were partially covered in Chapter II. Constructing the plasmids included several cloning steps where genes were copied or cut from a source of DNA and inserted into a plasmid. Once a plasmid was made, the long-term storage of the plasmid was in an *E. coli* strain such as DH5α that had been transformed with the plasmid and frozen at -80°C. When needed, the plasmid was isolated from *E. coli*.

### 1. SPECIFIC OBJECTIVES

To accomplish the major objective, there were five minor objectives to be completed. The five objectives were:

- To design seven plasmids for expression of AgNt84 *in planta*.
- To construct the seven plasmids.
- To transform *E. coli* with each of the seven plasmids
- To isolate each plasmid from *E. coli*
- To confirm that the correct plasmid was made.

The objective requiring the most thought was the design of the plasmid. An introduction into the design of the plasmids used in the research presented in this thesis is given below.

## 2. An Introduction to the Gateway System

Seven binary plasmids were designed for constitutive and inducible expression of AgNt84 and AgNt84-GFP *in planta*. To simplify clone construction, Gateway vectors were used to make the plasmids for transformation, first of *Agrobacterium rhizogenes* AR10 (R24E7), *A. rhizogenes* A4RS, or *Agrobacterium tumefaciens* LBA4404, and finally for transformation into plants. Gateway vectors allow directional cloning while selecting against undesired ligations by using the ccdB gene (Invitrogen 2002). The ccdB protein product interacts with DNA gyrase in certain strains of *E. coli*, preventing growth of the bacteria (Invitrogen 2002). Therefore, only the bacteria with the correct clones grow, facilitating the cloning process. The Gateway system requires two cloning steps referred to as the BP and LR reactions (Invitrogen 2002).

The first step in Gateway cloning is to amplify the sequence to be cloned, in this case *AgNt84* or *AgNt84-GFP*, using forward primers that contain the attB1site and reverse primers that contain the attB2 site. The second step in Gateway cloning is the BP reaction (**Figure III-1**, Invitrogen 2002). This reaction uses the PCR product from the first step and a donor vector containing the ccdB gene flanked by attP1 and attP2 sites. When the BP Clonase enzyme is added to the sample, this enzyme cuts and ligates the sites. The sites are paired based on the sequence of the att site. The attB1 site reacts with attP1 to form an attL1 site on the vector and an attR1 site on the byproduct (Invitrogen 2002). At the same time, attB2 reacts with attP2 to form an attL2 site on the vector and an attR2 site on the byproduct. The vector, called an entry clone, now contains the gene of interest flanked by attL sites, and the byproduct is a linearized strand of DNA containing the ccdB gene flanked by attR sites.

The LR reaction is the third step in the Gateway system (**Figure III-1**, Invitrogen 2002). The same entry vector can be used for several different LR reactions, which simplifies making multiple plasmid constructs. The LR reaction utilizes the entry vector,

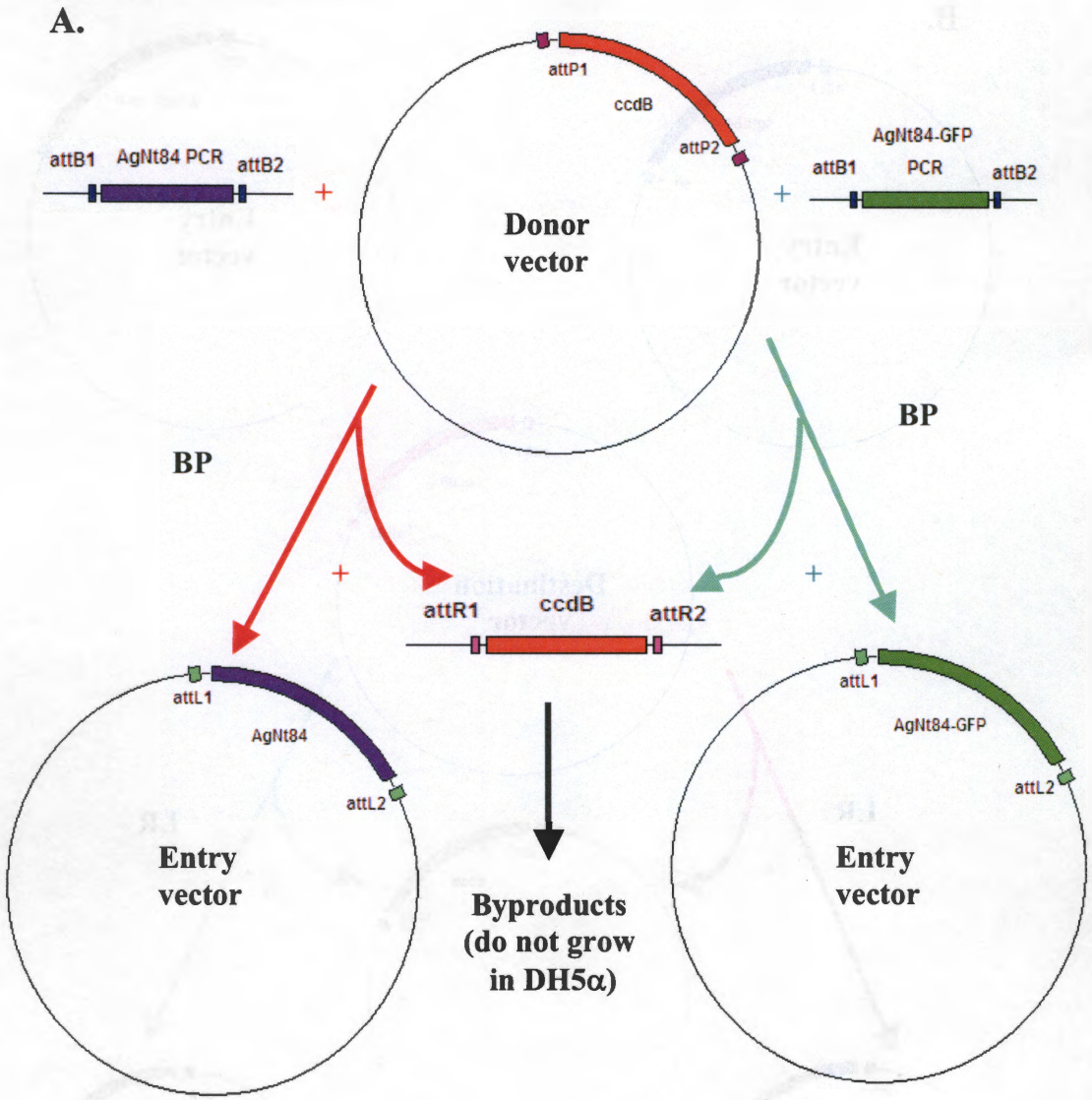


**FIGURE III-1: Diagram of the Gateway reaction with AgNt84 or AgNt84-GFP as the gene.** One side of the diagram follows the BP and LR reactions of AgNt84 (shown with red arrows and plus signs), and the other side of the diagram follows the BP and LR reactions of AgNt84-GFP (shown with green arrows and plus signs). **A.** The BP reaction. The PCR product and the donor vector undergo a BP reaction to form an entry vector and a linear byproduct containing the ccdB gene. The BP reaction byproduct does not grow in DH5α. **B.** The LR reaction. The entry vector and a destination vector undergo the LR reaction to generate the expression clone, which is the binary vector containing AgNt84 or AgNt84-GFP. A circular byproduct is made that does not grow in *E. coli*. For simplicity, the names of the plasmid used in the Gateway reaction are omitted. The diagram was made using information and diagrams based on the Gateway instruction manual from Invitrogen (2002).

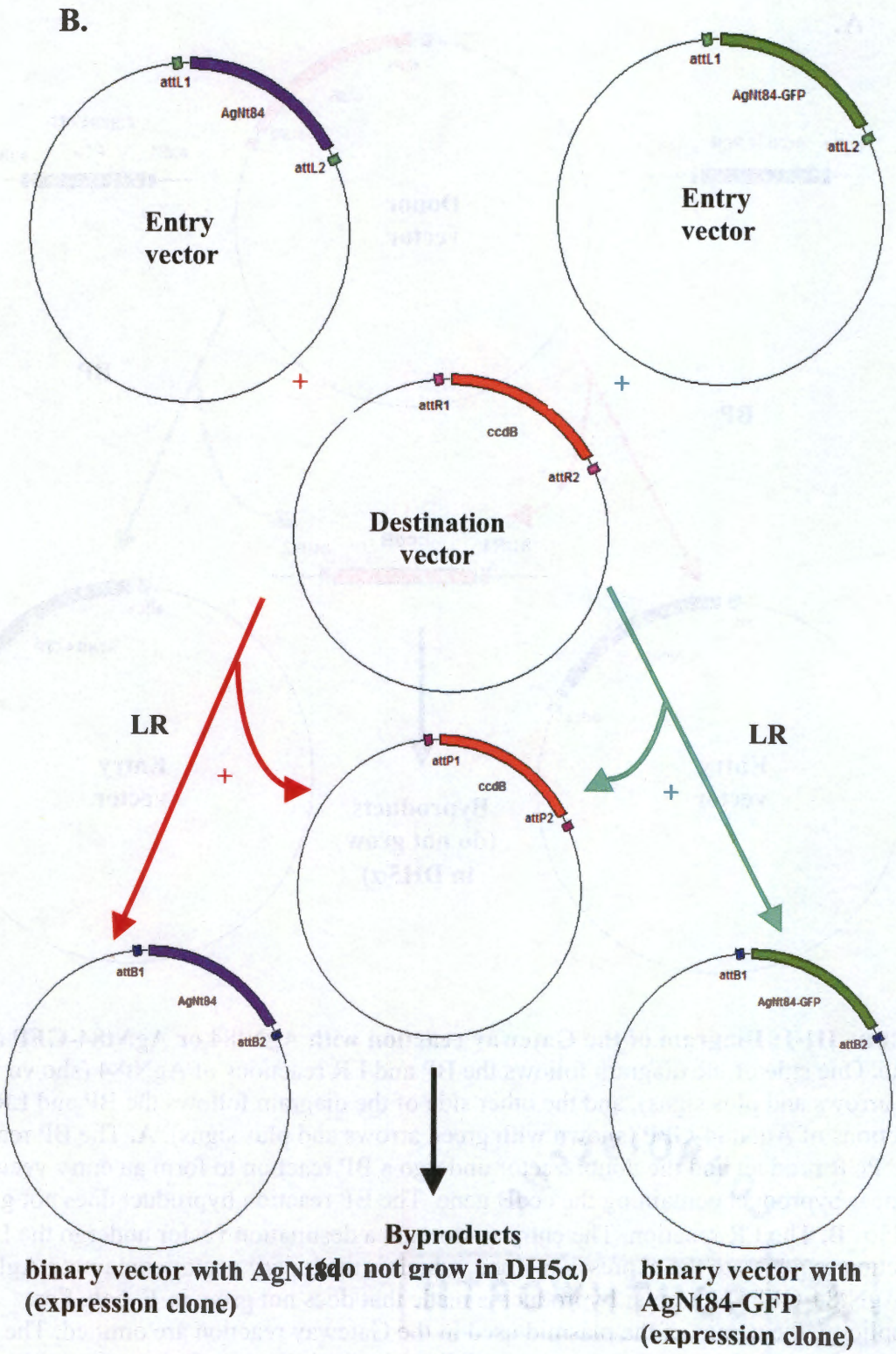


**FIGURE III-1:** Continued.

which contains the gene of interest flanked by attL sites, and the destination vector, which contains the ccdB gene flanked by attR sites. The destination vector is a different vector than used in the BP reaction. In this case, the vector is a binary vector. In addition, the enzyme used in this reaction is LR Clonase, which does the reverse reaction of BP Clonase. The attL1 site reacts with the attR1 site to create an attB1 site in the expression clone and an attP1 site in the byproduct. The attL2 site reacts with the attR2 site to generate an attB2 site in the expression clone and an attP2 site in the byproduct. The expression clone contains the binary vector with the gene of interest while the byproduct would be similar to the donor vector used in the BP reaction. Certain bacterial strains, such as DH5α, transformed with the byproduct should not grow because the ccdB gene is toxic to the bacteria.

### 3. Plasmids Used

The use of a constitutive promoter, an inducible promoter, and a reporter gene have several possible advantages and disadvantages when trying to accomplish major objectives of this research. With the Gateway system, construction of plasmids containing each type of promoter with and without a reporter gene could be completed quickly. Two destination vectors were used to obtain clones containing the constitutive 2X CaMV 35S promoter (Curtis and Grossniklaus 2000, http://www.unizh.ch/botinst/Devo_Website/curtisvector/index.html). The vector pMDC32 is a plasmid that contains the 2X CaMV 35S promoter without any reporter gene (Curtis and Grossniklaus 2000) (**Table III-1**). The plasmid with 2X CaMV 35S promoter and GFP-HDEL is pMDC202 (http://www.unizh.ch/botinst/Devo_Website/curtisvector/index.html). If AgNt84 was being targeted to the endoplasmic reticulum (ER), the HDEL tag would retain AgNt84-GFP-HDEL fusion protein in the ER. In addition, two destination vectors that contained inducible promoters were used. The plasmid pMDC7 contains the estradiol-inducible promoter, and pMDC30 has the heat-shock promoter (Curtis and Grossniklaus 2000). Both plasmids do not have a reporter gene (Curtis and Grossniklaus 2000).

Only one of the four destination vectors contained a reporter gene. However, plasmids containing AgNt84 and GFP were desired. A solution to this problem was to make an

**TABLE III-1: Binary vectors made for use in plant transformation.**

| Vectors Used: | Promoter Used: | Presence of: | | | Antibiotic Selection in: | |
|---|---|---|---|---|---|---|
| | | AgNt84 | GFP | HDEL Tag | Agrobacterium | Plants |
| pMDC7 | estradiol | Yes | No | No | Spectinomycin | Hygromycin |
| pMDC8 | estradiol | Yes | Yes | No | Spectinomycin | Hygromycin |
| pMDC30 | heat shock | Yes | No | No | Kanamycin | Hygromycin |
| pMDC30 | heat shock | Yes | Yes | No | Kanamycin | Hygromycin |
| pMDC32 | CaMV 35S | Yes | No | No | Kanamycin | Hygromycin |
| pMDC33 | CaMV 35S | Yes | Yes | No | Kanamycin | Hygromycin |
| pMDC202 | CaMV 35S | Yes | Yes | Yes | Kanamycin | Hygromycin |

AgNt84-GFP fusion by inserting AgNt84 into a plasmid containing GFP. Two entry vectors were made. One vector contained AgNt84, and the other vector contained AgNt84-GFP. In summary, three plasmids were used in the process of amplifying AgNt84 and AgNt84-GFP, and four destination vectors, two entry clones, and seven expression clones were used and/or made.

### 4. Nomenclature of Plasmids

Since several clones were made containing several different promoter genes with and without reporter genes, a nomenclature was devised to ease the identification and distinction of each of the clones from one another (**Table III-1**). All clones contained "pB" at the beginning. The next letter denotes the promoter used: "E" for estradiol-inducible promoter, "H" for heat-shock promoter, and "C" for the 2X CaMV 35S promoter. All clones then contain an "A" because the AgNt84 gene was inserted after the promoter. The last letters stand for the presence or absence of a reporter gene: "N" for no reporter, "G" for GFP reporter, and "GE" for GFP reporter containing an HDEL tag. Finally, the number "1" was added at the end in case the vectors would later be modified. Thus, the plasmids derived from pMDC7 containing the estradiol-inducible promoters would be pBEAN1 and pBEAG1. The vector pBEAN1 contained AgNt84, and pBEAG1 contained the AgNt84 fusion with a GFP reporter. The heat shock promoter constructs derived from pMDC30 were named pBHAN1 and pBHAG1. The constructs containing 2X CaMV 35S promoter stemming from pMDC32 were called pBCAN1 and pBCAG1, and the construct containing 2X CaMV 35S promoter made from pMDC202 was named pBCAGE1.

### 5. An Outline of Cloning Procedure

The procedure used for constructing the plasmids will be briefly outlined. There were several steps and plasmids used for constructing the seven plasmids. *AgNt84* was amplified from a clone previously made by Paul Twigg. The PCR product of *AgNt84* was used directly for the Gateway BP reaction. In addition, an *AgNt84* PCR product was

inserted into the vector AN59 which contains GFP. The *AgNt84-GFP* was amplified from the pAN59-AgNt84 plasmid.

At the BP reaction step, the PCR product of *AgNt84* or *AgNt84-GFP* was transferred to a Donor vector called pDONR/Zeo (Invitrogen), and the products of the reaction were transformed into *E. coli* (**Figure III-1A**). The entry clone containing *AgNt84* or *AgNt84-GFP* was then utilized for a LR reaction (**Figure III-1B**).

At the LR reaction step, both entry clones were used with the destination vectors pMDC7, pMDC30, and pMDC32 (**Figure III-1B**). The vector pMDC202 was combined only with *AgNt84*. Seven binary plasmids, or the expression clones, were made.

After constructing the plasmids, *E. coli* was transformed using the heat-shock method. Isolation of the plasmid was done following a mini-preparation protocol for plasmid DNA, and the plasmid was used for three purposes: confirmation of the correct plasmid, transformation directly into plant tissue, or transformation of *Agrobacterium*. Use of the plasmid for the latter two purposes is discussed in Chapter IV.

## B. Materials & Methods

### 1. Bacterial Strains

Library Efficiency *E. coli* DH5α (Invitrogen) was used for bacterial transformation of all the plasmids, except for propagating vectors containing the ccdB gene. Competent *E. coli* DB3.1 (Invitrogen) was used for propagation of these plasmids because it contains a mutated DNA gyrase not affected by the ccdB gene (Invitrogen 2002).

### 2. Plasmids and Gateway Kits

AN59 was received from Andreas Nebenführ, University of Tennessee, Knoxville. AN59 was provided in *E. coli* TOP10. Glycerol stocks of liquid cultures were made (Appendix A).

pAgNt84 had already been transformed in INV1αF' and made into a glycerol stock culture. pAgNt84 had been made previously in Beth Mullin's laboratory by a former graduate student in the lab, Paul Twigg.

The plasmids pMDC30, pMDC32, and pMDC202 were ordered from Mark Curtis, University of Zürich. The plasmids came on dry paper, and they were eluted with TE buffer (Appendix A). The plasmid pMDC7 was ordered from Nam-Hai Chua, Rockefeller University. The plasmid came as a lyophilized pellet. It was rehydrated in TE buffer. DB3.1 was transformed with each of the four plasmids, and a glycerol stock was made of each plasmid (Appendix C).

A Gateway PCR cloning kit containing pDONR/Zeo plasmid, the BP clonase enzyme kit, and the LR clonase enzyme kit was purchased from Invitrogen. pDONR/Zeo was transformed into DB3.1, and a glycerol stock was made.

## 3. PCR Amplification of AgNt84 for Gateway Reactions

### 3. a. Amplification of AgNt84 for Plasmids without a Reporter

The following primers were used to amplify *AgNt84* from pAgNt84. attB1 and attB2 sites are indicated by single and double underlining respectively.

Forward Primer: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTATTG TCTCCAATCCTCTTCATTG 3'

Reverse Primer: 5' GGGGACCACTTTGTACAAGAAAGCTGGGTATTT TGGTTGGTTTCAGTTTCGG 3'

In addition, a control PCR reaction was set up in which there was no pAgNt84 plasmid in the PCR reaction. A positive control was also done using primers and a template that were known to be amplified by PCR. The PCR was done following the steps outlined in Appendix B. The PCR products were cleaned using the QIAquick PCR Cleanup Kit (QIAGEN). The presence or absence of PCR products was checked by TAE agarose gel electrophoresis. The PCR product was used for the BP reaction.

### 3. b. PCR Amplification of AgNt84 Fused to a Reporter to Make Plasmids with a Reporter

The following primers were used to amplify *AgNt84* from pAgNt84, for cloning into AN59. The PCR primers were designed to engineer XbaI and BamHI recognition sites into the resulting amplified fragment.

Forward Primer: 5' CGATCTAGAATTGTCTCCAATCCTCTTCATTG 3'

Reverse Primer: 5' CGCGGATCCATTTTGGTTGGTTTCAGTTTCGG 3'

In addition, a control PCR reaction was set up in which there was no pAgNt84 plasmid in the PCR reaction. The amplification of AgNt84 was completed following the steps outlined in Appendix B. PCR products were cleaned using the QIAquick PCR Cleanup Kit (QIAGEN).

The PCR product was digested with the restriction enzymes XbaI and BamHI (Fisher Scientific). In addition, the plasmid AN59 was digested with the same restriction enzymes. A ligation reaction of the AgNt84 PCR product and AN59 was done using T4 DNA ligase (Invitrogen) according to the instructions supplied with the ligase (Appendix B). The product of the ligation reaction was cleaned using the Promega DNA Clean-up Kit. The resulting plasmid was called pAN59-AgNt84. DH5α was transformed with this plasmid, and the plasmid was isolated from DH5α. The proper insertion of the cloned fragment was checked by restriction enzyme digestion using XbaI and BamHI (both from Fisher Scientific) and TAE agarose gel electrophoresis. DNA sequencing of the region where AgNt84 was inserted confirmed the correct insertion and orientation of AgNt84.

Upon verification that one copy of AgNt84 was correctly oriented in AN59, the following primers were used to amplify *AgNt84-GFP* using pAN59-AgNt84 as a template. Again attB1 and attB2 sites are marked by single and double underlining respectively.

Forward Primer: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTATTG TCTCCAATCCTCTTCATTG 3'

Reverse Primer: 5' GGGGACCACTTTGTACAAGAAAGCTGGGTTTAC TTGTACAGCTCGTCCATGC 3'

In addition, a control PCR reaction was set up in which there was no pAgNt84 plasmid in the PCR reaction. The PCR was done following the steps outlined in Appendix B. The PCR products were cleaned using the QIAquick PCR Cleanup Kit (QIAGEN). The presence or absence of PCR products was checked by TAE agarose gel electrophoresis. The PCR product was used for the Gateway reaction.

### 4. Gateway BP and LR Reactions Using PCR Products

The PCR product of AgNt84 flanked with attB sites and pDONR/Zeo were used to create an entry clone named pENTRZeo-AgNt84 based on guidelines from the Gateway manual (Invitrogen 2002). The entry clone was created using the BP reaction outlined in Appendix B, and the entry clone was transformed into DH5α. Isolated plasmids of pENTRZeo-AgNt84 and each of the four destination vectors were used to create the expression vectors pBEAN1, pBHAN1, pBCAN1, and pBCAGE1. The destination vectors were pMDC7, pMDC30, pMDC32, and pMDC202, respectively. The expression vectors were generated using the LR reaction outlined in Appendix B.

The PCR product of AgNt84-GFP flanked with attB sites was treated the same as the PCR product of AgNt84 in the BP and LR reaction. The entry clone resulting from the BP reaction was named pENTRZeo-AgNt84GFP. For the LR reaction, only vectors pMDC7, pMDC30, and pMDC32 were involved in making the expression vectors pBEAG1, pBHAG1, and pBCAG1, respectively. pMDC202 was not combined with the AgNt84-GFP product.

### 5. Heat-shock Transformation of *E. coli*

Library Efficiency DH5α Chemically Competent *E. coli* (Invitrogen) was used for transformation of plasmid DNA except for reproducing plasmids containing the ccdB gene. The host used for these plasmids was competent DB3.1 *E. coli* (Invitrogen). In addition, a positive control (pUC19) and a negative control (no plasmid) were included. The heat-shock transformation protocol that was followed was supplied with the Library Efficiency Chemically Competent DH5α *E. coli.*

Aliquots from each transformation were spread on plates containing appropriate growth medium and antibiotics (**Table III-2**). Two different quantities were spread on separate plates, and they were 20 μL and 100 μL. For the positive control, 100 μL of the liquid medium was added to 900 μL of SOC for a 1:10 dilution, and 20 μL and 100 μL of the 1:10 dilution were plated on separate plates containing the appropriate antibiotics. The plates for the positive control were used to calculate the transformation efficiency.

**TABLE III-2: The bacterial media and antibiotics used to grow different strains of transformed *E. coli*.**

| Plasmid | *E. coli* Strain | Media* | Antibiotic selection |
|---|---|---|---|
| Positive (pUC19) | DH5α | LB | 100 μg/mL ampicillin |
| pAgNt84 | INV1αF' | LB | 100 μg/mL ampicillin |
| AN59 | TOP10 | LB | 100 μg/mL ampicillin |
| pAN59-AgNt84 | DH5α | LB | 100 μg/mL ampicillin |
| pDONR/Zeo | DB3.1 | Low-Salt LB | 30 μg/mL chloramphenicol<br>50 μg/mL zeocin |
| pENTRZeo-AgNt84 and pENTRZeo-AgNt84GFP | DH5α | Low-Salt LB | 50 μg/mL zeocin |
| pMDC30, pMDC32, and pMDC202 | DB3.1 | LB | 50 μg/mL kanamycin<br>30 μg/mL chloramphenicol. |
| pMDC7 | DB3.1 | LB | 50 μg/mL spectinomycin<br>30 μg/mL chloramphenicol |
| pBCAN1, pBCAG1, pBCAGE1, pBHAN1, and pBHAG1 | DH5α | LB | 50 μg/mL kanamycin |
| pBEAN1 and pBEAG1 | DH5α | LB | 50 μg/mL spectinomycin |

* The components of the bacterial medium listed are given in Appendix C.

## 6. Mini-prep of Bacterial Cultures to Recover Plasmid DNA

The method used for isolating plasmids was a modification of a protocol from the pGreen website (http://www.pgreen.ac.uk/). 1.5 mL of an overnight culture of either *E.coli* or *Agrobacterium* was transferred to microcentrifuge tubes. Each tube was centrifuged at 4000 rpm for 4 minutes. The supernatant was discarded, and the pellet was resuspended in 200 µL of Solution W (Appendix A). 200 µL of freshly prepared Solution P (Appendix A) were added into each microcentrifuge tube. Solution P was mixed in, and each tube was put on ice. 200 µL of ice-cold solution T (Appendix A) were then added to the tube, and the microcentrifuge tube was mixed again. The microcentrifuge tube was incubated on ice for 10 minutes. The tube was centrifuged for 5 minutes at 14,000 rpm. The supernatant was transferred to another microcentrifuge tube, and 500 µL of 24:1 chloroform/*iso*-amyl alcohol were added, and each microcentrifuge tube was vortexed until the contents in the tube were mixed. The tube was then centrifuged at 14,000 rpm for 1 minute. The upper aqueous phase was transferred to another tube while the bottom phase was discarded. 500 µL of isopropanol were added to the top phase. Sometimes, the tubes were placed in the –20°C freezer for a variable amount of time.

The tubes were centrifuged for 5 minutes at 14,000 rpm, and the supernatant was pipetted off the DNA pellet. 500 µL of ice cold 70% ethanol were added to the pellet to rinse the pellet, and the tube was centrifuged for 10 minutes at 14,000 rpm. The ethanol was pipetted off the pellet, and the drops of ethanol were taken out of the tube using a vacuum. Any remaining alcohol was allowed to evaporate. 40 µL of TE buffer (Appendix A) were added to the dried pellet. The tubes were kept on ice for 30 minutes to rehydrate the pellet. The DNA was mixed into the TE buffer by tapping.

Some of the purified plasmid DNA was digested with restriction enzymes to confirm the identity of the plasmid. Following restriction digestion, the bands were separated by TAE agarose gel electrophoresis. All the plasmids listed in **Table III-1** were isolated following this method. All the restriction enzymes except PacI and AscI came from Fisher Scientific. PacI and AscI came from New England Biolabs.

### 7. TAE Agarose Gel Electrophoresis

DNA was electrophoresed on a 0.8% agarose TAE minigel (Appendix A) at 100 volts in TAE buffer, until the dye in the loading buffer was around 2/3 of the way to the bottom of the gel. Following electrophoresis, the gel was placed in a plastic container and was covered with approximately 200 mL of electrophoresis buffer. 12.5 µL of 10 mg/mL stock solution of ethidium bromide was added to the container, and the solution was thoroughly mixed. The gel incubated in the ethidium bromide solution for 15 minutes in the dark while rocking. At the end of 15 minutes, the solution was poured off into a waste container, and the gel was rinsed briefly with water. Fresh water was added to the gel, and the gel was incubated in the water for 15 minutes while rocking. The water was poured off, and the gel was visualized under UV light.

## C. Results

### 1. Verification of PCR Products

#### 1. a. AgNt84 PCR Product for Gateway Cloning

To create binary plasmids containing AgNt84 without GFP or with GFP-HDEL, AgNt84 was amplified by PCR. The expected length of the PCR product was 388 bp, and the presence of the PCR product was checked by TAE agarose gel electrophoresis. The gel contained a band with the expected size of the PCR product (**Figure III-2A**), indicating that amplification of AgNt84 was successful and that the PCR product could be used for the BP reaction to generate an entry clone.

#### 1. b. Generation of AgNt84-GFP PCR Product for Gateway Cloning

There were three main steps that had to be done before performing the Gateway reactions to make binary plasmids with an AgNt84-GFP fusion. The first step was to amplify AgNt84 with primers that will allow ligation of the PCR product into AN59. The second step was to insert AgNt84 into AN59 by ligation to make the ligation product pAN59-AgNt84. The third step using PCR was to amplify AgNt84-GFP from

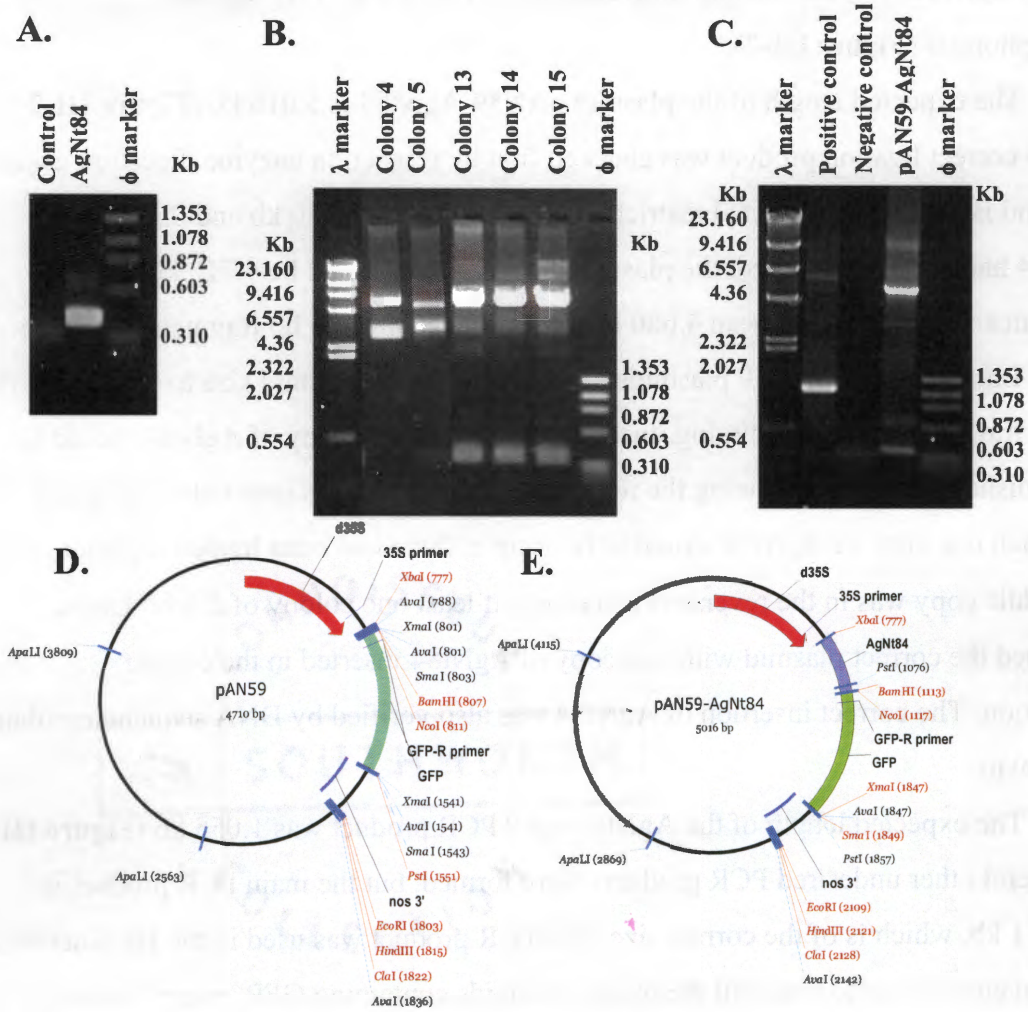


**FIGURE III-2: TAE agarose gel electrophoresis of PCR products and pAN59-AgNt84**. Band sizes of each marker are labeled to the side of the gels. **A.** Picture of the gel containing the AgNt84 PCR product with flanking Gateway attB1 and attB2 sites. Lane 1: control. Lane 2: AgNt84 PCR product with an expected fragment size of 388 bp. Lane 3: ϕX174 HaeIII marker. **B.** Picture of the gel containing the fragments of pAN59-AgNt84 digested with XbaI and BamHI. The expected fragment sizes are 4.680 kb and 0.336 kb. Lane 1: λ HindIII marker. Lanes 2-6: plasmids from colonies 4, 5, 13, 14, and 15 of *E. coli*, respectively. Lane 7: ϕX174 HaeIII marker. The presence of AgNt84 insert is confirmed by an approximately 366 bp fragment seen in colonies 13, 14, and 15 but not 4 or 5. **C.** Picture of the gel containing the AgNt84-GFP PCR product with flanking Gateway attB1 and attB2 sites. The expected fragment size of PCR product is 1.055 kb. Lane 1: λ HindIII marker. Lane 2: AgNt84 as a positive control. Lane 3: negative control. Lane 4: PCR of AgNt84-GFP. Lane 5: ϕX174 HaeIII marker. The positive control is roughly 300 bp. **D.** Plasmid map of AN59 used to design pAN59-AgNt84. **E.** Plasmid map of pAN59-AgNt84. The plasmid maps were made using Vector NTI, and the positions of the restriction sites are included on the maps.

pAN54-AgNt84. The second and third steps were verified by TAE agarose electrophoresis (**Figure III-2**).

The expected length of the plasmid pAN59-AgNt84 is 5.016 kb (**Figure III-2**), and the correct ligation product was checked first by restriction enzyme digestion using XbaI and BamHI. The expected restriction fragments were 4.680 kb and 0.336 kb. If AgNt84 had not been inserted, the plasmid size of AN59 would be 4.710 kb. The fragment sizes would have been 4.680 bp and 30 bp, so the 336 bp fragment would be absent. Ligation of two AN59 plasmids would increase the plasmid size to 9.420, and the 336 bp fragment would be missing. Inserting more than one copy of AgNt84 would be distinguished only by sequencing the region where AgNt84 was inserted. Ligation of more than one copy of AgNt84 would only occur if three had been ligated together, and the middle copy was in the reverse orientation. At least one colony of *E. coli* DH5α contained the correct plasmid with one copy of AgNt84 inserted in the correct orientation. The correct insertion of AgNt84 was also verified by DNA sequencing (data not shown).

The expected length of the AgNt84-GFP PCR product was 1.055 kb (**Figure III-2**). Several other undesired PCR products were formed, but the main PCR product is around 1 kb, which is of the correct size. The PCR product was used in the BP reaction to make an entry clone to make all the binary plasmids containing GFP, except for one.

### 2. The Presence of the Donor Vector and the Destination Vectors in *E. coli* Were Verified by Restriction Enzyme Digestion

*E. coli* DB3.1 was transformed with pDONR/Zeo, pMDC7, pMDC30, pMDC32, or pMDC202 upon receipt of the plasmids. The identity and presence of each of the plasmids was verified by running TAE agarose gel electrophoresis of DNA fragments from restriction digestion (**Figure III-3**).

pDONR/Zeo was digested with PstI and/or EcoRV for two reasons. Each enzyme had only one restriction enzyme site in the donor vector as well as the entry vectors, which is shown on the plasmid maps of the vectors (**Figure III-3**). These enzymes were

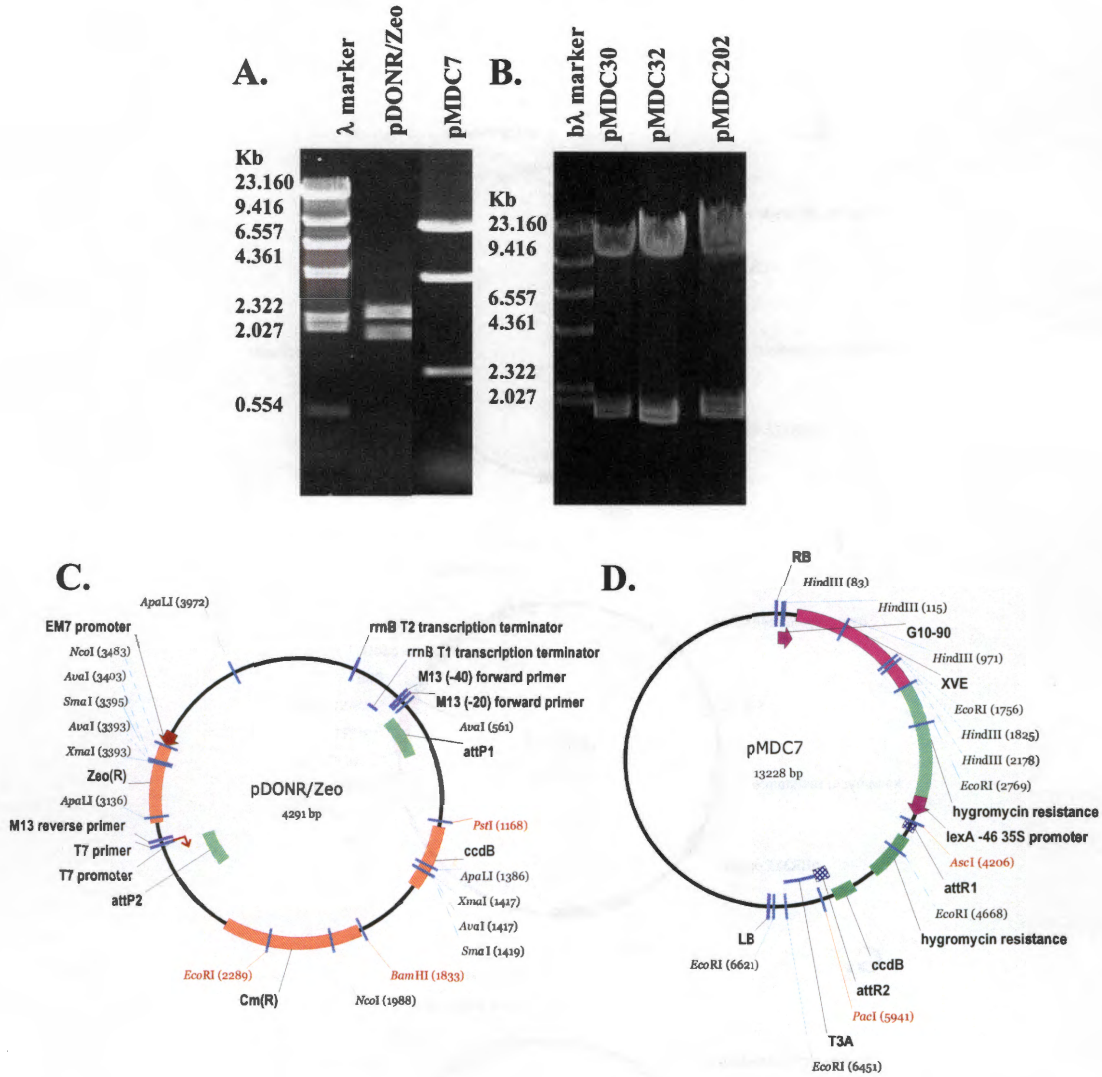


**FIGURE III-3: TAE agarose gel electrophoresis of pDONR/Zeo, pMDC7, pMDC30, pMDC32, and pMDC202. A.** Gel pictures of pDONR/Zeo digested with PstI and EcoRV and pMDC7 digested with BglII and EcoRV. Lane 1: λ HindIII marker. Lane 2: pDONR/ Zeo. The expected fragment sizes are 2.459 and 1.832 kb. Lane 3: pMDC7. The expected fragment sizes are 8.216 kb, 1.120 kb, and 3.892 kb. **B.** Gel pictures of pMDC30, pMDC32, and pMDC202 digested with AscI and PacI. Lane 1: λ HindIII marker. Lanes 2-4: pMDC30, pMDC32, and pMDC202, respectively. All have expected fragment sizes of approximately 10 kb and 1.7 kb. **C.** Picture of the plasmid map of pDONR/Zeo. **D.** Picture of the plasmid map of pMDC7. **E.** Picture of the plasmid map of pMDC30. **F.** Picture of the plasmid map of pMDC32. **G.** Picture of the plasmid map of pMDC202. The plasmid maps were made using Vector NTI. The positions of the restriction sites are shown on the maps of each plasmid.

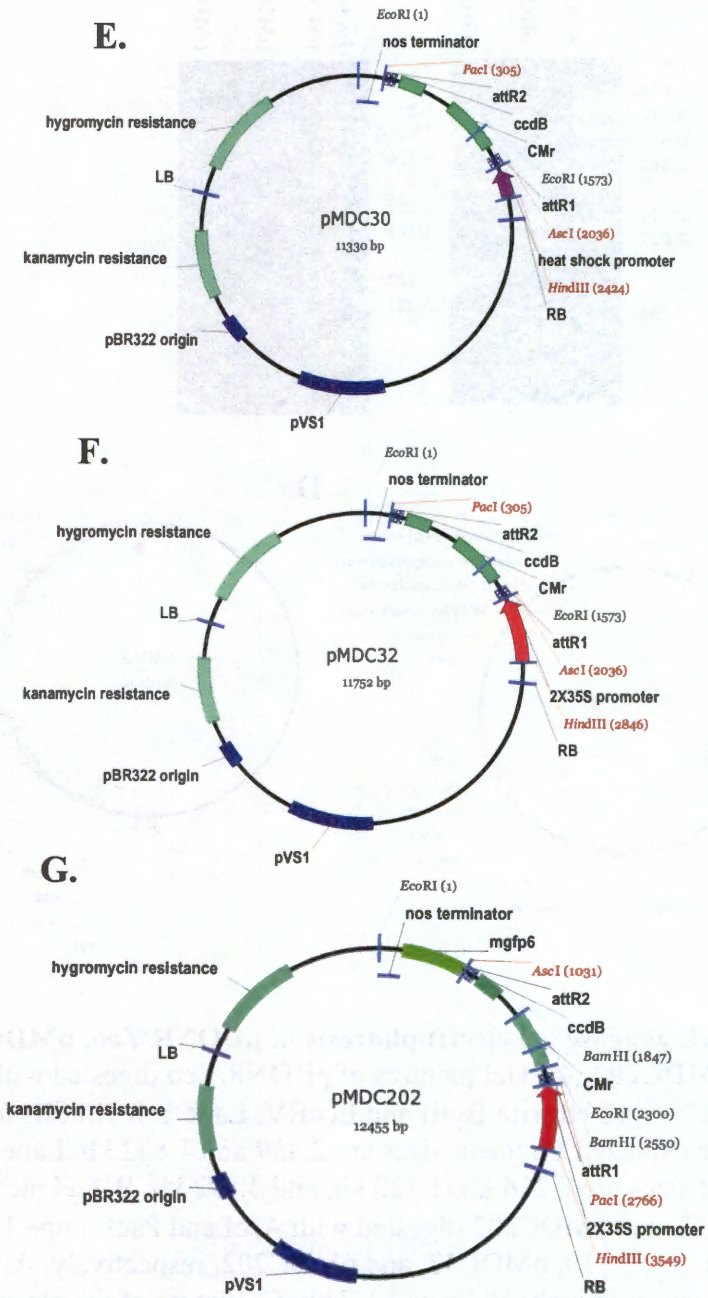


**FIGURE III-3:** Continued.

used to distinguish the donor vector from the entry vectors). The vector was 4.291 kb, so the expected fragment was 4.291 kb for either restriction enzyme used (picture of gel not shown). The expected fragments were 2.459 kb and 1.832 kb when both enzymes were used.

pMDC7 was digested with BglII and/or EcoRV. pMDC7 has two BglII sites and one Eco RV site (**Figure III-3**). The expected fragment sizes when both enzymes were used together were 8.216 kb, 1.120 kb, and 3.892 kb. The expected fragment sizes were 9.336 kb and 3.892 kb when BglII was used, and the fragment size when using EcoRV was 13.228 kb, which is the size of the plasmid (data not shown).

pMDC30, pMDC32, and pMDC202 were all digested with AscI and PacI. AscI and PacI resulted in the formation of two fragments that are about the same size for all three plasmids, and the fragment sizes were 10 kb and 1.7 kb (**Figure III-3**). This digestion was used to verify that the plasmids were present. This was necessary because these plasmids, when eluted from the paper on which they were sent, were not present at levels detectable by the fluorimeter. Later, the identity of the plasmids could be verified by restriction enzyme digestion of the seven expression vectors because inserting AgNt84 or AgNt84-GFP allowed differences to be detected among the plasmids.

Based on the restriction digestion of the plasmids, the results obtained supported that a plasmid was present.

### 3. Entry Clones from BP Reaction Were Made in the Proper Orientation

Two entry clones were made as a product of two different BP reactions. One reaction involved pDONR/Zeo and the PCR product of AgNt84, and the resulting entry vector was called pENTRZeo-AgNt84. The other reaction included pDONR/Zeo and the PCR product of AgNt84-GFP, and the entry vector was named pENTRZeo- AgNt84GFP. The correct orientation and number of inserts were verified by restriction enzyme digestion, and the fragments were separated by TAE agarose gel electrophoresis (**Figure III-4**). pENTRZeo-AgNt84 digested with PacI and EcoRV consisted of two fragments that are 2.249 kb and 0.149 kb, and digestion of only one of the enzymes would result in a

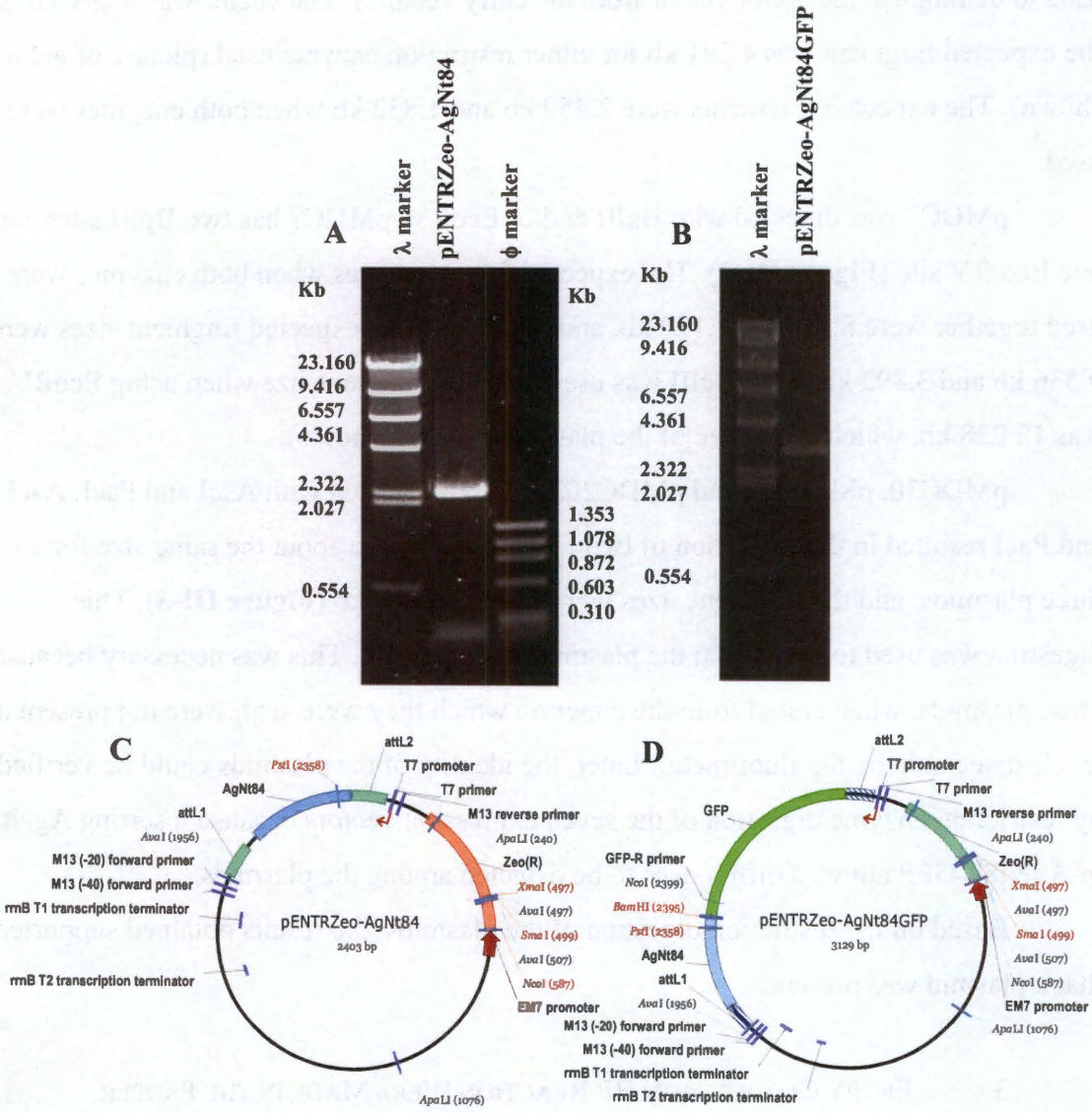


**FIGURE III-4: TAE agarose gel electrophoresis of pENTRZeo-AgNt84 and pENTRZeo-AgNt84GFP. A.** Picture of the gel containing pENTRZeo-AgNt84 digested with PstI and EcoRV. Lane 1: λ HindIII marker. Lane 2: pENTRZeo-AgNt84. The expected fragment sizes are 2.403 kb and 0.149 kb. Lane 3: ϕX174 HaeIII marker. **B.** Picture of the gel containing pENTRZeo-AgNt84GFP digested with BamHI. Lane 1: λ HindIII marker. Lane 2: pENTRZeo-AgNt84GFP. The expected fragment size is 3.129 kb. **C.** Picture of the plasmid map of pENTRZeo-AgNt84. **D.** Picture of the plasmid map of pENTRZeo-AgNt84GFP. The plasmid maps were made using Vector NTI. The positions of the restriction sites are shown on the maps of each plasmid.

fragment the size of the plasmid. pDONRZeo digested with PacI and EcoRV would have consisted of fragments 2.459 kb and 1.832 kb, and the use of one of the enzyme also would be the size of the plasmid (**Figure III-3**). pENTRZeo-AgNt84GFP was digested with BamHI. BamHI is present only once in pENTRZeo-AGNt84 and pDONR/Zeo (**Figure III-3** and **Figure III-4**, respectively). The size of the plasmid or the presence of more than one band would distinguish between correct and incorrect insertion of *AgNt84-GFP* into the donor vector. The plasmid size of pDONR/Zeo was 4.291 kb (**Figure III-3B**), and the plasmid size of pENTRZeo-AgNt84GFP was 3.129 kb (**Figure III-4**).

DNA sequencing using M13 forward and reverse primers was used to further confirm that only one copy of the insert was present and that it was oriented in the correct direction (data not shown). No sequence was obtained using the M13 reverse primer even though the site was present as determined by the sequence obtained from using the M13 forward primer in pENTRZeo-AgNt84 and pENTRZeo-AgNt84GFP.

### 4. Expression Clones from LR Reaction Were Made in the Proper Orientation

Seven LR reactions were completed to make seven binary vectors, which are also known in the Gateway system as the expression clones. Four of the expression clones were made using pENTRZeo-AgNt84 with pMDC7, pMDC30, pMDC32, or pMDC202. The resulting plasmids were named pBEAN1, pBHAN1, pBCAN1, and pBCAGE1, respectively. The three remaining expression clones were made by using pENTRZeo-AgNt84GFP with pMDC7, pMDC30, or pMDC32. These plasmids were named pBEAG1, pBHAG1, and pBCAG1, respectively.

Restriction enzyme digestion with HindIII and EcoRI could be used to distinguish the five different expression plasmids pBCAN1, pBCAG1, pBCAGE1, pBHAN1, and pBHAG1 (**Figure III-5** and **Figure III-6**). A 8.907 kb fragment was common among each of the five plasmids when digested with these two enzymes. This fragment is similar because these plasmids share a common backbone (Curtis and Grossniklaus 2000). A second smaller fragment was made when the plasmids were digested with HindIII and

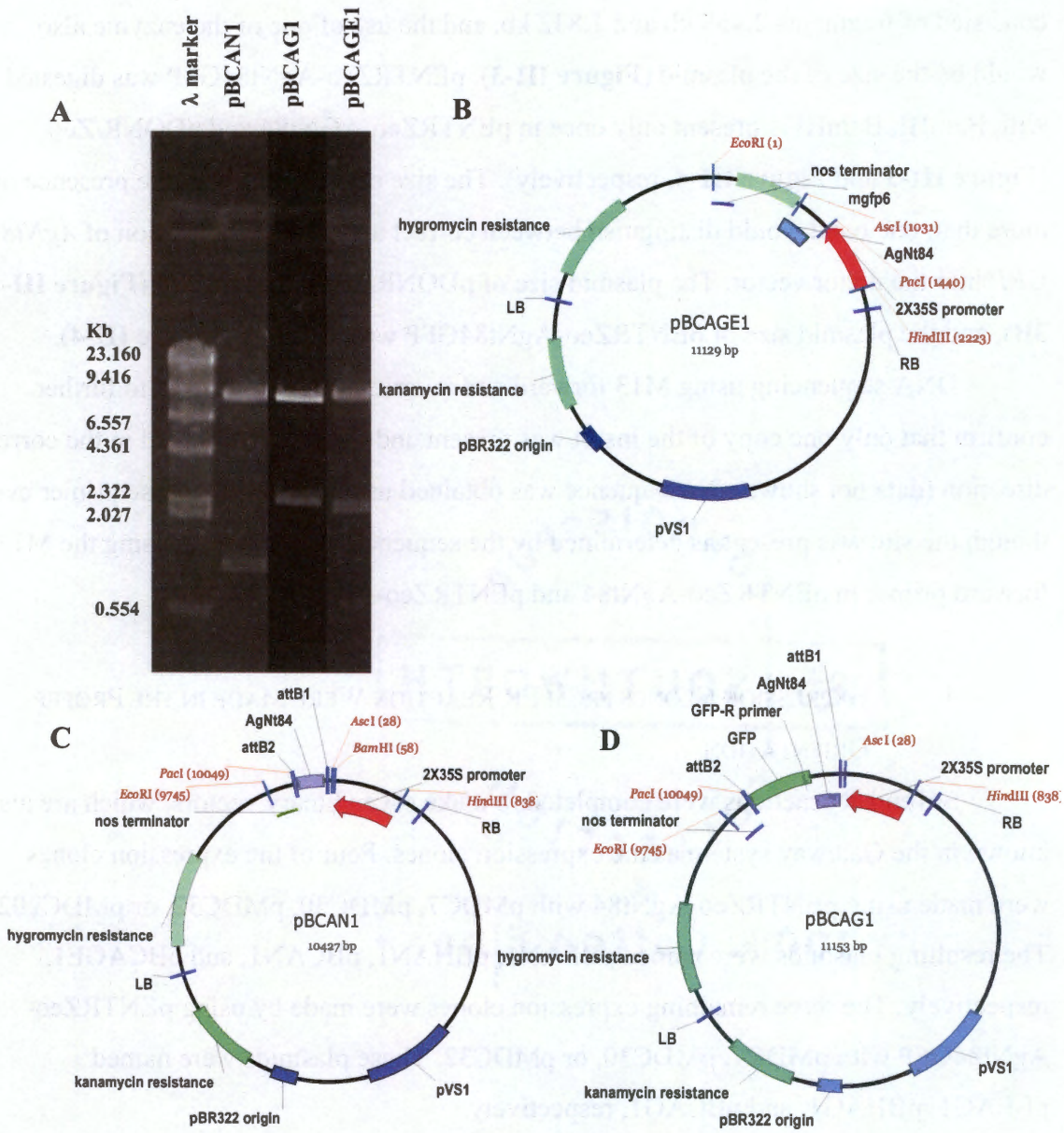


**FIGURE III-5: TAE agarose gel electrophoresis of pBCAN1, pBCAG1, and pBCAGE1. A.** Picture of the gel containing the restriction digestion of each plasmid with HindIII and EcoRI. Lane 1: λ HindIII marker. Lane 2: pBCAN1. The expected fragment sizes are 8.907 kb and 1.5 kb. Lane 3: pBCAG1. The expected fragment sizes are 8.907 kb and 2.246 kb. Lane 4: pBCAGE1. The expected fragment sizes are 8.907 kb and 2.224 kb. **B.** Picture of the plasmid map of pBCAGE1. **C.** Picture of the plasmid map of pBCAG1. **D.** Picture of the plasmid map of pBCAN1. The plasmid maps were made using Vector NTI. The positions of the restriction sites are shown on the maps of each plasmid.

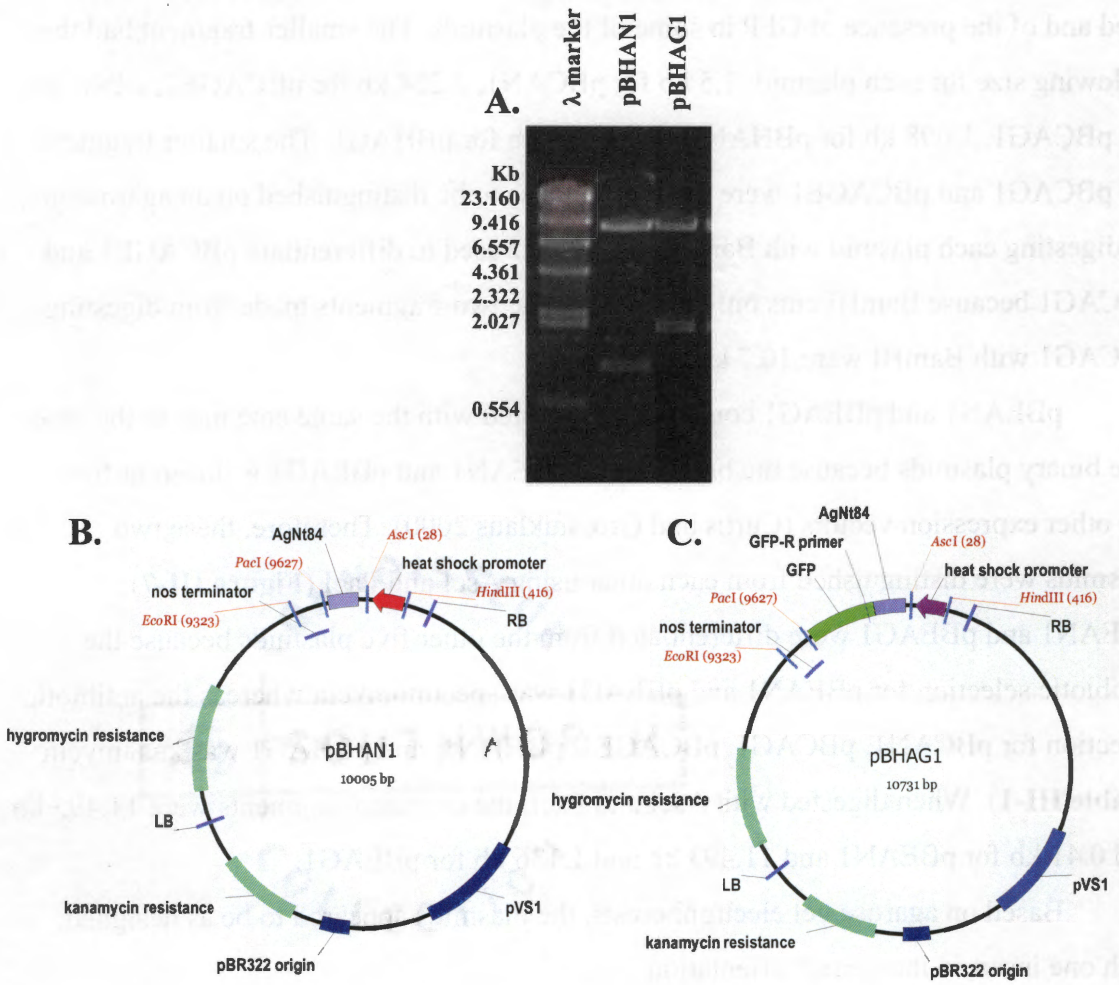


**FIGURE III-6: TAE agarose gel electrophoresis of pBHAN1 and pBHAG1. A.** Picture of the gel containing pBHAN1 and pBHAG1. Both are digested with HindIII and EcoRI. Lane 1: λ HindIII marker. Lane 2: pBHAN1. The expected fragment sizes are 8.907 kb and 1.5 kb. Lane 3: pBHAG1. The expected fragment sizes are 8.907 kb and 2.246 kb. **B.** Picture of the plasmid map of pBHAN1. **C.** Picture of the plasmid map of pBHAG1. The plasmid maps were made using Vector NTI, and the positions of the restriction sites are shown on the maps of each plasmid.

EcoRI. This fragment differed in size for each plasmid because of the different promoters used and of the presence of GFP in some of the plasmids. The smaller fragment had the following size for each plasmid: 1.5 kb for pBCAN1, 2.224 kb for pBCAGE1, 2.246 kb for pBCAG1, 1.098 kb for pBHAN1, and 1.824 kb for pBHAG1. The smaller fragment for pBCAG1 and pBCAGE1 were too close in size to be distinguished on an agarose gel, so digesting each plasmid with BamHI was further used to differentiate pBCAGE1 and pBCAG1 because BamHI cuts only pBCAG1. The two fragments made from digesting pBCAG1 with BamHI were 10.7 kb and 0.4 kb.

pBEAN1 and pBEAG1 could not be digested with the same enzymes as the other five binary plasmids because the backbone of pBEAN1 and pBEAG1 is different from the other expression vectors (Curtis and Grossniklaus 2000). Therefore, these two plasmids were distinguished from each other using AscI and PacI (**Figure III-7**). pBEAN1 and pBEAG1 were differentiated from the other five plasmids because the antibiotic selection for pBEAN1 and pBEAG1 was spectinomycin whereas the antibiotic selection for pBCAN1, pBCAG1, pBCAGE1, pBHAN1, and pBHAG1 was kanamycin (**Table III-1**). When digested with AscI and PacI, the expected fragments were 11.493 kb and 0.41 kb for pBEAN1 and 11.493 kb and 1.136 kb for pBEAG1.

Based on agarose gel electrophoresis, the plasmids appeared to be as designed, with one insert in the correct orientation.

## D. CONCLUSION

The construction of the seven expression plasmids and their transformation into *E. coli* were successful. As stated in the introduction to this chapter, there were four objectives to accomplish. The objectives were to design and make seven binary plasmids, to transform *E. coli* with the plasmids, to isolate the plasmids from *E. coli*, and to verify that the correct plasmids were made. With these objectives met, the next objective was to test the performance each expression plasmid *in planta* using methods to achieve both transient and stable transformation.

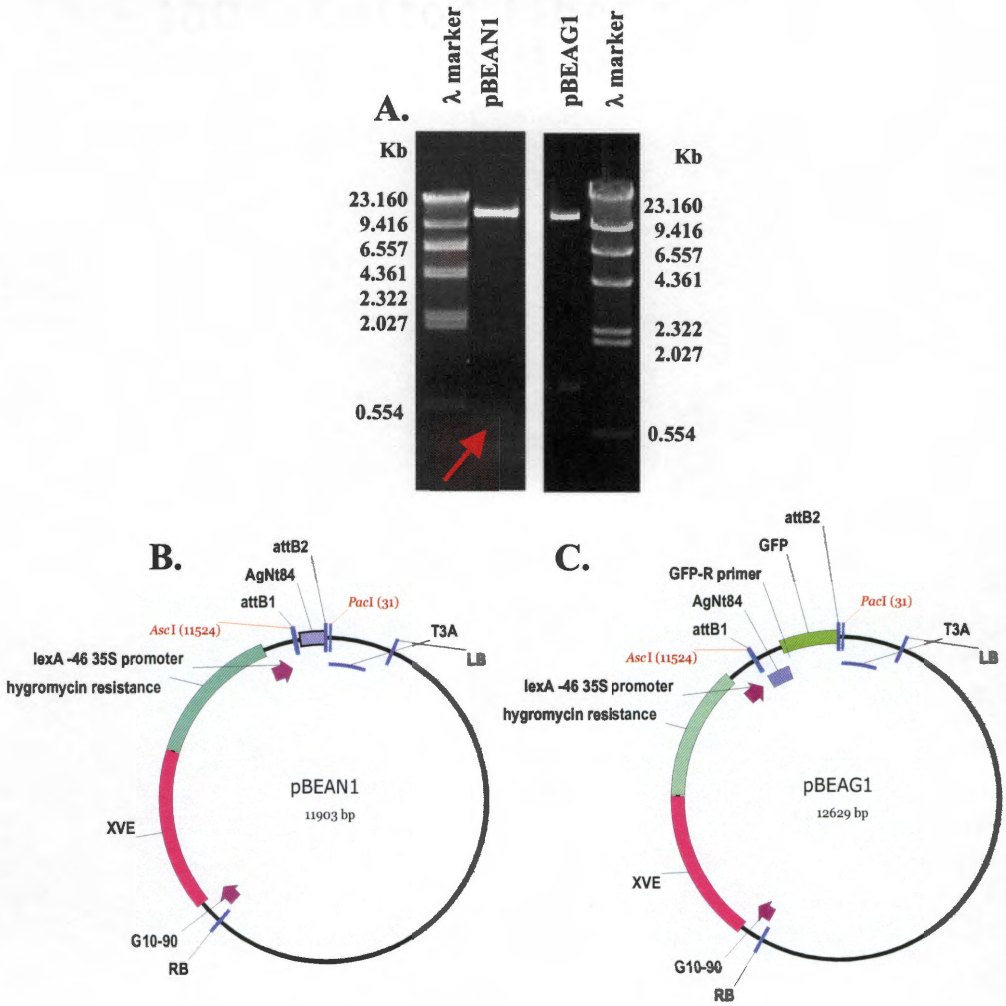


**FIGURE III-7: TAE agarose gel electrophoresis of pBEAN1 and pBEAG1. A.** Picture of the gel containing pBEAN1 and pBEAG1. Both are digested with AscI and PacI. Lane 1: λ HindIII marker. Lane 2: pBEAN1.The expected fragment sizes are 11.493 kb and 0.41 kb. A red arrow highlights the 0.41 kb fragment of pBEAN1. Lane 3: pBEAG1. The expected fragment sizes are11.493 kb and 1.136 kb. Lane 4: λ HindIII marker. **B.** Picture of the plasmid map of pBEAG1. **C.** Picture of the plasmid map of pBEAN1. The plasmid maps were made using Vector NTI, and the positions of the restriction sites are shown on the maps of each plasmid.

# CHAPTER IV

# PERFORMANCE OF THE EXPRESSION PLASMIDS IN PLANTA

## A. INTRODUCTION

The major purpose for making the seven binary plasmids discussed in Chapter III and for *E. coli* transformation was to eventually generate transgenic lines of tobacco with stable expression of AgNt84. *Agrobacterium*-mediated transformation was the method chosen for developing the transgenic tobacco lines. In addition, transient expression was used to check for gene expression before committing a lot of time to make stable transgenic lines. The two transient methods used were particle bombardment of onion epidermal cells and agroinfiltration of tobacco leaves.

Particle bombardment of onion epidermal cells is a method to achieve transient gene expression that can be used to quickly check plasmid constructs for GFP expression. This can be done before committing time to transform *Agrobacterium* with a plasmid followed by transformation of plants with *Agrobacterium* only to discover that GFP expression cannot be seen. Testing the plasmid using particle bombardment eliminates one of the possible reasons for no fluorescence. DNA is delivered directly into the cell first by coating tungsten or gold particles with the DNA, and shooting the particles into the cell using gas pressure, such as helium (Sanford et al., 1987). Once in the nucleus, the gene can be expressed.

The second method of transient transformation used was agroinfiltration of tobacco leaf epidermal tissue. Agroinfiltration is a method by which a liquid culture of *Agrobacterium* is used to inoculate tobacco by using pressure on the under side of the tobacco leaf. Pressure is applied to the leaf by a syringe or by pulling a vacuum (Batoko et al., 2000; Kapila et al., 1997). The use of agroinfiltration over stable transformation

also has the advantage that any positional effect of DNA integrating into the genome is not a concern because gene expression is from DNA not integrated into the genome (Kapila et al., 1997). In addition, this method provides a useful way to verify that a particular bacterial strain can transform the tobacco variety chosen for study.

*Agrobacterium*-mediated transformation is also used for stable transformation. Two types of *Agrobacterium* can be used, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *Agrobacterium tumefaciens* can be used to transform BY-2 suspension cell cultures and to transform tobacco tissue. With transformation of tobacco tissue, a new plant can be regenerated by inoculating, for example, a leaf piece with *A. tumefaciens*. With *A. rhizogenes*, hairy roots are made by inoculation of a wound site on the plant. The plant can be wounded at the hypocotyl, or the midvein on a leaf can be wounded (Wongsamuth and Doran, 1997; Medina-Bolívar and Cramer 2004).

Producing transgenic whole plants, hairy roots, and BY-2 suspension cell cultures was attempted because of the benefits of each type of transformation as discussed in Chapter II. *Agrobacterium tumefaciens* strain LBA4404, *Agrobacterium rhizogenes* strain AR10 (R24E7), and *Agrobacterium rhizogenes* strain A4RS were selected to be transformed with the binary plasmids designed in Chapter III so they could be used in plant transformation. *Agrobacterium rhizogenes* strain 15834 was later selected for generation of hairy roots.

Following transformation of *Agrobacterium*, there are two possible methods to verify that *Agrobacterium* contains the correct plasmid. The first method is to amplify a region of the binary plasmid by PCR. The second method is to isolate the plasmid, digest it with restriction enzymes, and separate the restriction fragments by agarose gel electrophoresis. With the plasmids used in this study, the first method may not provide as much useful information as the second method without carefully planning the primers. This is because the only difference between several of the plasmids is the presence of different promoters and the use of GFP. To distinguish between the seven plasmids, the promoter and of the AgNt84 or AgNt84-GFP region would have to be amplified. The second method is difficult to complete if restriction analysis is done using plasmid directly isolated from *Agrobacterium* for two reasons. First, the minipreparation method

for isolating plasmids also will isolate the Ti or Ri plasmid with the binary plasmid, complicating the digestion pattern of the binary plasmid. Also, the copy number of plasmids in *Agrobacterium* is low, making it difficult to isolate enough plasmid to analyze using miniprep methods. A method to overcome these problems is to isolate both plasmids from *Agrobacterium* and to transform *E. coli* with the plasmid mixture. *E. coli* containing only one plasmid type can be isolated following plating of the transformation mixture with the proper antibiotic selection. The integrity of the plasmid construct can now easily be confirmed by restriction digestion of the plasmid isolated from *E. coli* by the miniprep method.

### 1. Objectives

The two major objectives of this part of the research were:

1. To determine the subcellular targeting of AgNt84
2. To create a tobacco line stably expressing AgNt84

These objectives were to be accomplished through the following individual objectives:

- to transform onion epidermal cells by particle bombardment to verify GFP expression
- to transform *Agrobacterium tumefaciens* strain LBA4404, *Agrobacterium rhizogenes* strain AR10, and/or *Agrobacterium rhizogenes* strain A4RS with the seven binary plasmids
- to verify the presence of the correct plasmids in *Agrobacterium* by transforming the plasmid back into *E. coli*
- to transform tobacco leaves by agroinfiltration
- to transform tobacco plants and tobacco BY-2 cells with *Agrobacterium*

### 2. Outline of Procedure

In this section, a general description of the approaches used to achieve transformation is presented. Detailed procedures as well as a table summarizing the *Agrobacterium* strains, binary plasmids and transformation techniques used are given in the Materials and Methods section of this chapter.

GFP expression from pBCAG1, pBCAGE1, pBEAG1 and pBHAG1 was checked by particle bombardment of onion epidermal cells. When expression was confirmed, *Agrobacterium* strains, except for A4RS, were transformed with each of the plasmids by electroporation. Transformation through triparental mating was attempted with A4RS. Some of the transformed *Agrobacterium* were checked for the correct plasmid by transforming *E. coli* with the binary plasmid from *Agrobacterium*.

Negative and positive controls were set up for both *A. rhizogenes* AR10 and *A. tumefaciens* LBA4404-mediated transformation. The negative controls were the *Agrobacterium* strains without a binary plasmid. The positive controls were the *Agrobacterium* strains transformed with a modified pBIN20 plasmid constructed to express an endoplasmic reticulum-targeted form of GFP driven by the CaMV35S promoter. The *A. tumefaciens* LBA4404 positive control, AN160, was received from Andreas Nebenführ. The same plasmid was isolated from the TOP10 *E. coli* strain AN101, also provided by Andreas Nebenführ, and was transformed into AR10 resulting in the A. rhizogenes strain AR10 AN101.

*Agrobacterium rhizogenes* AR10, AR10 pBCAN1, AR10 pBCAG1, AR10 pBCAGE1, AR10 pAN101, A4RS, and 15834 were used in attempts to form hairy roots. The leaf disk method commonly used for *Agrobacterium tumefaciens* transformation was applied to all the *A. rhizogenes* strains except 15834 and AR10 pAN101. The second method involved wounding the hypocotyl of tobacco with a needle that had liquid *Agrobacterium* in it and placing a small amount of *Agrobacterium* in the wound site. The only strain used for this technique was AR10 pAN101. The last method used was wounding the midvein of a tobacco leaf with a scalpel dipped into an *Agrobacterium* colony. The tobacco plants used for this method of inoculation were both wild-type and transgenic. The wild-type plants were inoculated with AR10 pBCAG1, AR10 pBCAGE1, AR10, and 15834. The transgenic plants came from T10, T12, and T17 seeds provided by Mentewab Ayalew. These plants had previously been transformed with *Agrobacterium tumefaciens* strain GV3850 containing the binary plasmid pBINAgNt84 (**Figure IV-1**). Expression of AgNt84 RNA in these three tobacco lines is discussed in Chapter V. The transgenic plants were inoculated with 15834.

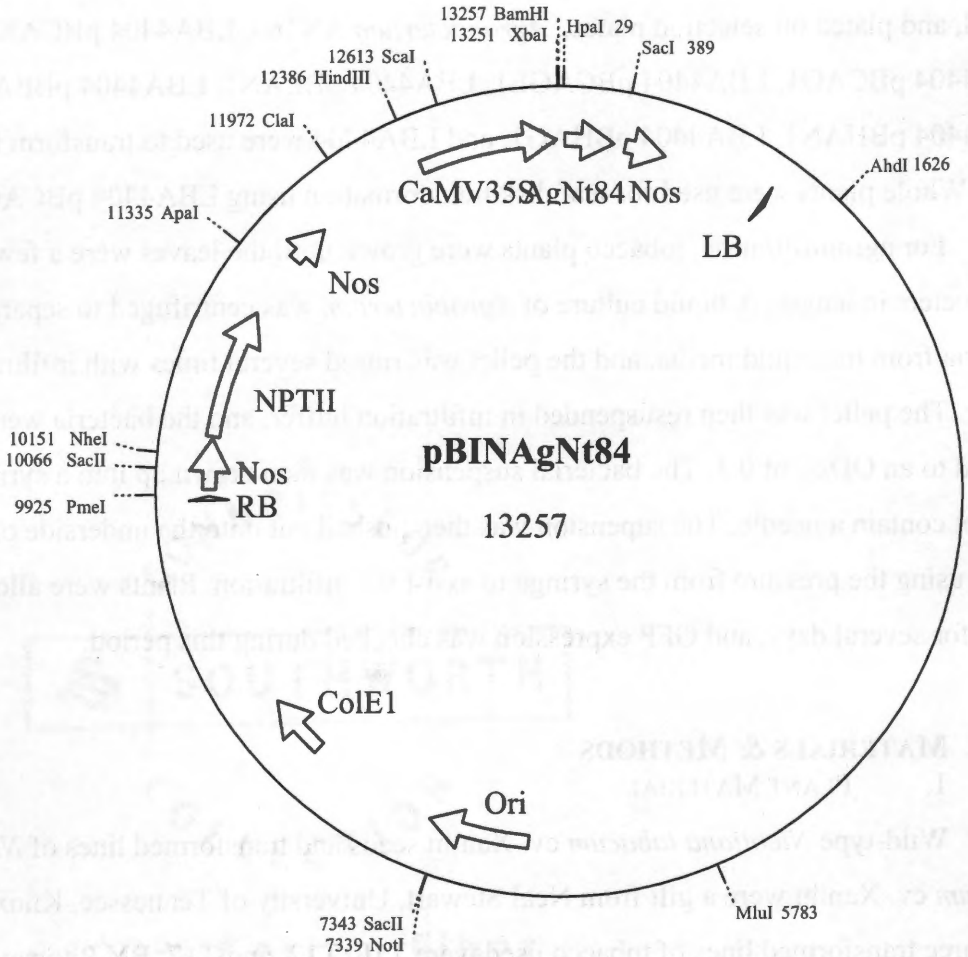


**FIGURE IV-1: Plasmid map of pBINAgNt84.** The binary plasmid pBINAgNt84 was made by Mentewab Ayalew, and it was transformed into *A. tumefaciens* GV3850 by Mentewab. Seed lines from plants transformed with this strain of *Agrobacterium* were used. This binary plasmid is similar to pBCAN1 in that this plasmid contains the CaMV 35S promoter driving the expression of AgNt84 without a reporter gene.

*Agrobacterium tumefaciens* was used to transform tobacco BY-2 suspension cells and tobacco plants. The BY-2 cells were co-cultivated in *Agrobacterium* for two days, rinsed, and plated on selection plates. *Agrobacterium* AN160, LBA4404 pBCAN1, LBA4404 pBCAG1, LBA4404 pBCAGE1, LBA4404 pBEAN1, LBA4404 pBEAG1, LBA4404 pBHAN1, LBA4404 pBHAG1, and LBA4404 were used to transform BY-2 cells. Whole plants were used for leaf disk transformation using LBA4404 pBCAG1.

For agroinfiltration, tobacco plants were grown until the leaves were a few centimeters in length. A liquid culture of *Agrobacterium* was centrifuged to separate the bacteria from the liquid media, and the pellet was rinsed several times with infiltration buffer. The pellet was then resuspended in infiltration buffer, and the bacteria were diluted to an $OD_{600}$ of 0.3. The bacterial suspension was then drawn up into a syringe that did not contain a needle. The supension was then pushed out onto the underside of a leaf while using the pressure from the syringe to assist the infiltration. Plants were allowed to grow for several days, and GFP expression was checked during this period.

## B. Materials & Methods

### 1. Plant Material

Wild-type *Nicotiana tabacum* cv. Xanthi seeds and transformed lines of *Nicotiana tabacum* cv. Xanthi were a gift from Neal Stewart, University of Tennessee, Knoxville. The three transformed lines of tobacco used were T10, T12, and T17. BY-2 tobacco cells were a gift from Andreas Nebenführ University of Tennessee, Knoxville. Onions were purchased at the local grocery store

### 2. Bacterial Strains

*E. coli* strain TOP10 with AN101, *Agrobacterium tumefaciens* strain LBA4404, and AN160 were a gift from Andreas Nebenführ, University of Tennessee, Knoxville. *Agrobacterium rhizogenes* strain A4RS was a gift from Didier Bogusz, IRD, Montpellier. *Agrobacterium rhizogenes* strain AR10 (R24E7) was a gift from Gary Stacey, University of Missouri. *E. coli* pRK2012 was a gift from Albrecht von Arnim, University of Tennessee, Knoxville. *A. rhizogenes* strain 15834 was a gift from Fabricio Medina-

Bolívar, Virginia Polytechnic Institute and State University. *E. coli* pRK2012, *E. coli* TOP10 with AN101, *A. rhizogenes* AR10, *A. rhizogenes* 15834, *A. tumefaciens* LBA4404, and AN160 were received on plates whereas A4RS was received as agar slants. A4RS was streaked on selective plates. A colony of each strain except for 15834 was picked from selective plates. The colony was grown in liquid culture with the appropriate antibiotics to make glycerol cultures for long-term storage (Appendix C).

## 3. Particle Bombardment of Onion Epidermal Cells

### 3. a. Materials Used for Particle Bombardment

The materials used for bombarding onion epidermal cells were kindly provided by Albrecht von Arnim, University of Tennessee, Knoxville, and the technique was carried out in Dr. von Arnim's laboratory with the assistance of Chitra Subramanian, using her protocol.

### 3. b. DNA Preparation

Tungsten particles already washed in ethanol and suspended in water were used. The particles were vortexed for 15 minutes. 10 µL were transferred to a new microfuge tube. 500 ng of plasmid DNA in water were added to the tungsten particles, and the microfuge tube was mixed. The DNA was one of the following binary plasmids: pBCAG1, pBCAGE1, pBEAG1, or pBHAG1. 20 µL of 4 M $CaCl_2$ was added, and the tube was mixed. 8 µL of 0.1 M spermidine was added, and the tube mixed. The tube was incubated on ice for 10 minutes. The microfuge tube was centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded. The tube was vortexed to get the pellet off the side, which simplifies resupension in ethanol. 150 µL of 70% ethanol was added to the pellet, and the tube was mixed first by tapping and then by vortexing. Any remaining pellet particles were removed from the side of the tube by scraping with a pipette tip. The microfuge tube was centrifuged as before, and the supernatant was discarded. 150 µL of 100% ethanol was added to the pellet, and the tube was mixed by vortexing. The microfuge tube was spun as before, and the supernatant was removed. The pellet was resuspended in 6 µL of 100% ethanol. The suspension was placed evenly in the center of

a microcarrier. Each microcarrier was dried in a round petri dish that contained a piece of filter paper and Dri-Rite.

### 3. C. Preparation of Onion Tissue for Particle Bombardment

An onion bulb was cut into four sections. The epidermis on the inner side of the onion leaf was peeled off using tweezers. Epidermal tissue bombarded with pBCAG1, pBCAGE1, or pBHAG1 was placed on round petri plates containing solidified MS media without sucrose (Appendix D). For the onion tissue bombarded with pBEAG1, the tissue was placed on MS media with 2 μL 17-β estradiol added to induce gene expression (Appendix D). The side that had been facing outwards on the onion was placed facing the MS media. The outermost and the innermost leaves of the onion were not used.

### 3. D. Bombardment of Onion Epidermal tissue

The helium pressure tank attached to the particle gun was opened, and the pressure of the tank was set to 1300 psi. The vacuum pump was turned on, and the particle gun was turned on last.

The rupture disk was inserted first, and the microcarrier was inserted next. The microcarrier was inserted so that the DNA was facing down. The MS plate containing the onion tissue was placed in the chamber with the lid off, and the chamber door was closed.

The vacuum was pulled and held. The fire switch was held down until the rupture disk burst, and the vacuum was released. The onion tissue was placed in the dark at room temperature, except for onion tissue transformed with pBHAG1. For pBHAG1, the onion tissue was placed in a 37°C incubator to induce gene expression. The tissue was checked for GFP fluorescence approximately 24 hours after bombarding the tissue.

### 4. Electrocompetent *Agrobacterium* Cells

The method for preparing electrocompetent *Agrobacterium* cells was adapted from a protocol on the pGreen website (http://www://pgreen.ac.uk/). *Agrobacterium* were grown on agar plates for 2 days at 28°C. A colony of *Agrobacterium* was picked, and the colony was inoculated into 5 mL of appropriate media with the correct antibiotics.

*Agrobacterium rhizogenes* strains A4RS and AR10 (R24E7) were grown in *Agrobacterium* medium (Appendix C), and *Agrobacterium tumefaciens* strain LBA4404 was grown in LB media (Appendix C). The 5-mL cultures were grown from early morning to 3:00 in the afternoon. At this time, the 5-mL cultures were transferred to 100 mL of media containing the correct antibiotics. The media for the *A. rhizogenes* strains consisted of *Agrobacterium* media supplemented with 1 g/L of glucose. The media for *A. tumefaciens* strain was L Broth (Appendix C). The 100 mL cultures grew overnight at 28°C while shaking at 225 rpm.

The cells were harvested the following morning. The 100 mL culture was separated into several centrifuge tubes that could fit in a Sorvall SS-34 rotor. The cells were spun at 4°C for 5 minutes at 5000 rpms. All the centrifuge tubes and samples were kept on ice after this step. The supernatant was discarded, and the pellets for each strain of *Agrobacterium* were combined into 1 tube by resuspending all the pellets into 50 mL of 10% ice-cold glycerol. The tube was centrifuged at the same speed, time, and temperature as before. The glycerol rinse was repeated, and the pellet that resulted from this final rinse was resuspended into 1 mL of ice-cold 10% glycerol. From this 1 mL solution, 10 aliquots of 100 µL were made, and these aliquots were frozen in liquid nitrogen and were stored at -80°C.

### 5. ELECTROPORATION OF *AGROBACTERIUM* WITH DNA

*Agrobacterium* strains were electroporated using an Electroporator II from Invitrogen belonging to Albrecht von Arnim. Frozen glycerol cultures of electrocompetent *Agrobacterium* were thawed on ice, and cuvettes for the electroporator were placed on ice to chill. 1 µL of DNA was placed into each cuvette, and between 40 and 50 µL of the electrocompetent cells were placed onto the DNA in the cuvette. The cells were pulsed in the electroporator at 50 µF, 1800 Vmax, and 150 Ω. One mL of media was placed into the cuvette, and the contents of the cuvette were mixed by pipetting up and down and transferred to a Falcon tube. As before, the medium for LBA4404 was LB, the medium for A4RS and AR10 (R24E7) was *Agrobacterium* medium (Appendix C). The Falcon tubes were placed in a shaker at 28°C to shake at 200

rpm for 2 to 4 hours. 10 µL and 100 µL of the *Agrobacterium* in the Falcon tubes were spread onto separate plates containing the appropriate antibiotics. The plates were incubated at 28°C for 2 to 3 days.

An attempt was made to electroporate each of the plasmids into *A. tumefaciens* LBA4404, *A. rhizogenes* AR10, and *A. rhizogenes* A4RS except that no attempt was made to transfer pBEAN1 and pBEAG1 into A4RS because of a conflict with antibiotic selection.

### 6. TRIPARENTAL MATING OF *AGROBACTERIUM RHIZIGENES* STRAIN A4RS

This protocol was received with *E. coli* pRK2012 from Albrecht von Arnim. Liquid cultures of *E. coli* pRK2012 and *E. coli* DH5α transformed with either pBCAN1 or pBCAG1 were grown in LB with 50 µg/mL kanamycin. *Agrobacterium rhizogenes* strain A4RS was grown in *Agrobacterium* medium containing 50 µg/mL rifampicin and 500 µg/mL spectinomycin. An overnight liquid culture of the bacteria was used. In a microfuge tube, 100 µL of *E. coli* pRK2012, 200 µL A4RS, and 100 µL DH5α were mixed. Also, three controls were made. One control omitted *E. coli* pRK2012. Another control omitted *E. coli* DH5α, and the last control omitted A4RS. A sterile square of PVDF membrane that had been cut into a 1-cm square was placed on each LB plate used, and each bacterial mixture was placed on its own plate. The plates were left open so that the medium from the bacteria could dry. The plates were placed in a 30°C incubator for a day once they had become dry.

On the following day, the bacteria were washed off into a small sterile petri plate using 1 mL of LB medium. With each bacterial mixture, a dilution was made where 100 µL of the bacteria suspension was added to 900 µL of LB medium, and 20 µL was plated on selective medium. The plates were incubated at 30°C for 3 days.

It was not realized until later that the PVDF membrane should have been treated before use so that the liquid bacterial media would have absorbed through the membrane. Instead, the cells were spread on the PVDF membrane so the liquid media would dry.

7. MINI-PREPARATION OF 15834 AND PCR AMPLIFICATION OF THE ROL C GENE ON THE RI PLASMID OF 15834

A colony of 15834 grown on nutrient agar plates (Appendix C) was inoculated into 5 ml of nutrient broth (Appendix C) and grown overnight. Plasmid DNA was isolated using the QIAprep Miniprep Kit (QIAGEN). This DNA was amplified with the following forward and reverse primers using the same settings as with the amplification of AgNt84. (Appendix B):

Forward Primer: 5' TGTGACAAGCAGCGATGAGC 3'

Reverse Primer: 5' GATTGCAAACTTGCACTCGC 3'

The PCR reaction was verified by agarose gel electrophoresis as in Chapter III, and the agarose gel was stained as in Chapter III. A picture of the gel was taken. Several colonies were chosen for verification.

8. TOBACCO SEED STERILIZATION

The protocol for tobacco seed sterilization was from Mentewab Ayalew, University of Tennessee, Knoxville. A microfuge tube containing tobacco seeds was filled with 10% Clorox solution containing 0.1% Tween 20. The tube was mixed by rotating on a shaker for 5 to 15 minutes, and the bleach was subsequently pipetted off the seeds. The tube was filled with 70% ethanol, and was inverted several times to mix. The ethanol remained on the seeds for no longer than a minute. The seeds were rinsed 3 times with sterile water. Wildtype tobacco seeds were resuspended in MS media with 3% sucrose (Appendix D) and plated on the same media solidified with Phytagel (Sigma). Seeds from transgenic lines were resuspended in the same media plus 200 μg/mL of kanamycin and were plated on that media solidified with Phytagel (Appendix D).

9. TRANSIENT TRANSFORMATION OF TOBACCO LEAF EPIDERMIS USING *AGROBACTERIUM*

The method used for agroinfiltration of tobacco leaf epidermis was based on the protocol from Batoko et al. (2000). 3-week-old sterile tobacco plants were placed in

containers of vermiculite. They were kept watered, and were periodically irrigated with ¼ strength modified Hoagland's medium (Appendix D).

Around 1.5 mL of an overnight liquid culture of *Agrobacterium rhizogenes* AR10 or *Agrobacterium tumefaciens* LBA4404 was transferred to a microfuge tube, and the microfuge tube was centrifuged at 4,000 rpm for 8 minutes. The supernatant was discarded. The pellet was washed in infiltration buffer (Appendix D) two times without centrifugation between the rinses. The pellet was resuspended in 1 mL of the infiltration buffer. If more than one microfuge tube was processed from the same *Agrobacterium* strain with the same plasmid, the bacteria were combined and diluted with infiltration buffer until the $OD_{600}$ was 0.3. The *Agrobacterium* was placed into a syringe without the needle attached. The syringe held 1 mL of the bacterial suspension. The underside of a tobacco leaf was marked with a permanent marker near the site where the *Agrobacterium* would be inoculated. The syringe was placed in contact with the leaf tissue, and the 100 µL of the liquid *Agrobacterium* suspension was applied with gentle pressure to the underside of the leaf. The plants were allowed to grow for several days.

## 10. Transformation of Nicotiana tabacum "Xanthi" with *Agrobacterium rhizogenes*

### 10. a. Method 1: Leaf Disk Transformations

The protocol followed was based on a protocol received from Mentewab Ayalew. This protocol is used for *A. tumefaciens*, but it was adjusted for *A. rhizogenes* by omitting the hormones since hormones are not necessary for regeneration of hairy roots. Tobacco plants were grown in Magenta boxes until they reached the top of the Magenta box. Several pieces of tissue were cut from a tobacco leaf, and several leaves from each plant were used. Each piece of tissue was dipped into a liquid culture AR10 or A4RS that had not been transformed with a binary vector. In addition, as a control, some leaf pieces were dipped into uninoculated *Agrobacterium* medium (Appendix C) (**Table IV-1**). The leaf pieces were allowed to drain, and the pieces were then placed on MS agar in round petri plates (Appendix D). The plates were then wrapped in parafilm and placed in a dark

**TABLE IV-1: Methods of tobacco transformation and the *Agrobacterium* strains used with each method.**

| Strain | Method 1: Leaf disc (without blotting tissue) | Method 1: Leaf disc (blotting tissue) | Method 1: Leaf disc (cut leaf with razor dipped in liquid *Agrobacterium)* | Method 2: Hypocotyl wounding | Method 3: Midvein wounding |
|---|---|---|---|---|---|
| AR10 | Used | Used | Used | | Used |
| A4RS | Used | | | | |
| AR10 with pBCAN1 | | Used | Used | | Used |
| AR10 with pBCAG1 | | Used | Used | | Used |
| AR10 with pBCAGE1 | | Used | Used | | Used |
| AR10 with AN101 | | Used | Used | Used | |
| LBA4404 with pBCAG1 | Used | | | | |
| 15834 | | | | | Used |
| no AR10 | | | Used | | |

drawer at room temperature. After two to three days, the plant pieces were transferred to MS medium supplemented with 500 µg/mL carbenicillin.

In a variation of this protocol the tobacco leaf pieces were blotted dry after being dipped into the *Agrobacterium* cultures. The rest of the protocol was the same as above. *Agrobacterium* used with this variation were: AR10, AR10 with pBCAN1, AR10 with pBCAG1, and AR10 with pBCAGE1 (**Table IV-1**).

In a final variation, instead of dipping leaf pieces into the liquid culture, a razor blade dipped into the AR10 culture was used to cut the tobacco tissue. The rest of the protocol was followed as above. The treatments carried out using this variation were: no AR10, AR10, AR10 with pBCAN1, AR10 with pBCAG1, AR10 with pBCAGE1, and AR10 AN101 (**Table IV-1**).

It was discovered after the protocol had been followed a few times that the MS medium used in the above protocol should have contained 3% sucrose.

### 10. B. Method 2: Wounding of the Hypocotyl

3 to 4 week old tobacco seedlings were inoculated with *A. rhizogenes* by wounding the hypocotyl (**Table IV-1**). Two to 3 colonies from a 2 day-old plate of *A. rhizogenes* AR10 transformed with AN101, were resuspended in 1 mL of *Agrobacterium* medium (Appendix C). The bacterial mixture was pipetted into a microcentrifuge tube, and the tube was centrifuged for 5 minutes at 14,000 rpm. The supernatant was taken off, and the pellet was suspended in 1 mL of *Agrobacterium* medium. The solution was drawn up into a syringe with a 26G needle that was then used to pierce the region below the cotyledons. The hypocotyls were pierced once with the needle, and a few drops of *A. rhizogenes* AR10 containing AN101 was placed on the wound site. The plants were accidentally cut in half by the needle. After 3 days, the tobacco plants were transferred to MS media with antibiotic selection (50 µg/mL kanamycin and 500 µg/mL carbenicillin).

The following steps were not done because hairy roots did not appear at the wound site, but the steps are included to document the complete method. From 14 to 21 days after inoculation of the seedlings with *Agrobacterium*, hairy roots should be growing from the wound site. These roots should be cut, and grown in MS medium with

appropriate antibiotics. The roots should be grown at 27°C on an orbital shaker at 120 rpm. The hairy roots should be subcultured every 14 days in 250 mL flasks containing 50 mL of Gamborg's B5 medium, pH 5.7 (Appendix D). For long-term growth of hairy root cultures, it has been shown that roots grow better in B5 media than in MS media (Wangsamuth and Doran 1997).

### 10. C. Method 3: Wounding of the Midvein of the Petiole

The method described was based on Medina-Bolívar and Cramer (2004), which describes in detail an *Agrobacterium*-mediated transformation protocol resulting in the formation of hairy roots. In this protocol, the petiole of the leaf is inoculated with transformed *A. tumefaciens* by wounding, and the transgenic plant that arises has the midvein of the leaf inoculated with untransformed *A. rhizogenes* (Medina-Bolívar and Cramer 2004). This method optimizes producing transformed roots because antibiotic selection can also result in untransformed roots growing on the media if only *A. rhizogenes* is used (Medina-Bolívar and Cramer 2004). However, the use of hygromycin as a plant selection in the step involving transformation with *A. tumefaciens* has a few problems. Hygromycin can act as a growth regulator by increasing the size of the calli formed, and hygromycin lengthens regeneration time (Medina-Bolívar, personal communication). Thus, only the second part of this method was used to generate hairy roots by transforming wild-type tobacco using *A. rhizogenes* AR10 containing pBCAG1 or pBCAGE1. These two vectors contained the AgNt84 fusion to a reporter gene, and expressing GFP in roots visually discriminated between transformed and untransformed roots. The second part of this method was also used to generate hairy roots by transforming transgenic tobacco expressing AgNt84 using *A. rhizogenes* 15834 as well as using *A. rhizogenes* AR10. *A. rhizogenes* ATCC 15834 was grown on nutrient agar medium (Appendix C) at room temperature while AR10 was grown on *Agrobacterium* medium.

The plants were grown to the same size as in Method 10A.The leaf was separated from the rest of the plant by cutting with a scalpel blade. The blade was used to trim off half of the petiole of the leaf so that the tissue closest to the stem node was removed. The

blade was dipped into a single colony of *Agrobacterium*, and the blade was used to make an incision in the center of the leaf's midrib. The blade was sterilized with a flame before proceeding to the next leaf, colony, or strain. The *Agrobacterium* strains used were AR10, AR10 with pBCAN1, AR10 with pBCAG1, AR10 with pBCAGE1, and 15834 (**Table IV-1**). The leaves were placed on B5 medium containing 2% sucrose. For simplicity, this medium will be referred to as modified B5 medium. Putative hairy roots were visible at the wound site within 2 to 3 weeks.

Plates of tobacco from the transformation with the 15834 strain were badly contaminated, so the putative hairy roots were not excised from the leaf tissue. For some of the transformants of the AR10, some of the roots were treated further. The hairy roots were cut off the petiole using a scalpel, and the roots were transferred to modified B5 medium supplemented with 600 µg/mL cefotaxime and 50 µg/mL hygromycin. A small section of the roots was cut to check for the expression of GFP. The roots were then placed at 28°C for two weeks, and the roots were then moved to a new plate with antibiotics. However, the roots were still contaminated with *Agrobacterium*. After the third transfer onto plate containing cefotaxime, the growth of *Agrobacterium* appeared to be killing the tobacco roots.

The rest of the protocol was not followed because *Agrobacterium* growth could not be stopped, but the steps will be included to complete the protocol. Had *Agrobacterium* growth been stopped, the roots should be placed onto modified B5 medium supplemented with 50 µg/mL hygromycin and grown for 2 weeks. Between 6 and 10 root tips derived from the same root should be transferred into a 50 mL Erlenmeyer flask. The flask should contain 10 mL of modified B5 medium with hygromycin, and the flask should be placed in a 28°C shaker at 90 rpm in the dark. When the roots reach the subculturing stage, they should be moved to a petri dish containing modified B5 medium at a volume where the roots will be submerged so they do not dehydrate. Twenty 1-cm long root tips should be transferred to a 250-mL flask containing 50 mL of modified B5 medium with hygromycin. The roots should be placed in the shaker as before, and they should be subcultured every 12 days.

## 11. Growth of Tobacco BY-2 Suspension Cell Cultures

BY-2 suspension cell cultures were grown in media described in Nagata et al. (1992), which was named BY-2 media (Appendix D). The suspension cell cultures were grown in the dark at 27°C while shaking at 120 rpm. Callus cultures were grown at 27°C in the dark.

BY-2 suspension cells were kept growing by subculturing. When the original culture was a suspension cell culture, 400 µL of the 1-week-old suspension cell culture were added to 20 mL BY-2 media. Alternatively, the suspension cells were made from growing calli, or calli were made from suspension cells. For growing a suspension culture from callus tissue, a small amount of the tissue was cut from the edge of a 3-week-old callus and added into 20 mL of BY-2 media. For growing calli from suspension cells, a small drop of suspension culture was added to a plate of BY-2 media containing Phytagel (Appendix D). The cells were spread by rotating the plate. The calli were made fresh around every three weeks.

## 12. Transformation of BY-2 Suspension Cells with *Agrobacterium tumefaciens*

### 12. a. Method A

The first protocol used for transformation of BY-2 cells is from Bonifacino et al. (2004). Bacteria used were LBA4404, AN160, LBA4404 pBCAGE1, LBA4404 pBCAN1, LBA4404 pBCAG1, LBA4404 pBEAN1, LBA4404 pBEAG1 and a control with no bacteria. The procedure of transformation was listed in **Table IV-2**. After the cells were plated on media, the plates were placed in the 28°C incubator for 3 to 4 weeks. After this period, the cells were overgrown with *Agrobacterium*, and the calli could not be transferred to another plate, which should have been done four times before subculturing into liquid medium. Another method was tried.

### 12. b. Method B

The second protocol that was followed was from Andreas Nebenführ. Bacteria used were LBA4404, LBA4404 pBCAN1, LBA4404 pBCAG1, LBA4404 pBCAGE1,

**TABLE IV-2: Steps used in the two methods of BY-2 transformation.** The similarities are listed in the column labeled Steps, and the differences in the two methods are listed in the columns labeled Method A and Method B.

| Steps | Method A | Method B |
|---|---|---|
| 1. LBA4404 was grown overnight as a liquid culture. | No other specifications were given. | Bacteria was diluted in morning to an $OD_{600}$=1. |
| 2. BY-2 cells were placed into a sterile petri dish. | The amount of cells added was 1 mL, and 4-day-old cells were used | The amount of cells added was 5 mL of 3-day-old cells, and 5 µL of a 20 mM stock of acetosyringone was mixed in. |
| 3. *Agrobacterium* was added to the plate and mixed. | The amount of *Agrobacterium* was 50 µL. | The amount of *Agrobacterium* was 25 µL and 100 µL. |
| 4. Plates were kept in the dark for 2 days at 28°C. | | |
| 5. *Agrobacterium* and BY-2 cells were moved to a container and rinsed. The cells were mixed by inversion, and the cells were allowed to settle before taking off the rinse solution. | The container was a microfuge tube, and the cells were rinsed 3 times with 1 mL of BY-2 medium. | The container was a sterile, disposable 15 mL centrifuge tube (Fisher). The plate was rinsed with 7 mL of BY-2 medium, and the rinse was added to the centrifuge tube. The cells were then washed a total of 3 rinses with BY-2 medium. Finally, the cells were then rinsed with BY-2 medium containing 500 µg/mL carbenicillin. |
| 6. The cells were resuspended in medium and plated. The cells were spread | The resuspension was in 1 mL of BY-2 medium, and all the cells were spread on BY-2 medium plates with 100 µg/mL carbenicillin and 50 µg/mL hygromycin. For AN160, the antibiotic selection was 50 µg/mL kanamycin. | The cells were resuspended to a final volume of 12 mL. The BY-2 medium contained 500 µg/mL carbenicillin. Hygromycin was initially at 50 µg/mL, but hygromycin was reduced to 30 µg/mL. |

LBA4404 pBEAN1, LBA4404 pBEAG1, LBA4404 pBHAN1, LBA4404 pBHAG1 and a control with no bacteria. The steps of the protocol are listed in **Table IV-1**. The hygromycin selection was initially 50 μg/mL hygromycin. Once the cells were plated, the plates were placed in the 28°C incubator for 3 to 4 weeks. After 4 weeks, the calli were transferred to fresh plates using toothpicks. Because the growth of the calli was slow, the cells were transferred onto plates that contained 30 μg/mL hygromycin and not 50 μg/mL hygromycin. In addition, extraneous contamination occurred with some of the transformed lines, and the method was repeated on those lines using 30 μg/mL hygromycin in the plates after rinsing the BY-2 cells.

13. MICROSCOPY

The microscopes used to check for fluorescence were those in the Nebenführ lab or in the Microscopy Facility. The microscope in the Microscopy Facility was a Nikon Eclipse E600 fluorescence microscope. The digital camera used to take the picture was a QIMAGING camera. The objectives used were 10X or 20X. The filter used for fluorescent microscopy was B-2A, which had an excitation wavelength between 450 and 490 nm. Nebenführ's microscope was a Zeiss axiovert 200M, and the digital camera was Hamamatsu Orca-ER. The objectives used were 20X/0.5 or 63X/1.4.

Onion cells transformed by particle bombardment were visualized using the epifluorescence microscope in the Nebenführ lab. The tissue was mounted on a microscope slide with water.

Tobacco leaves transformed by agroinfiltration were visualized using the epifluorescence microscope in the Nebenführ lab as well as the confocal and epifluorescence microscope in the Microscopy Facility. John Dunlap assisted with the confocal microscopy pictures. The tissue was mounted on a slide using water when using the epifluorescence microscope, and the tissue was held on a slide by double sticking tape when using the confocal microscope.

Hairy roots were visualized using the epifluorescence microscope in the Microscopy Facility. The roots were treated as with the onion cells by mounting the tissue in water.

A part of a small callus of BY-2 cells transformed with LBA4404 or LBA4404 pBCAG1 was separated from the remaining callus with a small spatula. The piece of callus was added to 200 µl of BY-2 medium in a microfuge tube, and the tube was tapped to separate the callus into individual cells. Some of the solution containing the resuspended callus was placed onto a microscope slide to observe GFP fluorescence.

### 14. TRANSFORMATION OF TOBACCO WITH *A. TUMEFACIENS* LBA4404 CONTAINING PBCAG1

Tobacco plants were grown in Magenta boxes as in Method 10A. A colony of *A. tumefaciens* LBA4404 transformed with pBCAG1 was grown for two days in 100 ml YEB medium (Appendix C). LBA4404 cells were pelleted from the 100 mL culture by centrifugation at 5000 rpm for 5 minutes. The supernatant was discarded, and 50 mL of DBI medium (Appendix D) was used to resuspend the pellet. The liquid LBA4404 suspended in DBI medium was transferred to a petri plate to ease submersion of tobacco pieces. Tobacco leaf tissue was cut into small pieces with a scalpel and added to the LBA4404 suspension. After the last piece was added, the leaf pieces remained in the bacterial suspension for 30 minutes. The pieces were taken out of the suspension, and the tissue was allowed to briefly drain before plating.

The tissue was placed on MS plates with 3% sucrose for 3 days. Later when this method of transformation was attempted by Mentewab Ayalew, the tissue was placed on DBI plates for 3 days. After 3 days, the leaf pieces from each attempt were transferred to DBI plates containing 200 µg/mL kanamycin and 500 µg/mL timentin.

Regeneration of plants did not occur, and the remaining steps in the procedure are included to complete the protocol. After 3 weeks on DBI plates with antibiotics, the regenerated shoots should be cut off from the leaf tissue and placed in Magenta boxes containing MS media with 3% sucrose, containing 200 µg/mL kanamycin, and 500 µg/mL timentin. Once the roots appear, the plants could be grown in pots. These plants could have been used: to check GFP fluorescence, to obtain seeds, and to check RNA expression using Northern blots.

## C. RESULTS

### 1. GFP EXPRESSION IN ONION EPIDERMAL CELLS

Onion epidermal cells transformed with pBCAG1, pBCAGE1, pBHAG1, or pBEAG1 showed GFP expression 24 hours after transformation (**Figure IV-2**). When looking at the tissue at lower magnification, GFP appeared throughout the cell. Cells transformed with pBHAG1 (picture not shown) and pBEAG1 showed weak expression of GFP, and the exposure time when taking pictures was increased to see the expression. There were fewer expressing cells in the transformation with pBHAG1 and pBEAG1. At higher magnification, cells transformed with pBCAG1 showed the presence of GFP in what appears to be the endoplasmic reticulum (**Figure IV-3**). AgNt84 was not visually detected in the cytosol. The location of GFP expressed from pBCAGE1 was similar to that from pBCAG1 (picture not shown). In addition, cells transformed with pBCAGE1 showed GFP traveling to what is likely to be the plasmadesmata, which can be seen in the fluorescence microscope as regularly spaced highly fluorescent regions at higher magnification. However, movement of GFP through the plasmadesmata could not be detected in the neighboring cell. The movement to the plasmadesmata was likely to be through the endoplasmic reticulum.

In summary, AgNt84-GFP could be seen inside the expressing cells and predominantly within the endoplasmic reticulum, but not in the extracellular matrix. It is possible that GFP cannot fluoresce outside the cell under the conditions used, but a control to check GFP fluorescence outside the cell was not designed or expressed in the onion epidermal cells.

### 2. TRANSFORMATION OF *A. RHIZOGENES* STRAINS AR10 (R24E7) AND LBA4404

Several attempts to transform *Agrobacterium tumefaciens* strain LBA4404 and *Agrobacterium rhizogenes* strain AR10 (R24E7) with binary plasmids were made. LBA4404 was able to grow on antibiotic selection when each of the seven plasmids were used to transform LBA4404. However, colonies of AR10 grew when transformed with pBCAN1, pBCAG1, or pBCAGE1 but not with pBEAN1, pBEAG1, pBHAN1 or

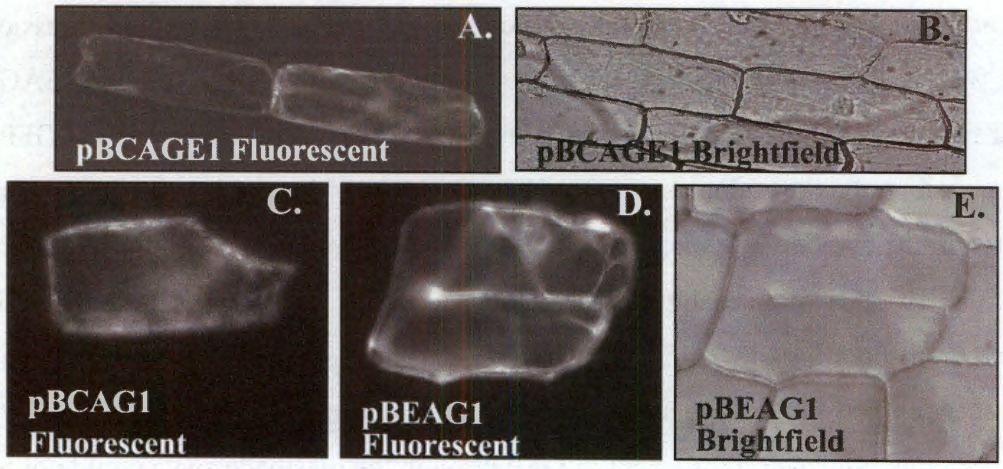


**FIGURE IV-2: Expression of GFP in onion epidermal cells transformed with pBCAG1, pBCAGE1, or pBEAG1.** All the pictures are at 20X magnification on a fluorescence microscope. The brightfield picture is below the fluorescent picture. Pictures of both **A** and **B** were taken under the same conditions, but picture C.) had a longer exposure time. **A.** An onion cell from the transformation with pBCAG1 expressing AgNt84-GFP. **B.** Two onion cells from the transformation with pBCAGE1 expressing AgNt84-GFP. The brightfield picture is below the fluorescent picture. **C.** Two onion cells from the transformation with pBEAG1 expressing AgNt84-GFP. The expression of AgNt84-GFP was induced with estradiol.

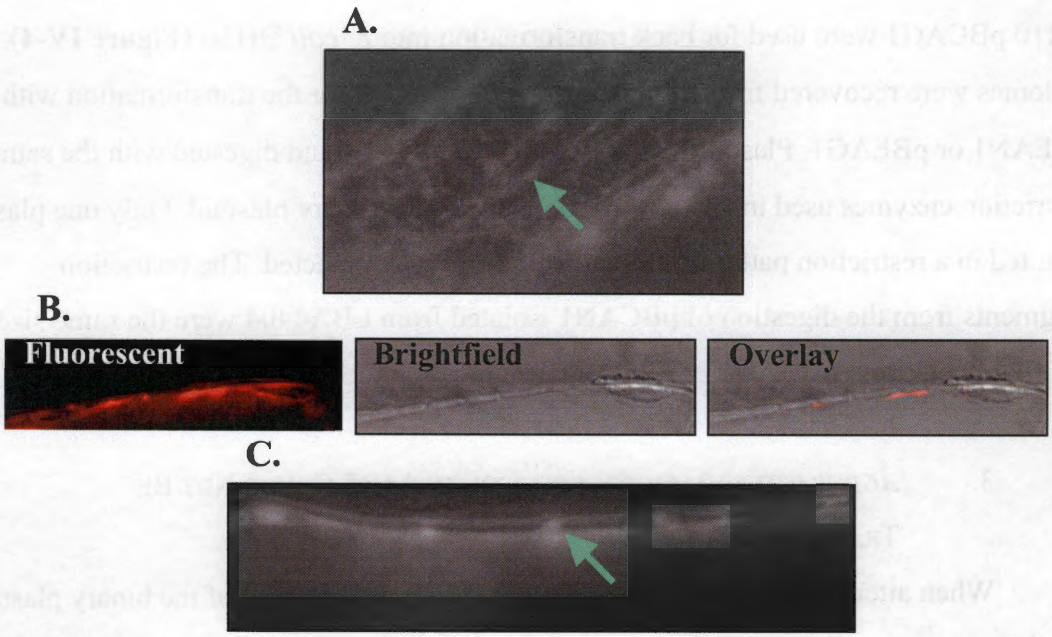


**FIGURE IV-3: Expression of GFP in onion epidermal cells transformed with pBCAG1 and pBCAGE1 at a higher magnification.** Pictures were taken at 63X magnification. **A.** Expression of onion cells transformed with pBCAG1 of AgNt84-GFP in the endoplasmic reticulum (indicated by an arrow). Onion cells transfomed with pBCAGE1 showed the same expression pattern as the cells transformed with pBCAG1 (picture not shown). **B.** Onion cells transformed with pBCAG1 showing that expression of AgNt84-GFP detected in the cell. Picture on left is a fluorescent picture. Picture in the middle is a brightfield picture. Picture on the right is an overlay of the two pictures. Fluorescence was labeled with the color red. **C.** GFP expression in an onion cell transformed with pBCAGE1. The region indicated by an arrow shows the accumulation of the AgNt84-GFP fusion protein in the region likely to be the plasmadesmata.

pBHAG1. In addition, AR10 was transformed with pAN101, which is a binary plasmid that can be used as a positive control in *Agrobacterium*-mediated transformation in case expression of AgNt84 was not detected.

Plasmids from LBA4404 transformed with each of the seven plasmids and from AR10 pBCAG1 were used for back transformation into *E. coli* DH5α (**Figure IV-4**). Colonies were recovered from all transformations, except for the transformation with pBEAN1 or pBEAG1. Plasmids were isolated from DH5α and digested with the same restriction enzymes used in Chapter III to identify each binary plasmid. Only one plasmid resulted in a restriction pattern different from what was expected. The restriction fragments from the digestion of pBCAN1 isolated from LBA4404 were the same size as the fragments from pBCAG1 or pBCAGE1, indicating the plasmid was not pBCAN1.

### 3. *Agrobacterium rhizogenes* Strain A4RS Could Not Be Transformed

When attempts were made to transform A4RS with several of the binary plasmids by electroporation, the few colonies that grew were very small and took longer than three days to grow. These colonies appeared to be colonies of A4RS, but transformation was not likely because of the small size of the colonies. Triparental mating was tried. However, no colonies of A4RS grew from this attempt.

### 4. GFP Expressed in the Cells of Tobacco Leaves

Transient transformation of tobacco leaf tissue was done using the following: LBA4404, AR10, LBA4404 with pBCAG1, AR10 with pBCAG1, or AR10 with pBCAGE1. Fluorescence microscopy pictures showed that the tobacco plants transformed with LBA4404 or AR10 contained only autofluorescence (**Figure IV-5**). On the other hand, LBA4404 with pBCAG1, AR10 with pBCAG1, and AR10 with pBCAGE1 resulted in GFP fluorescent cells surrounded by autofluorescent tissue. GFP expression in tobacco transformed with AR10 containing pBCAGE1 could not be visualized clearly until 5 days after transformation. GFP fluorescence was not detected in LBA4404 pBCAGE1.

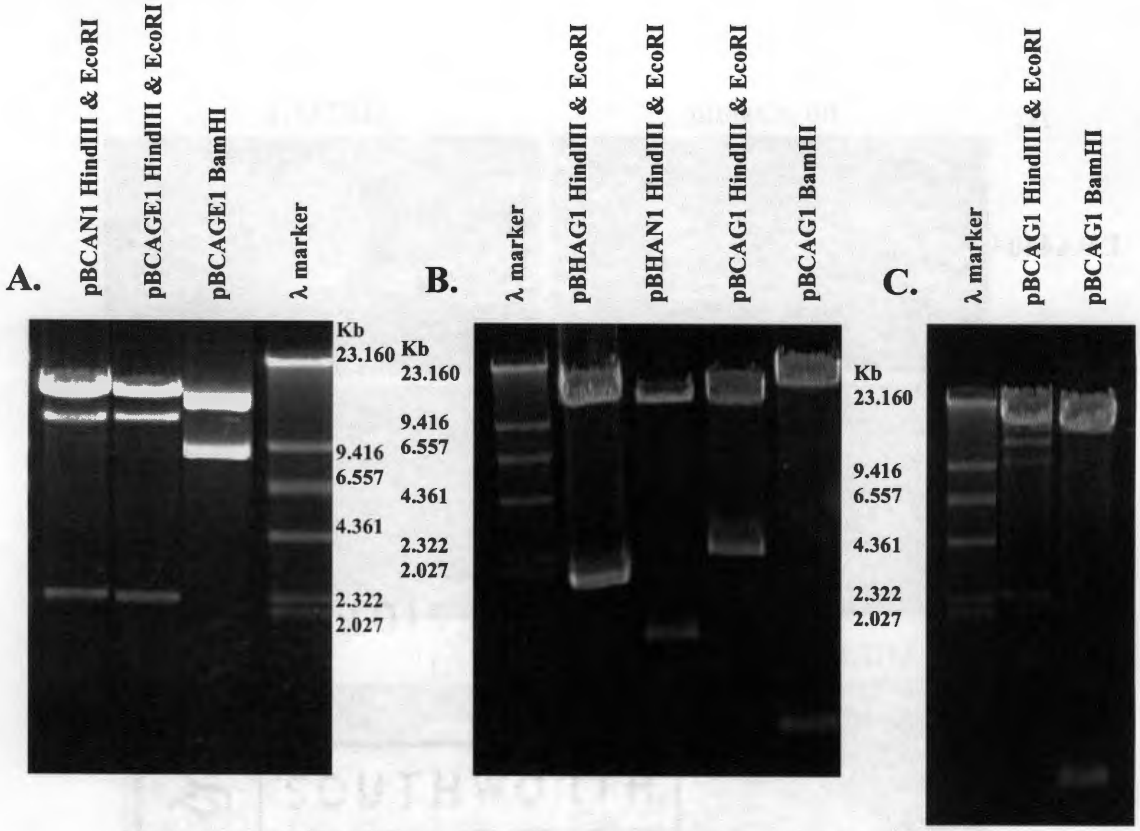


**FIGURE IV-4: TAE agarose gel electrophoresis of the binary plasmids back transformed into *E. coli*.** The restriction enzymes used are listed below the plasmid name, and the enzymes are the same as the enzymes used in Chapter III. Fragment sizes of the λ Hind III marker are labeled to the side of the gel. **A.** pBCAN1, pBCAG1, and pBCAGE from LBA4404. Lane 1: pBCAN1 digested with Eco RI and Hind III. Expected fragment sizes are 8.9 and 1.5 kb. Smaller fragment seen is 2.2 kb, which is same size for pBCAGE1 or pBCAG1, indicating that the plasmid was not pBCAN1. Lane 2: pBCAGE1 digested with Eco RI and Hind III. Expected fragment sizes are 8.9 and 2.2 kb. Lane 3: pBCAGE1 with Bam HI, which does not cut pBCAGE1. Lane 4: λ Hind III marker. **B.** pBHAG1 and pBHAN1 from LBA4404. Lane 1: λ Hind III marker. Lane 2: pBHAG1 digested with Eco RI and Hind III. Expected fragment sizes are 8.9 and 1.8 kb. Lane 3: pBHAN1 digested with Eco RI and Hind III. Expected fragment sizes are 8.9 and 1.1 kb. Lane 4: pBCAG1 digested with Eco RI and Hind III. Expected fragment sizes are 8.9 and 2.2 kb. Lane 5" pBCAG1 digested with Bam HI. Expected fragment sizes are 10.7 kb and 0.4 kb. **C.** pBCAG1 from transformed AR10 (R24E7). Lane 1: λ Hind III marker. Lane 2: pBCAG1 digested with Eco RI and Hind III. Expected fragment sizes are 8.9 and 2.2 kb. Lane 3: pBCAG1 digested with Bam HI. Expected fragment sizes were 10.7 kb and 0.4 kb.

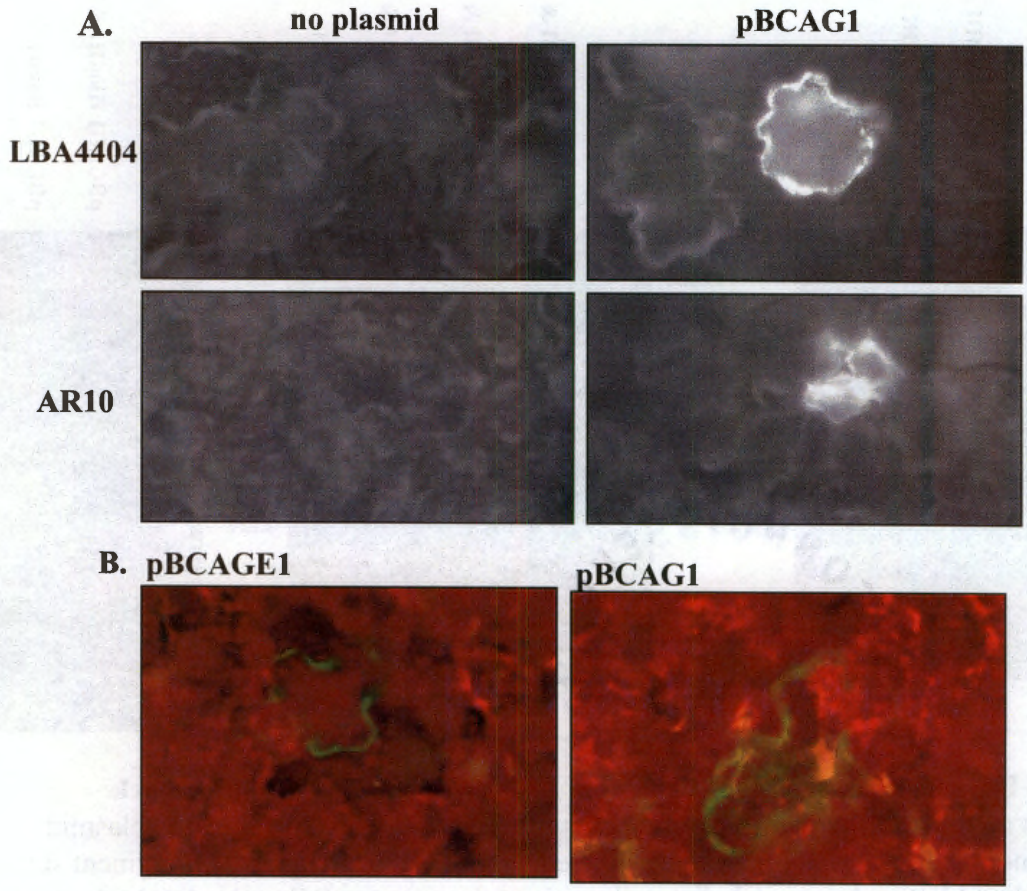


**FIGURE IV-5: GFP Expression in tobacco leaf epidermal cells transiently transformed with *A. tumefaciens* LBA4404 pBCAG1, *A. rhizogenes* AR10 pBCAG1, or *A. rhizogenes* AR10 pBCAGE1.** Figure A is a black and white image of tobacco leaf tissue 2 days after transformation, and Figure B is an image of tobacco leaf tissue 5 days and 9 days after transformation taken with a color camera. Pictures were taken at 10X magnification. **A.** A picture of tobacco leaves transformed with LBA4404, LBA4404 with pBCAG1, AR10, and AR10 with pBCAG1. The faint outline of tobacco cells is autofluorescence. All four pictures were taken at 3 days after transformation. **B.** Tobacco tissue transformed with AR10 containing either pBCAGE1 or pBCAG1. These pictures were made from leaf sections cut from the same plants as before 5 days and 9 days after transformation, respectively. In one of the cells from the pBCAG1 transformation, GFP expression can be detected within the cell and surrounding the nucleus. An arrow identifies the cell being described, and the cell was enlarged to the right of the picture. Pictures in **B** were taken by Beth Mullin.

Transformation was successful using either *Agrobacterium* strain, and AR10 could be used for hairy root transformation of tobacco. Batoko et al. (2000) mentions that GFP fluorescence could be detected between 1 and 3 days following inoculation. However, in these experiments expression of GFP 1 day post inoculation was only faintly detectable (**Figure IV-6**). GFP fluorescence was readily detectable from 3 to 9 days after transformation. Furthermore, GFP fluorescence in AR10 pBCAG1 was mainly detected around the external edges of the cell, but GFP fluorescence in AR10 pBCAG1 could be detected around the nucleus and within the ER of the cell after several days.

### 5. Several Colonies of 15834 Contain the Rol C Gene

The Ri plasmid is a plasmid necessary for *A. rhizogenes*-mediated transformation of plants, and the Ri plasmid from 15834 does not contain antibiotic selection. The presence of the Ri plasmid was verified by amplifying the Rol C gene, which is included on the Ri plasmid (Medina-Bolivar, personal communication). Several colonies were amplified by PCR, and the PCR products were detected by electrophoresis (**Figure IV-7**). The expected band size was 490 bp. All the colonies, except for one colony, showed amplification of a band of the correct size as expected.

### 6. Stable Transformation of Tobacco with *A. rhizogenes* Appeared to Be Successful But Tissue Could Not Be Used

Three different methods of transformation were used with AR10 to achieve transformed hairy roots. The first method was a variation of the leaf disk method of transformation. Initially, tobacco tissue was not blotted dry with sterile filter paper after dipping into the *Agrobacterium*, but the tissue was blotted dry in later transformation attempts. Eventually this method involved dipping a razor blade in liquid *Agrobacterium* suspension before cutting the leaf tissue. The second method involved wounding the hypocotyl with a needle and placing a drop of liquid culture onto the wound site. The last method was wounding the midvein of the leaf with a scalpel dipped into a colony of *Agrobacterium*.

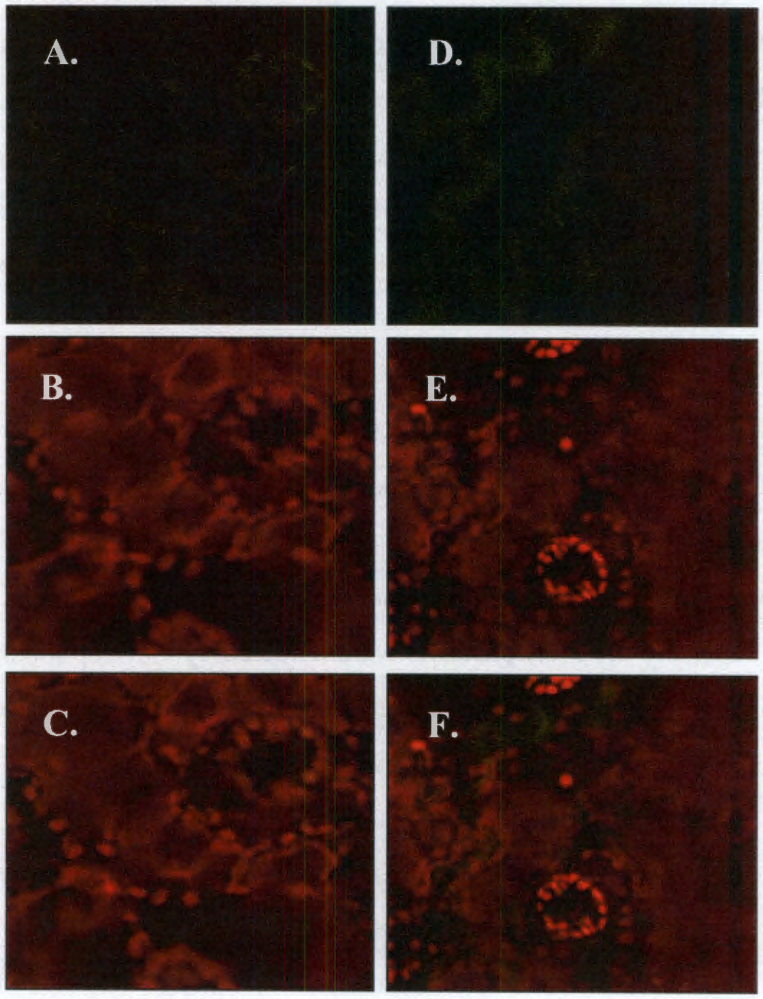


**FIGURE IV-6: Pictures of tobacco epidermal leaves transiently transformed with *A. tumefaciens* LBA4404 pBCAG1 after 1 day after inoculation.** Tobacco leaves were transformed the day before the picture was taken. Pictures shown are taken by a confocal microscope. The pictures were taken using the same settings. **A.** Picture from leaf tissue transformed with LBA4404in the range that GFP fluoresces. **B.** Picture from leaf tissue transformed with LBA4404 of the background autofluorescence. **C.** An overlay of **A** and **B**. **D.** Picture from leaf tissue transformed with LBA4404 pBCAG1in the range that GFP fluoresces. **E.** Picture from leaf tissue transformed with LBA4404 pBCAG1 of the background autofluorescence. **F.** An overlay of **D** and **E**. There is a slight increase in the fluorescence of **F** when compared to **C**.

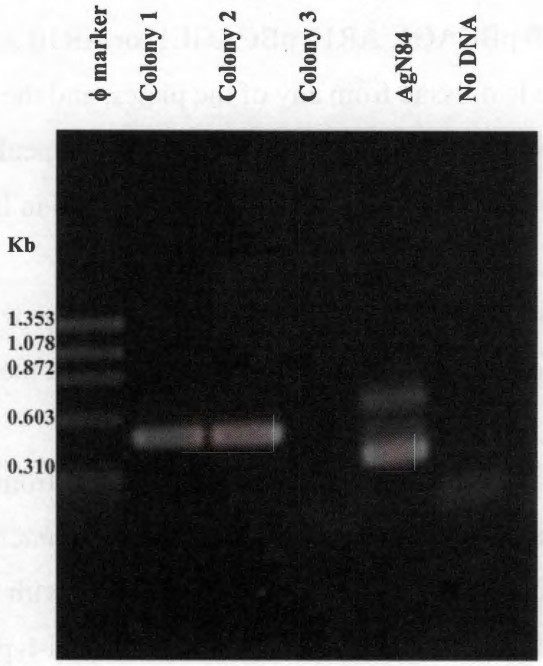


**FIGURE IV-7: TAE agarose gel electrophoresis of the PCR product from the Rol C gene on the Ri plasmid of *A. rhizogenes* 15834.** DNA from several colonies of 15834 was used in PCR amplification of the RolC gene to verify that 15834 contained the Ri plasmid. Lane 1: ϕX 174 Hae III marker. The sizes of the fragments are given to the side of the gel. Lanes 1-3: colonies of 15834. The expected PCR fragment is 490 bp. The Rol C gene is amplified in colonies 1 and 2, but colony 3 is not amplified. L5: AgNt84 amplified using Gateway primers. The expected fragment size is 388 bp.

Method 1 was tried without success. Plant tissue that had not been blotted dry with filter paper died of *Agrobacterium* overgrowth. The plant tissue facing the cover became overgrown with *Agrobacterium* within a few days after inoculation, so the pieces were inverted and moved to an unused region of the plate to decrease the *Agrobacterium* growth. The appearance of the leaf pieces worsened, and hairy roots did not form. In cases where the tissue had been blotted dry, the tissue sometimes became overgrown with AR10, AR10 pBCAN1, AR10 pBCAG1, AR10 pBCAGE1, or AR10 AN101. However, no roots would form from the leaf tissue from any of the plates, and the tissue not overgrown with *Agrobacterium* would begin to die 3 weeks after inoculation. The same behavior happened when the leaf tissue was cut with a razor dipped in liquid *Agrobacterium*.

In Method 2, roots also did not appear on the hypocotyl at the wound site. However, roots appeared in regions that had not been wounded, and they were not transformed.

With Method 3, hairy roots did appear from the wound site from all the transformation attempts within 3 weeks after inoculation with *Agrobacterium*. These attempts included: AR10 with wild-type tobacco, AR10 pBCAG1 with wild-type tobacco, AR10 pBCAGE1 with wild-type tobacco, 15834 with wild-type tobacco, 15834 with T10, 15834 with T12, and 15834 with T17. However, overgrowth of *Agrobacterium* could not be stopped, and the root tissue began to die after a small period of time.

Success of transformation was determined by looking for GFP fluorescence in the roots. Mild autofluorescence could be seen in tobacco roots transformed with AR10 (**Figure IV-8**), the fluorescence seen in tobacco roots transformed with AR10 pBCAG1 was noticeably brighter than the background seen in roots transformed with AR10. Fluorescence in the root hairs could only be detected in the fluorescence microscope with the tissue transformed with AR10 pBCAG1 but not with AR10. Roots transformed with AR10 pBCAGE1 did not show expression of GFP (data not shown). Other roots of the transformation with AR10 pBCAGE1 grew, but the contamination was too severe to check for GFP expression.

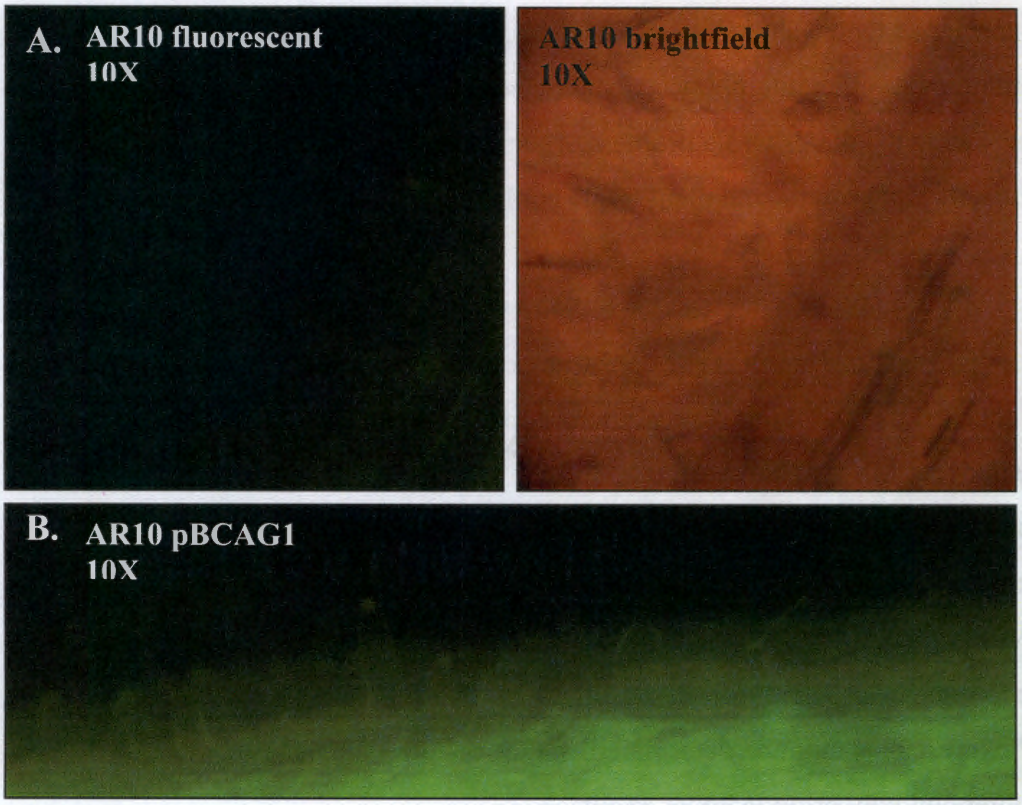


**FIGURE IV-8: GFP Expression in tobacco roots transformed with *A. rhizogenes* AR10 pBCAG1.** Pictures were taken on a fluorescence microscope. The same exposure time was used for the fluorescent pictures. **A.** Pictures of a tobacco root transformed with the control, or AR10. Pictures are at 10X magnification. The fluorescent picture and the brightfield picture are labeled accordingly. Autofluorescence can be detected in the root. However, the root hairs are not visible in the fluorescent picture, but they can be seen in the brightfield. **B.** Pictures of a tobacco root transformed with AR10 pBCAG1. Pictures are at 10X magnification. The fluorescence detected in AR10 pBCAG1 is stronger than the autofluorescence in AR10. The root hairs can be seen by their fluorescence.

### 7. Transformation of Tobacco with *A. tumefaciens* Was Not Successful

Tobacco leaves were cut and dipped into LBA4404 pBCAG1 in an attempt to make whole transgenic plants expressing AgNt84-GFP. This was done initially with the assistance of Mentewab Ayalew. However, plant tissue was dying within a week post inoculation. Another attempt was made by Mentewab Ayalew, and *Agrobacterium* overgrowing the plates was a problem. The plant tissue was dying because of the *Agrobacterium* growth, and plant tissue could not be regenerated.

### 8. BY-2 Cells Transformed with pBCAG1 Showed GFP Expression

GFP expression in BY-2 cells transformed with LBA4404 pBCAG1 or LBA4404 was checked using a fluorescence microscope (**Figure IV-9**). Both cells had an ill appearance. Cells that had been transformed with LBA4404 showed fluorescence in the vacuole, which is not seen in healthy BY-2 cells. In addition, both cells were plasmolyzed, so the cells were not healthy or living. However, cells transformed with LBA4404 pBCAG1 did not show fluorescence in the vacuole, and the color of the fluorescence was different from the LBA4404 control. Whether the fluorescence was GFP expression cannot be determined, and it was possible that other calli may be healthier and may provide better results.

## D. Conclusions

### 1. Particle Bombardment of Onion Epidermal Cells

Transient expression of some of the binary plasmids was successful, showing that the cloning steps made a protein product that could be detected by GFP fluorescence. In addition, the transient method of transformation gave further information. The pathway for a protein targeted extracellularly involves the protein to be first in the endoplasmic reticulum before going to the Golgi apparatus. Detecting AgNt84-GFP in the endoplasmic reticulum supports the possibility that the protein is targeted extracellularly because the endoplasmic reticulum is the first organelle that a protein would be present in when following the secretory pathway. However, other proteins that do not end in the

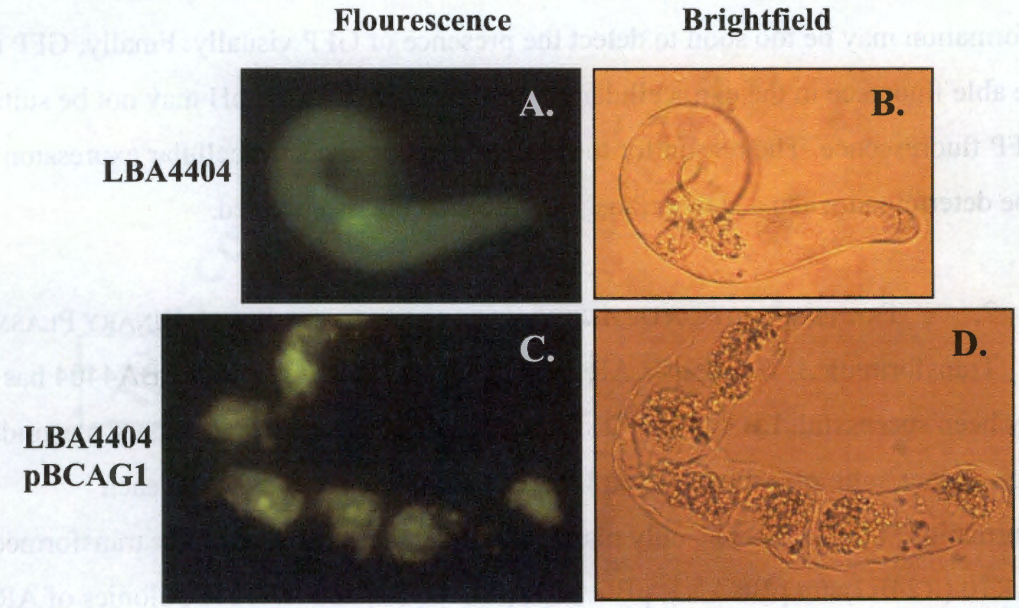


**FIGURE IV-9: GFP Expression in BY-2 cells transformed with *A. tumefaciens* LBA44O4 pBCAG1.** Pictures were taken on a fluorescence microscope. Exposure time was the same for the fluorescence pictures. **A.** The fluorescence picture of a BY-2 cell transformed with LBA4404. **B.** The brightfield picture of the same BY-2 cell transformed with LBA4404. **C.** The fluorescence picture of BY-2 cells transformed with LBA4404 pBCAG1. **D.** The brightfield picture of the same BY-2 cells transformed with LBA4404 pBCAG1. Pictures were taken by Beth Mullin.

plasma membrane are also targeted to the endoplasmic reticulum, so other possibilities about AgNt84's location have not been discarded. A result complicating interpreting possible extracellular targeting was that fluorescence outside the cell could not be detected. This conclusion was based on the picture of the onion cell transformed with pBCAG1 where the fluorescent picture and the brightfield picture were overlayed. There are at least three possible explanations for why GFP was not detected extracellularly. The first explanation is that the protein might not be targeted extracellularly. The second explanation is that looking for GFP fluorescence extracellularly the day after transformation may be too soon to detect the presence of GFP visually. Finally, GFP may not be able fluoresce in the extracellular environment because the pH may not be suitable for GFP fluorescence. The reason for the lack of detection of extracellular expression may be determined when a stable transgenic tobacco line is obtained.

### 2. Transformation of *Agrobacterium* with the Seven Binary Plasmids

Transforming *A. rhizogenes* AR10 (R24E7) and *A. tumefaciens* LBA4404 has largely been successful. LBA4404 transformed with each of the seven binary plasmids grew on proper antibiotic selection, and frozen cultures of a colony from each transformation were made. The only plasmids that have been successfully transformed into AR10 (R24E7) are pBCAN1, pBCAG1, pBCAGE1, and AN101. Colonies of AR10 (R24E7) transformed with pBEAN1, pBEAG1, pBHAN1, or pBHAG1 were not obtained.

The transformation of *A. rhizogenes* A4RS was unsuccessful with the one attempt at triparental mating and several tries at electroporation. Transforming A4RS was done as a precaution against the possibility that AR10 (R24E7) may not transform tobacco since the only published research of AR10 (R24E7) dealt with *Lotus japonicus* (Stiller et al., 1997). On confirmation that AR10 (R24E7) could transform tobacco by agroinfiltration, attempts at trying to transform A4RS were stopped.

Back-transformation of plasmids isolated from transformed *Agrobacterium* into *E. coli* was used to verify that transforming the correct binary plasmid into *Agrobacterium* was successfully carried out. LBA4404 transformed with each of the

seven plasmids was used for back-transformation. Colonies of *E. coli* grew when transformed with the binary plasmids from LBA4404 transformed with pBCAN1, LBA4404 transformed with pBCAG1, LBA4404 transformed with pBCAGE1, LBA4404 transformed with pBHAN1, or LBA4404 transformed with pBHAG1. The reason that *E. coli* transformation with the other two plasmids, pBEAN1 and pBEAG1, did not result in any colonies is because of a small amount of DNA in the minipreps. The small concentration of DNA may be because of the low copy number of these plasmids in LBA4404. Except for the back-transformation of pBCAN1, restriction enzyme digestion of the minipreps of all of these plasmids resulted in the correct fragment lengths. The fragment lengths of the one colony of pBCAN1 had the same lengths as pBCAG1 or pBCAGE1. It is most likely that the transformation of pBCAN1 was contaminated with pBCAG1 or pBCAGE1 during one of the steps since the three plasmids contain the same bacterial and plant selection. In addition, *E. coli* was transformed with the binary plasmid from AR10 transformed with pBCAG1. In addition, *E. coli* was transformed with the binary plasmid from AR10 transformed with pBCAG1, and this was successful as well.

### 3. Agro-infiltration of Tobacco Epidermal Tissue

As described above, tobacco leaves were infiltrated with AR10 (R24E7), AR10 pBCAG1, AR10 pBCAGE1, LBA4404, LBA4404 pBCAG1, or LBA4404 pBCAGE1. Leaves infiltrated with AR10 pBCAG1, AR10 pBCAGE1, or LBA4404 pBCAG1 showed fluorescence of GFP, but little information about the localization of the protein was gained. Upon initial observation of tobacco plants expressing AgNt84, GFP expression was detected as a clear outline of the region on the edge of the cell, and this pattern was true for AgNt84-GFP and AgNt84-GFPHDEL. The expression of protein fusion after several days visibly showed GFP expression as strands within the cell. In particular, GFP expression was detected close to the nucleus but not within the nucleus. This expression was most likely from the presence of the protein fusion in the endoplasmic reticulum. The endoplasmic reticulum can be close to the nucleus, and AgNt84-GFP was shown to be expressed in the endoplasmic reticulum of onion cells bombarded with pBCAG1.

The agroinfiltration of tobacco with LBA4404, LBA4404 pBCAGE1, or AR10 resulted in cells that did not show GFP fluorescence. It is likely that if more tissue had been analyzed over a longer period, agroinfiltration with LBA4404 pBCAGE1 would have resulted in tissue expressing GFP. It is unlikely that something is wrong with the construct because transformation of onion cells with pBCAGE1 was successful.

Batoko et al. (2000) mentions that GFP expression can be seen between 1 and 3 days after transformation, but expression of AgNt84-GFP in tobacco epidermal leaves was detected as long as 9 days after transformation. According to Voinnet et al. (2003), the brief time for expression is due to gene silencing. The prolonged expression of AgNt84-GFP could be because expression does not induce gene silencing or because of enhanced stability of the GFP fusion protein.

Voinnet et al. (2003) mentions that expressing the transgene is optimal on day 3. However, this was not known when confocal microscopy was attempted. In this case, confocal microscopy could have been used as late as 9 days after transformation. Information on the targeting of the protein may have been obtained had confocal microscopy been tried at least 3 days after transformation.

### 4. *A. RHIZOGENES* 15834 CONTAINS THE ROL C GENE

Successful PCR amplification of the Rol C gene in 15834 shows that 15834 contains the Ri plasmid. In addition, one colony did not result in amplification of the Rol C gene, suggesting that the Ri plasmid was not present or amplified. The other colonies could be selected for use in transforming tobacco.

### 5. TOBACCO TRANSFORMATION WITH *A. RHIZOGENES*

Initially, the leaf disk method and hypocotyl wounding of tobacco did not result in transformed hairy roots. One possible reason is misunderstanding the information in the protocol. The protocol mentioned that the tissue was to be placed on MS medium with hormones used in *A. tumefaciens* transformation of tobacco to regenerate the shoot, but the hormones would not be needed for *A. rhizogenes* transformation. MS medium was made without sucrose following recipes in tissue culture books that did not include

sucrose. Addition of sucrose would have helped the growth of new tissue. Sucrose would especially be necessary in the leaf disk method since the leaf tissue was kept in the dark and was therefore not able to photosynthesize. This technique might have been successful if sucrose had been added. It is also possible that B5 medium would have been a more suitable medium to use. Wongsamuth and Doran (1997) report that root cultures grown in MS media with 3% sucrose do not last long, and long-term culturing of hairy roots is done in B5 medium with 3% sucrose. In addition, Medina-Bolivar and Cramer (2004) use B5 medium with sucrose added to it to grow tobacco leaves transformed with *A. rhizogenes* 15834.

Regardless of the problems mentioned above with maintaining transformed tissue, wounding the midvein of a target tobacco leaf with a scalpel dipped into a colony of *Agrobacterium* appeared to lead to transformation. How to prevent overgrowth of *Agrobacterium* remains unresolved. If hairy roots are important to have, obtaining transgenic plants by *A. tumefaciens* transformation should be accomplished first, and the transgenic plants should then be transformed with *A. rhizogenes*. Advantages to hairy root transformation exist which would make this a worthwhile objective for future work.

### 6. TOBACCO TRANSFORMATION WITH *A. TUMEFACIENS*

The error in the first attempt at tobacco transformation with LBA4404 pBCAG1 is likely due to the leaf tissue being plated on MS medium with sugar instead of DBI medium. The plant tissue was not beginning to rapidly divide while co-incubating with *Agrobacterium* before being placed on selection plates. These plates did not grow *Agrobacterium*, and the leaf tissue did not begin to regenerate.

The error in the next attempt at tobacco transformation is most likely that the density of LBA4404 was initially too high. Possible solutions to this problem are to dilute LBA4404 or to transform a less virulent strain of *A. tumefaciens* with the binary plasmid.

### 7. BY-2 TRANSFORMATION WITH *A. TUMEFACIENS*

BY-2 cells continued to grow slowly. In addition, several cells on the initial plates did not grow on selection. Therefore, the calli appear transformed despite the slow

growth. However, the slow growth of the calli prevents these transformed cells from being currently used in metal uptake experiments, and fluorescence microscopy of BY-2 cells had yet to confirm expression of the fusion protein in the cells analyzed.

## 8. Subcellular Targeting of AgNt84

For a protein, the pathway to the plasma membrane and export from the cell is through the endoplasmic reticulum and the Golgi apparatus. Proteins targeted to the mitochondria or chloroplasts come from the cytosol and not from the endoplasmic reticulum (Alberts et al., 2002). Tamura et al. (2003) found that GFP was degraded in the vacuoles, so the GFP fused to AgNt84 might not fluoresce in the vacuoles if targeted there. Onion cells bombarded with AgNt84-GFP constructs showed expression in the endoplasmic reticulum, a location consistent with extracellular targeting.

## 9. Future Objectives

The objectives that have been completed are:

- to transform onion epidermal cells by particle bombardment
- to transform *Agrobacterium tumefaciens* strain LBA4404 with the seven binary plasmids.
- to transform tobacco leaves by agroinfiltration

The objectives that have been partially completed are:

- to transform *Agrobacterium rhizogenes* strain AR10 with the seven binary plasmids
- to transform tobacco plants and tobacco BY-2 cells with *Agrobacterium*
- to verify the presence of the correct plasmid in *Agrobacterium* by transforming the plasmid back into *E. coli*
- to learn about the subcellular targeting of AgNt84

Of these three objectives, transforming AR10 may not be important since 15834 can be used to generate hairy roots from tobacco plants. The only objective that was not finished was:

- to transform *Agrobacterium rhizogenes* strain A4RS

Problems with completing some of these objectives lead to suggestions for further objectives and modifications of the last major objective. Suggestions for future objectives are:

- to transform tobacco with LBA4404 containing pBCAG1, pBCAGE1, pBEAG1, and/or pBHAG1 by the leaf disk method to be used for determining the subcellular targeting of AgNt84
- to use antiAgNt84 and/or antiGFP antibodies to discover whether the protein is targeted extracellularly
- to transform tobacco again by agroinfiltration with LBA4404/AR10 containing pBCAG1 to detect the subcellular targeting of AgNt84-GFP at a higher magnification using confocal microscopy
- to transform transgenic tobacco lines expressing AgNt84 with *A. rhizogenes* 15834

If agroinfiltration is repeated, microscopy should be done when the cells have expressed the fusion protein for several days since GFP could not be detected by confocal microscopy after 1 day following transformation. In the last objective, the use of hairy roots in finding out the subcellular targeting of AgNt84 is not necessary, and whole plants can be made for that objective. However, there are advantages to using hairy roots in metal uptake experiments, which make further endeavors into generating hairy roots valuable.

Transformation of tobacco with *Agrobacterium* containing the AgNt84 constructs discussed above did not result in transgenic lines for metal uptake experiments. Therefore, metal uptake experiments were done using transgenic lines constructed by Mentewab Ayalew in Dr. Neal Stewart's laboratory. The final major objective was:

- to determine if transgenic plants expressing AgNt84 have an increase in cadmium uptake compared to wild-type plants.

This objective is discussed in detail in the next chapter.

# CHAPTER V

# SHORT-TERM CADMIUM UPTAKE EXPERIMENTS

## A. INTRODUCTION

### 1. OBJECTIVE

The final major objective of this research was: to determine if transgenic plants expressing AgNt84 have an increase in cadmium uptake and accumulation compared with wild-type plants. This objective required verification that a transgenic line was expressing AgNt84 and the design of experiments to determine cadmium levels in transgenic and wild-type plants following their exposure to cadmium.

### 2. METHODS USED FOR DETECTING CADMIUM CONCENTRATION AND LOCATION

Several methods can be used to determine the concentration of cadmium in tobacco plants, and they include radioactive and nonradioactive methods. The primary method used in this study was inductively coupled argon plasma spectrometry (ICP). Using this technique, measurements were accurate without the complications of using radioactive materials. Another method used was dithizone staining of tobacco tissue, which gives a rough estimate of cadmium concentration and location.

For ICP analysis, the tissue sample must be in solution. For the tobacco samples used in this experiment, the tissue was dry ashed, dissolved in a mixture of strong acids, and analyzed in a dilute acid solution. The solution is introduced into the ICP instrument by pumping into the nebulizer, which atomizes the solution. The sample moves into the argon torch where the hot plasma ionizes the atom(s) in the sample. An array of CCD detectors recognizes the light emissions. The information collected by the detectors is used to calculate the concentration of cadmium.

Dithizone staining is a method to histochemically detect where cadmium is located within plant tissue. When in the presence of cadmium, dithizone, a dark violet crystal, changes color by forming a dithizonate complex with cadmium (Maevskaya et al., 2001). The color intensity of the dithizonate complex depends on the concentration of the metal in the plant tissue. In Maevskaya et al. (2001), the color range is given for gametophytic tissue. A light pinkish-red color is visible below 1 mM of the dithizone complex. A red color is visible around 1 mM of the dithizone complex. A dark red color is visible when dithizone complex was greater than 1 mM. Pielichowska and Wierzbicka (2004) also report that the dithizonate complex ranges from dark red color to a black color in the cadmium hyperaccumalator *Biscutella laevigata*, a vascular plant in the *Brassicaceae* family. Although using dithizone is not quantitative relative to ICP analysis, this method assisted in determining the location of cadmium in the plant.

### 3. Outline of Procedure

The transgenic lines described in Chapter IV needed to be checked for expression of AgNt84 mRNA. One to two plants were selected from each tobacco line for northern blot analysis.

Plants were grown hydroponically for metal uptake experiments. If agar was present in the media and could not be completely rinsed off the roots it might trap cadmium, resulting in increased variation and inaccurate quantitation of cadmium levels.

A literature search was conducted to identify protocols for the hydroponic growth of tobacco plants. Protocols involving the use of only liquid media used expensive containers to grow plants. Furthermore, several protocols involved growing plants in vermiculite or perlite and adding media to the plants. There were two problems with this arrangement. The use of vermiculite or perlite would cause difficulties in cleaning the plants, and the cadmium contaminated vermiculite or perlite would result in a huge volume to discard as hazardous waste. Using only liquid reduces the volume of cadmium waste because the liquid can be passed through a Chelex resin column, which will bind cadmium removing it from the liquid. After cadmium is collected on the resin, it can be

eluted from the resin in a small volume of liquid or the entire batch of resin can be disposed of as hazardous waste.

Xiang and Oliver (1998) describe a method in which *Arabidopsis* plants are grown in flasks containing a small volume of medium. In the protocol, the seeds are placed in the flasks, and when the seeds germinate the seedlings are submerged. A variation of this method was tried using wild-type tobacco. Instead of starting with seeds, seedlings that were approximately 2 weeks old were placed into liquid culture. In another variation of this method, a hydroponic apparatus was designed so the shoots remained above the solution. Tobacco plants were hydroponically grown for 2 weeks in MS media prior to the cadmium uptake experiments. The plants were then placed in MES buffer containing 200 μM $Cd(NO_3)_2 \cdot 4H_2O$. MES buffer is found to increase cadmium uptake because the buffer does not contain molecules that cadmium would form complexes with, preventing uptake of cadmium into the plant (Nedelkoska and Doran 2000b).

The process of dithizone staining was fast. The plant tissue was incubated for several minutes in the stain, and the excess dithizone was rinsed off the tissue. The tissue was checked histochemically for differences in dithizonate coloration.

In ICP spectrometry, the plant tissue is processed before use. The tissue was rinsed several times, and the root tissue and shoot tissues were separated. The tissue was dried, and future calculations were based on the dry mass. The dried tissue was then dry ashed for several hours. The dry ash was dissolved, brought up to volume, and filtered. The filtered solutions were subjected to ICP analysis.

## B. Materials & Methods

### 1. Plant Material

*Nicotiana tabacum* cv. "Xanthi" wild-type seeds and T1 seeds from three transgenic tobacco lines transformed with pBINAgNt84 were a gift from Mentewab Ayalew in Neal Stewart's lab, University of Tennessee, Knoxville. The transgenic lines were named T10, T12, and T17.

## 2. Northern Blot Analysis of Tobacco Lines

### 2. a. Small-scale RNA Preparation of Plant Samples

Glass beads were added to a 1.7 mL microfuge tube to the 150 µL line. Approximately 200 mg of 3-week-old tobacco plant tissue was placed in the microfuge tube, and the tube was placed in liquid nitrogen. The tube was taken out of the liquid nitrogen and mixed for 10 seconds using a Silamat S5 Ivoclar Vivadent machine. The tube was immediately placed back into liquid nitrogen. The tube was mixed as before, and again placed in liquid nitrogen. The remaining protocol for isolation of RNA was the protocol that was supplied with the Concert™ Plant RNA Reagent (Invitrogen) (Appendix B). The quantity and quality of RNA were checked by reading the $OD_{260}$ and $OD_{280}$. RNA was stored at –80°C.

### 2. b. Electrophoresis of Isolated RNA

A 1.2 % agarose formaldehyde gel was prepared as follows. 0.48 g agarose, 37 mL RNAase-free water, and 2mL of 20X MOPS buffer (Appendix A) were combined in a flask and melted by heating in a microwave. The flask was cooled to around 65°C, and 0.4 µL of a 10 mg/mL stock of EtBr and 2.16 mL of 37% formaldehyde were mixed in. The gel was poured into a gel casting container and allowed to solidify. The gel was transferred to an electrophoresis tank. 20X MOPS buffer that had been diluted to 1X MOPS buffer was used to fill the tank.

15 µg of each RNA sample was loaded into a well in the gel, and RNA was separated by electrophoresis using 90V for approximately 1 hour. The gel was visualized on a UV illuminator, and a picture was taken.

### 2. c. Overnight Blotting

The gel was transferred to a small container and was rinsed several times. The first rinse was in 0.05 N NaOH while rocking on a shaker for 20 minutes. NaOH was discarded, and the gel was rinsed in $dH_20$ briefly. The third rinse was in 20X SSC (Appendix A) while rocking on a shaker for 45 minutes. The SSC was discarded, and the gel was ready to begin the transfer of RNA onto a membrane.

The transfer stacks were prepared. A small tray was filled about halfway with 10X SSC, and the gel casting container was positioned with the bottom facing upward in the SSC. A wick was made by cutting 3 MM paper into a cross and folding the legs down over the bottom of the gel casting container. The cross was cut so that there were no open regions and that the edges of the legs were flush with each other. Three sheets from the same material were cut larger than the gel, wetted with 10X SSC, and placed on top of the wick. The gel had been trimmed so that the lanes containing the RNA samples were the only lanes to be transferred, and one edge of the gel was cut off at an angle to identify the orientation of the gel. A sheet of Hybond N membrane and 3 sheets of 3 MM paper were cut the same dimensions as the gel. The gel was positioned in the tray with the bottom up, and the gel was surrounded by parafilm so that the movement of SSC would occur only through the gel. The membrane was wetted with 10X SSC and positioned on top of the gel. The 3 sheets of 3 MM paper cut to the size of the gel were placed on top of the membrane. Care was taken to remove any bubbles under the gel and the membrane when they were added onto the stacks. Several layers of paper towels were placed on top so that the depth of the paper towels was approximately 5 cm deep, and a glass plate was placed on top of the paper towels. A heavy container was place on top of the glass plate to evenly weigh down the paper towels.

The RNA was transferred to the membrane overnight. The papers, SSC, and gel were discarded. The membrane was rinsed in 6X SSC and blotted dry with paper towels. RNA was crosslinked to the membrane using a Stratalinker® UV Crosslinker at energy 1200 J. The membrane was air dried in the middle of two sheets of 3MM paper. The membrane was used immediately for hybridization or was placed in a drawer to be used the following day.

### 2. D. Hybridization of AgNt84 Probe

The membrane was prehybridized for at least 2 hours in prehybridization buffer (Appendix A). The prehybridization buffer was warmed to 65°C prior to use, and the volume used was approximately 0.2 mL/cm$^2$. The membrane was rotated in a roller bottle at 65°C during the incubation.

AgNt84 probe was radiolabeled while the membrane was prehybridizing. The handling of radioactive materials and solutions was done by Mentewab Ayalew. The RadPrime DNA labeling system (Invitrogen) was used with $^{32}$P dCTP with a specific activity of 6000 Ci/mmol (Perkin Elmer). 25 ng of pAgNt84 was added to water so that the final volume was 23.5 µL in a microfuge tube. The tube was boiled for 5 minutes and then cooled on ice. 1 µL of dATP, 1 µL of dTTP, 1 µL of GTP, and 20 µL of labeling buffer were added to the microfuge tube. 2.5 µL of $^{32}$P dCTP was mixed into the microfuge tube, and 1 µL of Klenow was mixed in. The microfuge tube was briefly centrifuged. The microfuge tube was kept at 37°C for 15 minutes, and stop buffer was added to the tube. The probe was cleaned by passing the probe over a NucAway Spin column (Ambion).

The microfuge tube was placed in boiling water for 5 minutes to denature the probe. The microfuge tube was transferred to ice, and the tube was incubated on ice for 5 minutes. The entire probe was added to 5 mL of hybridization buffer. The hybridization buffer was warmed to 65°C prior to use. The prehybridization buffer on the membrane was discarded, and the hybridization buffer containing the radiolabeled probe was added to the membrane. The membrane hybridized overnight at 65°C while rotating in a roller bottle.

In the morning the probe was discarded, and the membrane was washed six times. The first two washes were in wash solution 1 pre-warmed to 65°C (Appendix A). The next two washes were in wash solution 2 pre-warmed to 65°C (Appendix A). The final two washes were in wash solution 3 pre-warmed to 65°C (Appendix A). Each wash was done at 65°C while rotating for 15 minutes in a roller bottle.

### 2. E. Picture of Membrane with the Phosphoimager

After the membrane was washed six times, the membrane was blotted dry and wrapped in plastic wrap. The membrane in the plastic wrap was placed in an empty cassette for a phosphoimager, and the cassette was exposed overnight. The following morning, the cassette was scanned with a phosphoimager to obtain a picture of the membrane.

### 3. Hydroponic Growth of Tobacco

Wild-type tobacco seeds were sterilized as in Chapter IV, and the seeds were plated on filter paper with water. The plants were allowed to germinate over a 2-week period. The seedlings were placed in modified half-strength MS media (Appendix D). The volume of media and the flask size used were a 250-mL flask containing 80 mL of media, a 500-mL flask containing 80 mL of media, and a 500-mL flask containing 160 mL of media. The flasks were rotated on a shaker at 80 rpm. The plants were kept under continuous light. It was later discovered that a miscalculation of the sucrose occurred, and the sucrose should have been at 2%, or 20 g/L, not 0.2%, or 2 g/L. After a few days, the leaves of the tobacco plants appeared to be watery and bruised, and the roots of the plants were being ripped off. It is not known if the watery appearance would have occurred if the correct amount of sucrose had been added, but shaking the plants and submerging the plants in media did not appear to be keeping the plants healthy.

A method that allows the plants to remain in an upright position was developed (**Figure V-1**). With the new arrangement for growing plants, the method for germinating and hydroponically growing the plants was changed. The seeds were sterilized as in Chapter IV, but the seeds were plated on round petri plates containing MS media with 3% sucrose and 0.4% Phytagel (Appendix D). For the transgenic lines, 200 μg/mL of kanamycin was added to the MS media. A filter paper disk was added to the top of the media to prevent the roots from growing into the media. The idea to use filter paper on a plate came from an *Arabidopsis* transformation protocol in Limpens et al. (2004). The plants remained on the plates for 3 weeks. The transgenic seeds were from the T1 generation, so the plants not containing the AgNt84 construct would not grow on kanamycin selection or did not grow past the cotyledon stage before dying. 3-week-old plants were transferred to holes in a round autoclaved disk cut from a pipette tip box. Each plant was placed in a separate hole so its roots were suspended below the disk and its leaves above the disk. The disk was kept above the solution using 3 microfuge tubes, and the tubes were kept in place by wrapping with a rubber band. The disk was autoclaved in a deep petri dish containing 200 mL of MS media with 3% sucrose

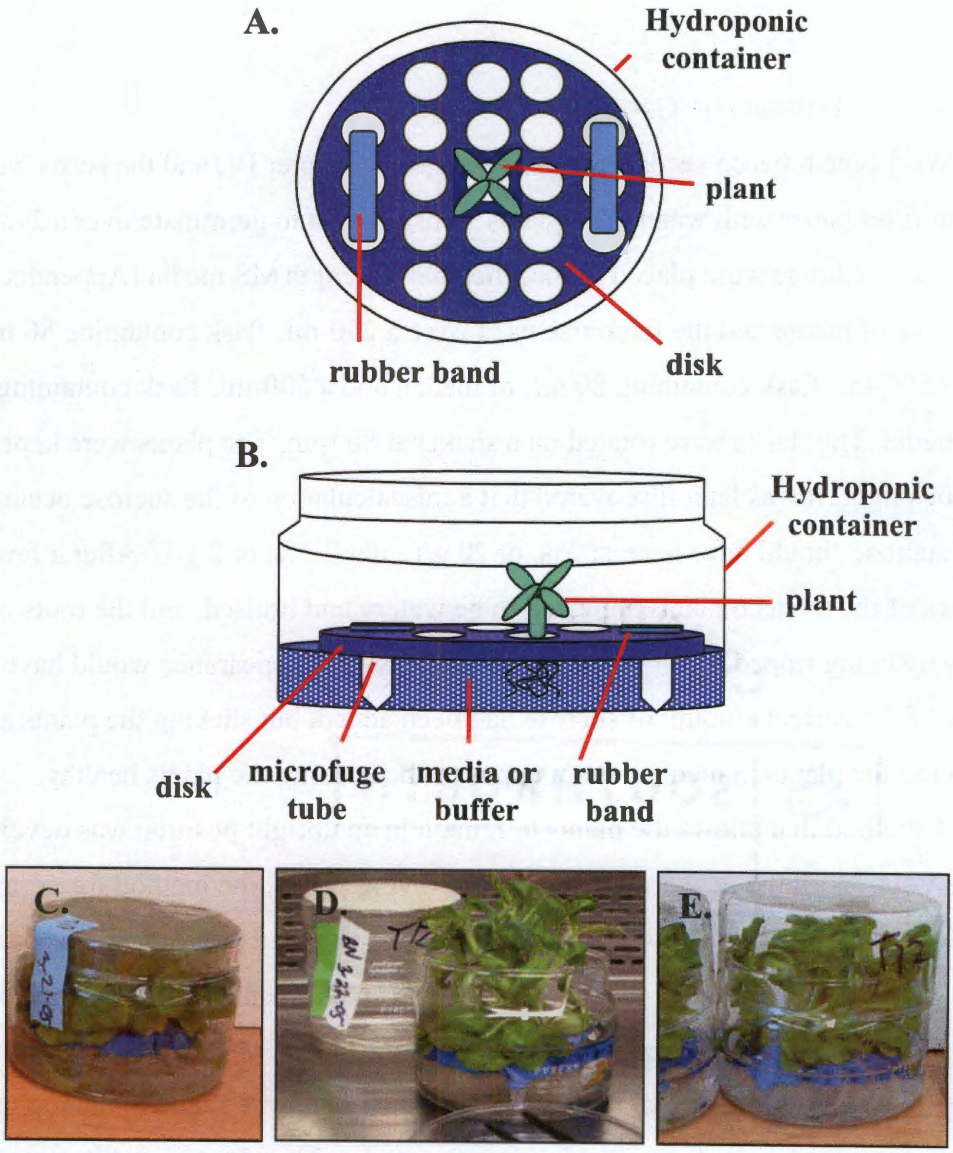


**FIGURE V-1: Hydroponically grown tobacco plants.** The first two figures are diagrams of the setup of the hydroponic containers, and the remaining pictures are of the plants at various steps in the metal uptake experiment. **A.** A diagram of the disk in the container. The view is through the opening of the petri dish container. The disk can easily be moved in and out of the container, which facilitates rinsing the plants. Each plant is placed in a hole in the disk. **B.** A diagram of the setup of the hydroponic container. The disk is above the buffer or media so that the roots are in contact. In addition, the microfuge tubes hold up the disk to allow the disk to remain above the buffer or media. **C.** Picture of tobacco plants hydroponically grown in MS. **D.** Tobacco plants in the process of being rinsed with water in the laminar flow hood. **E.** Tobacco plants in MES buffer containing 200 μM $Cd(NO_3)_2 \cdot 4H_2O$. A bigger lid was used to accommodate the size of the plants. The picture was taken after the plants were rinsed with water.

(Appendix D). Using this system it was possible to transfer plants from one solution to another without disturbing the roots by carefully lifting the disk out of one container and placing it into a second container. The seedlings remained in MS media for two weeks before being used for cadmium-uptake experiments.

### 4. Acid-Washing of Glass Containers for Cadmium-uptake Experiments

All glassware was acid-washed before use in cadmium-uptake experiments. The glassware was thoroughly washed and rinsed 3 times in $ddH_2O$. Glassware was filled with 0.1 N HCl, and glassware remained in HCl (Sigma) for at least 1 hour. HCl was discarded, and glassware was rinsed with $ddH_2O$ 5 times. Glassware was dried before using.

### 5. Incubation with Cadmium

Hydroponically grown tobacco plants were used for cadmium uptake experiments. The plants were rinsed to clean the roots of residual MS media (**Figure V-1**). The disk holding the plants was carefully lifted out of the culture dish and transferred to another container with approximately 250 mL of $ddH_2O$. The plant roots were kept in the water for 10 minutes, and were then transferred to another container of $ddH_2O$ again by lifting the disk out of one container and placing it into a new container. Five rinses in $ddH_2O$ were completed. 4 to 6 plants were taken out for drying, and pieces of root tissue were selected for dithizone staining. The plants were moved to 200 mL 5 mM MES buffer containing 200 μM $Cd(NO_3)_2 \cdot 4H_2O$ (Appendix A). The solution in which the plants were incubated was based on Nedelkoska and Doran (2000b). The rinses were done in the laminar flow hood to keep the plants axenic.

Following a one day incubation in $Cd(NO_3)_2 \cdot 4H_2O$, the plants were taken out of 5 mM MES buffer with 200 μM $Cd(NO_3)_2 \cdot 4H_2O$, and were rinsed five times in $ddH_2O$ as before, and again the rinses were done in the laminar flow hood. The same number of plants was removed for drying, and root tissue was selected for dithizone staining. Plants

were placed back in the original 5 mM MES buffer with 200 μM $Cd(NO_3)_2 \cdot 4H_2O$ that they were in before the rinse.

The remaining plants were rinsed on day 3 of the metal uptake experiments. The disk containing the plants was taken out of 5 mM MES buffer with 200 μM $Cd(NO_3)_2 \cdot 4H_2O$, and the plant roots were rinsed five times in $ddH_2O$ as before except the rinses were done at a lab bench. The same number of plants was selected for drying, and root tissue was selected for dithizone staining.

### 6. Dithizone Staining of Tobacco Roots

Dithizone staining of tobacco plants was done as outlined in Maevskaya et al. (2001) and Seregin et al. (2004). Tobacco roots and shoots at day 1 and day 3 of the metal uptake experiments in addition to tobacco roots incubated in 5 mM MES without cadmium were used for dithizone staining.

Plant tissue, rinsed in $ddH_2O$ as described above, was incubated in dithizone stain solution for 10 to 15 minutes (Appendix D). The stain solution was discarded, and the plant tissue was rinsed with the dithizone destain solution (Appendix D) until the dithizone destain solution was clear. The sample was covered with $dH_2O$ and mounted on a slide.

### 7. Drying of Tobacco Samples

Tobacco plants selected for drying were separated into shoot tissue and root tissue. Roots and shoots from each sample were placed in separate Pyrex flasks, and the fresh mass was taken. The tissue was dried for two days or until no change in dry mass occurred. Once the sample was dried, the dry mass of the tissue was measured. The samples were then ready to be dry ashed.

### 8. Dry Ashing and ICP Analysis

The dried plant material was placed into a muffle furnace, and the temperature was set to gradually increase to approximately 520 °C. The temperature was maintained

for approximately 8 hours. The samples were allowed to cool before removing them from the muffle furnace.

10 mL of 1 N $HNO_3$ (Fisher) was added to each sample, and the samples were heated on a hot plate. $HNO_3$ was slowly evaporated. The sample was allowed to cool, and the sample was dissolved with 10 mL of 1 N HCl. The sample was heated until the solution was close to boiling. At this time, the sample was transferred to a 100 mL volumetric flask. The original container holding the sample was rinsed three times with deionized water, and the rinses were added to the volumetric flask. The volume was brought up to the 100-mL volume mark on the volumetric flask. The sample in the volumetric flask was filtered using Whatman No. 42 filter paper. ICP analysis was used to determine cadmium concentration.

### 9. Statistical Analysis of ICP Results

Cadmium concentration in samples analyzed with ICP was recorded in units of mg/L. The concentration of cadmium was converted to the unit μg cadmium/g dry plant mass.

The effect that time and tobacco line had on the concentration of cadmium was analyzed using analysis of variance (ANOVA) with mixed models. The ANOVA design used was a completely randomized design (CRD) factorial with two treatment factors. The two treatment factors were time and tobacco line. The equation for the design used was $y_{ijk}=\mu+T_i+D_j+ T*D_{ij} + R(T*D)_{ijk}$ where T was the tobacco line, D was the day the sample was taken, and R was the replicate. The program used for analysis was SAS 9.1. The macro DandA.sas designed and copyrighted by Arnold Saxton, University of Tennessee, Knoxville was also used.

## C. Results

### 1. Northern Analysis Shows that T10 Expresses AGNT84

The small RNA (4S, 5S & 5.8S RNAs) from line T12 was not visible in one of the agarose gels, and the small RNA from T12 in the other agarose gel was barely

detectable (**Figure V-2**). The small RNA from T17 and T10 was more abundant than in T12. In addition, RNA from line T17 showed some degradation.

A hybridization band was detected in T10 but not in T12 or T17 when the membrane was hybridized with a radioactive probe. The band in T10 corresponded to the region where AgNt84 RNA is expected to have been, so T10 was the only transgenic tobacco line that showed expression of AgNt84 RNA.

### 2. Dithizone Staining of Tobacco Roots

Plants that had been incubated in MES buffer containing cadmium were stained with dithizone to visualize the location and intensity of cadmium staining. In addition, a control was included where plants were incubated in MES buffer without cadmium. As stated in the introduction, the color of the dithizonate complex was dependent on the concentration of cadmium, and the color ranged from the absence of color to light red color to black. The lighter dithizonate color indicates that less cadmium complexes with dithizone whereas the darker dithizonate color indicates that more cadmium complexes with dithizone. In tobacco plants not incubated in cadmium, there was no formation of dithizonate, which was indicated by the absence of any red color (**Figure V-3**). Since dithizone also complexes with other metals such as lead (Maevskaya et al., 2001), this control showed that the coloration seen in plants incubated in cadmium was due to the addition of cadmium.

When tobacco plants were incubated in cadmium for 1 day, the red color of dithizonate was visible (**Figure V-4**). Although there was a range in the degree of dithizone staining for each line of tobacco, tobacco roots from lines T10 and T12 stained darker than wild-type tobacco. T17 generally was darker than wild-type tobacco, but T17 stained lighter than the other transgenic lines. In addition, not all of the cells showed staining. Dithizonate complexes appeared in lines along the cell wall in every tobacco line, and the dithizonate complexes also appeared as spots. The reddish spots appeared along the cell wall or within the cell. The black spots appeared once per cell, and the dark spot might coincide with the nucleus. Tobacco tissue incubated for 3 days in cadmium

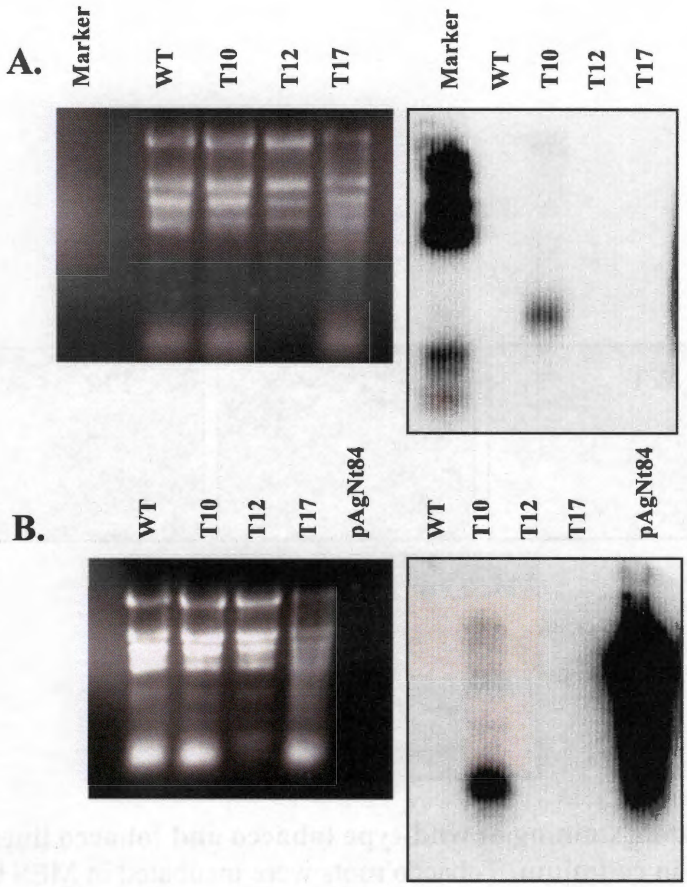


**FIGURE V-2: Expression of AgNt RNA was detected in T10 by northern analysis.** Pictures to the left are of the formaldehyde gels showing total RNA, and pictures to the right are from the membrane radiolabeled with AgNt84 probe. **A.** RNA gel and membrane. Lane 1: DNA ladder. Lane 2: wild-type tobacco. Lane 3: T10. Lane 4: T12. Lane 5: T17. The positive control, pAgNt84, was cut off because the amount loaded was miscalculated. **B.** RNA gel and membrane. Lane 1: wild-type tobacco. Lane 2: T10. Lane 3: T12. Lane 4: T17. Lane 5: pAgNt84. This gel contained the same RNA for T10 and T17 as in **A.**, but the RNA for T12 came from a different tobacco plant. Pictures were taken by Mentewab Ayalew.

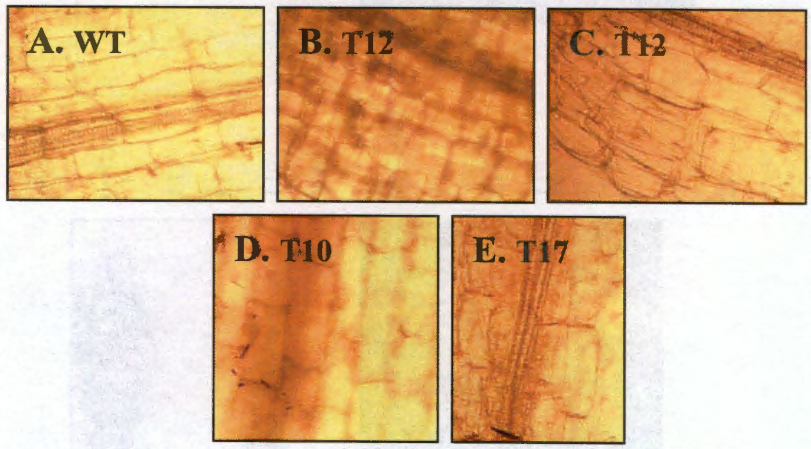


**FIGURE V-3: Dithizone staining of wild-type tobacco and tobacco lines T10, T12, and T17 not incubated in cadmium.** Tobacco roots were incubated in MES buffer without cadmium for up to 3 days. Day 1 and day 3 roots had the same appearance. Pictures of only day 3 plants are shown. Small linear black lines (indicated by arrows) are dithizone crystals that were not washed away in the rinses. **A.** Root from wild-type tobacco. **B.** and **C.** Roots from tobacco line T12. **D.** Roots from tobacco line T10. **E.** Roots from tobacco line T17.

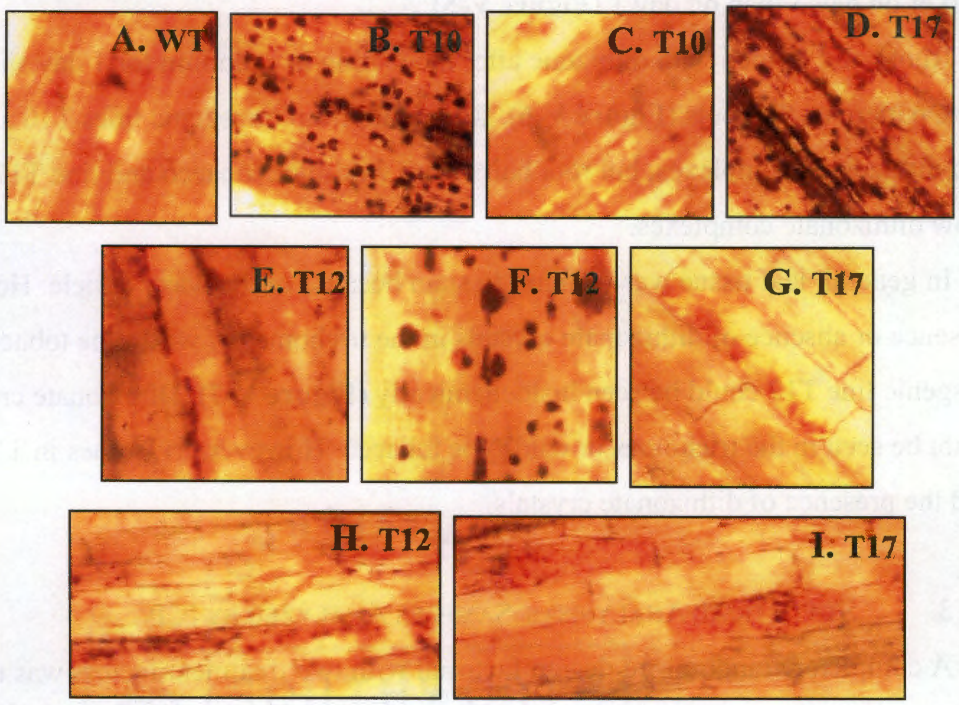


**FIGURE V-4: Dithizone staining of wild-type tobacco and tobacco lines T10, T12, and T17 incubated in cadmium for 1 day.** Figures **E** and **F** are at 20X magnification, which were at a higher light intensity than the other pictures taken. The other pictures are at 10X magnification. **A.** wild-type tobacco roots. Dithizonate was detected in tobacco tissue mainly as red colored lines corresponding to the region around the cell wall. The picture taken is the extreme color seen in wild-type tobacco. **B.** and **C.** T10 roots. Dithizonate was detected as black spots seen in the tissue or as reddish lines. The reddish lines were similar to those seen in the extreme cases of wild-type tobacco. The black coloration indicates a higher concentration of dithizonate than the red coloration. **D., G.,** and **I.** T17 roots. Dithizonate was sometimes detected as the black spots seen in **E.**, but the tissue was commonly seen as red spots within certain cells. These spots were throughout the cell (as in **I.**) or along the edge of the cell wall (as in **G.**). **E., F.,** and **H.** T12 roots. Dithizonate was detected as black spots as in T10 (**F.**), red spots as in T17 (**H.**), and red lines as in wild-type tobacco (**E.**). The red spots in T12 shown in **H.** appear along the edges of the cell wall as in T17 (**G.**). In addition, the black spots of dithizonate appeared within the cell at 20X magnification with the light intensity increased (**E.**). The black spots might be at the location of the nucleus since a majority of the cells appeared to contain one black spot per cell. Two black spots might be the nuclei from neighboring cells.

was not drastically different from day 1 even though tobacco tissue stained with dithizone was darker on day 3 than on day 1 (**Figure V-5**).

Root hairs stained with dithizone after incubating in cadmium for 1 day showed the same pattern as the roots (**Figure V-6**). Root hairs from transgenic lines T10 and T12 showed dithizonate complexes, but root hairs from wild-type tobacco and line T17 did not show dithizonate complexes.

In general, leaf tissue was difficult to stain because of the waxy cuticle. However, the presence or absence of dithizonate crystals in the trichomes of wild-type tobacco and of transgenic line T12 could be seen histochemically (**Figure V-7**). Dithizonate crystals could not be seen in the trichomes in wild-type tobacco. However, trichomes in T12 showed the presence of dithizonate crystals.

### 3. Statistical Analysis of Samples Analyzed by ICP

A completely randomized design factorial with two treatment factors was used to analyze the cadmium concentration in tobacco samples that had been calculated from the results obtained from ICP (**Table V-1**). Background (or blank) levels of cadmium could be detected in some of the replicates before the addition of cadmium, and variability within each replicated could be seen. Statistical difference was found in the effect of the tobacco line on cadmium concentration for roots ($p < 0.05$; **Table V-2**).), but the tobacco line did not affect cadmium concentration in shoots ($p > 0.7$). The effect of day or the interaction between the tobacco line and the day did not affect cadmium concentration in roots or shoots ($p > 0.9$). The least square means are the means for each treatment that had been adjusted for the other variables in the model. The least square means is used because the raw means would not be as accurate since unbalanced data were collected.

When statistical significance was found in the effect of tobacco line in root tissue, LSD mean separation was used to compare means to find means that were statistically different from each other (**Table V-3**). In LSD mean separation, a value is calculated, known as the least significant difference, which is used to compare two means. If the difference between the two means is greater than the least significant difference value, the means are considered statistically different. Assignment of identical letters between

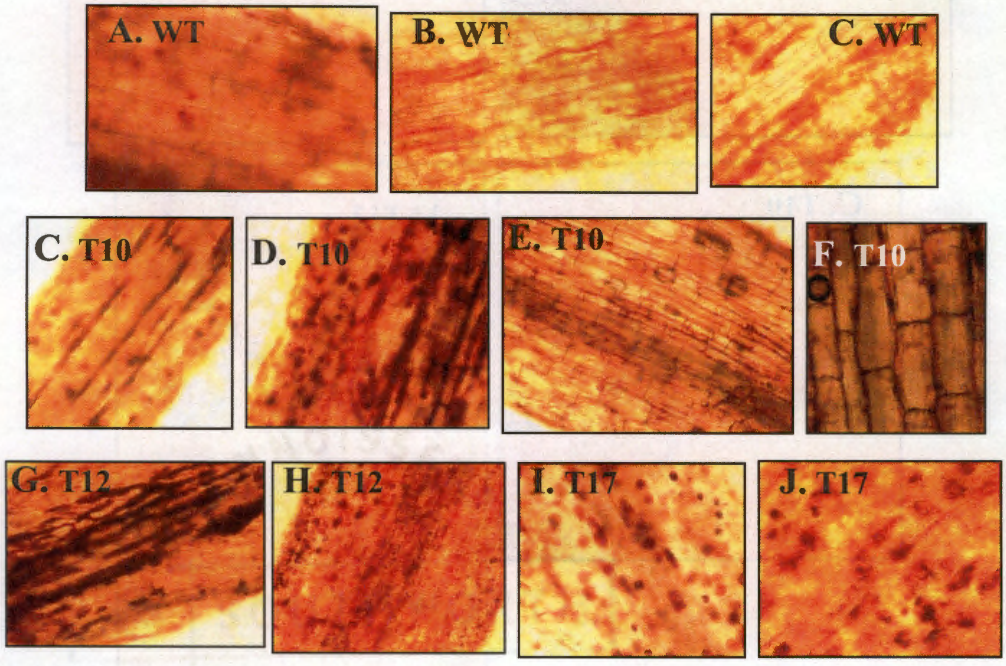


**FIGURE V-5: Dithizone staining of wild-type tobacco and tobacco lines T10, T12, and T17 incubated in cadmium for 3 days**. Figures **A.**, **B.**, and **C.** are at 20X magnification. The other pictures are at 10X magnification. **A.**, **B.**, and **C.** are wild-type tobacco. Dithizonate was detected in tobacco tissue as red colored lines as in day 1, but dark red spots were also seen. **D.**, **E.**, **F.**, and **G.** are T10 roots. Dithizonate was sometimes detected as the black lines (**D.** and **E.**) similar in shape to wild-type tobacco. The dark coloration indicates the presence of more dithizonate than seen in wild-type tobacco. In older tissue of T10 showing less dithizonate, the lines of dithizonate staining were clearly associated with the cell wall of each cell (**F.** and **G.**). **H.** and **I.** pictures of T12. In less mature regions, dark lines of dithizonate staining were detected, and dark red to black spots were detected in the older regions of tobacco roots. In general, the stain did not stain old tissue as well as young tissue. **J.** and **K.** are T17 roots. Dark red coloration of dithizonate was detected as spots in T17, but they were not as black as the other two transgenic lines.

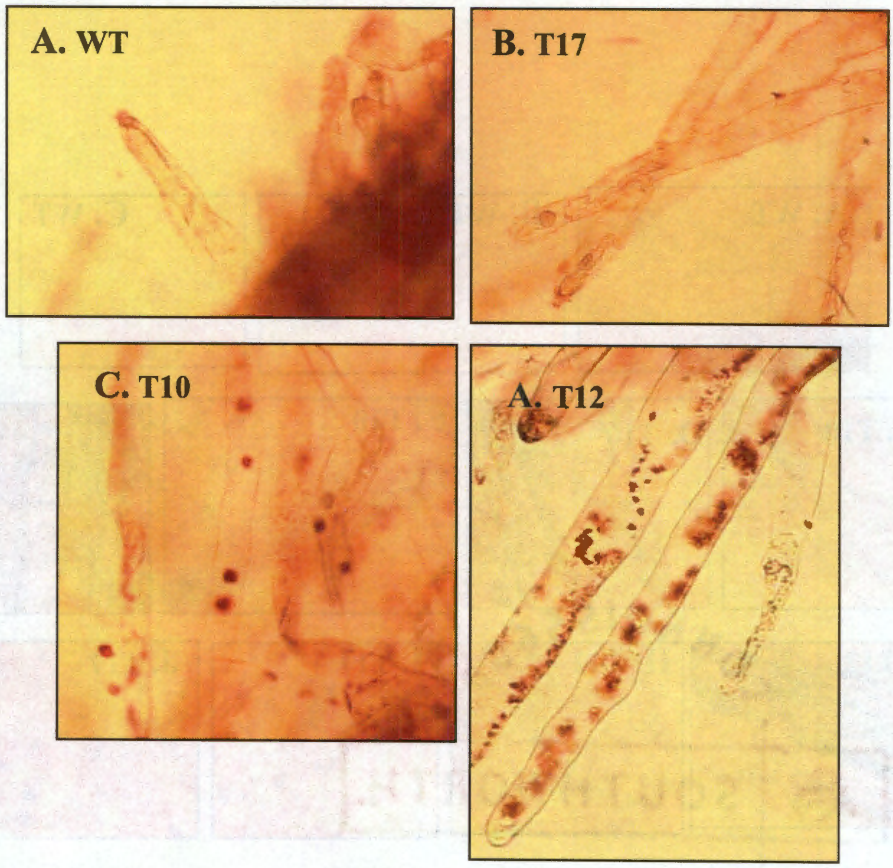


**FIGURE V-6: Dithizone staining of the root hairs of wild-type tobacco and tobacco lines T10, T12, and T17 incubated in cadmium for 1 day.** Pictures of the root hairs are at 20X magnification. **A.** Wild-type root hairs. Brown dithizonate crystals were not present in root hairs. **B.** T17 root hairs. As with wild-type tobacco, dithizonate crystals were not detected on day 1. **C.** T10 root hairs. Brown dithizonate crystals were present in many root hairs. However, the crystals were limited to one to two big spots in the root hairs. **D.** T12 root hairs. Several brown dithizonate crystals were present in many root hairs. Picture of **D.** was taken by Beth Mullin.

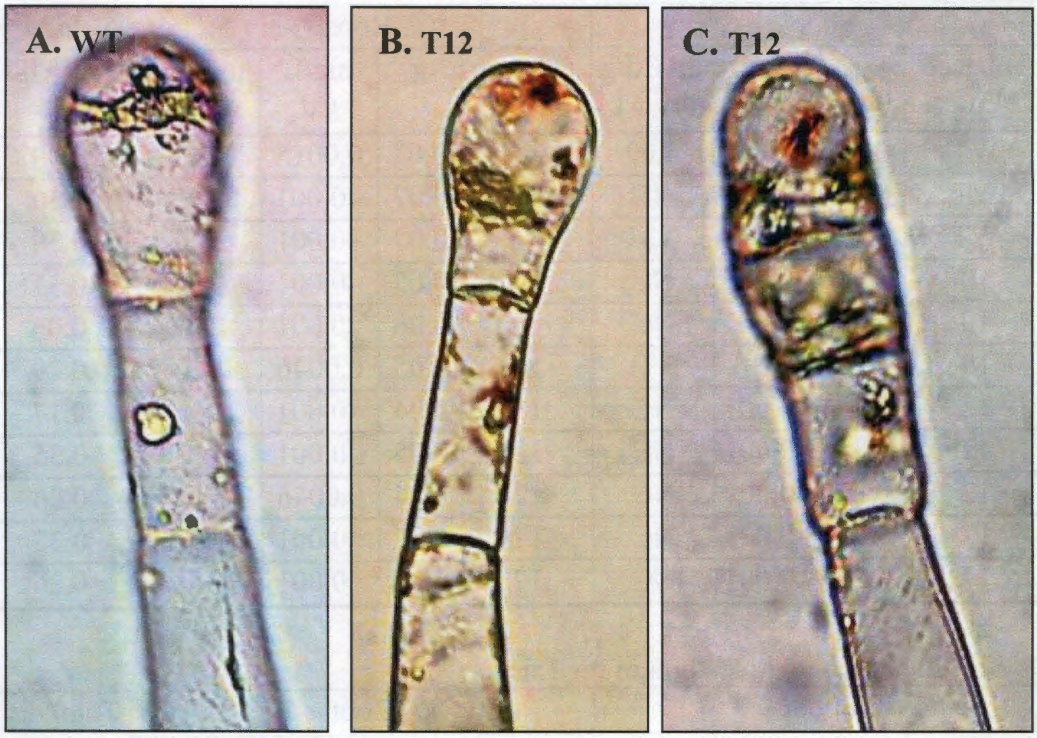


**FIGURE V-7: Dithizone staining in the trichomes of wild-type tobacco and tobacco lines T10, T12, and T17 incubated in cadmium for 1 day.** Pictures are at 20X magnification. **A.** wild-type tobacco trichome. Dithizonate crystals were not present in the trichome of wild-type tobacco. **B.** and **C.** T12 trichomes. In both pictures, a reddish black dithizonate crystal was detected at the tip of both trichomes. In addition, formation of a crystal was detected in one cell (**B.**) or two cells (**C.**) down from the top.

**TABLE V-1: Data collected from the cadmium uptake experiments of T10, T12, T17, and wild-type tobacco.** Blank areas indicate where sample was lost during processing, and NC indicates where no calculation was done because the amount of cadmium was below detection levels. **A.** Blank samples. **B.** Day 1 samples. **C.** Day 3 Samples.

**A.** Blanks – No incubation with cadmium.

| Shoot | | | | Root | | | | Total dry mass (g) |
|---|---|---|---|---|---|---|---|---|
| Tobacco Line | g | mg/L Cd | µg Cd/g dry wt. | Tobacco Line | g | mg/L Cd | µg Cd/g dry wt. | |
| WT | 0.41 | 0.017 | 4.09 | WT | 0.07 | 0.0015 | 2.14 | 0.48 |
| WT | 0.43 | <0.001 | NC | WT | 0.07 | <0.001 | NC | 0.50 |
| WT | 0.32 | 0.001 | 0.31 | WT | 0.07 | <0.001 | NC | 0.39 |
| 10 | 0.26 | <0.001 | NC | 10 | 0.05 | <0.001 | NC | 0.31 |
| 10 | 0.06 | <0.001 | NC | 10 | 0.06 | <0.001 | NC | 0.06 |
| 10 | 0.24 | 0.014 | 5.71 | 10 | 0.05 | 0.0046 | 9.2 | 0.29 |
| 12 | 0.13 | <0.001 | NC | 12 | 0.04 | <0.001 | NC | 0.17 |
| 12 | 0.30 | <0.001 | NC | 12 | 0.05 | <0.001 | NC | 0.35 |
| 12 | 0.22 | <0.001 | NC | 12 | 0.04 | 0.0046 | 11.5 | 0.26 |
| 17 | 0.30 | <0.001 | NC | 17 | 0.07 | <0.001 | NC | 0.37 |
| 17 | 0.23 | <0.001 | NC | 17 | 0.03 | <0.001 | NC | 0.26 |

**B.** Day 1 – Incubation with cadmium for 1 day.

| Shoot | | | | Root | | | | Total dry mass (g) |
|---|---|---|---|---|---|---|---|---|
| Tobacco Line | g | mg/L Cd | µg Cd/g dry wt. | Tobacco Line | g | mg/L Cd | µg Cd/g dry wt. | |
| WT | 0.30 | 4.879 | 1626 | WT | 0.06 | 4.880 | 8133 | 0.36 |
| WT | 0.19 | 0.988 | 520 | WT | 0.05 | 3.669 | 7338 | 0.24 |
| WT | 0.23 | 0.333 | 145 | WT | 0.08 | 3.334 | 4168 | 0.31 |
| WT | 0.16 | 0.311 | 194 | WT | 0.04 | 2.181 | 5453 | 0.20 |
| WT | 0.21 | 1.226 | 584 | WT | 0.05 | 6.070 | 12140 | 0.26 |
| 10 | 0.33 | 1.686 | 511 | 10 | 0.07 | 6.000 | 8571 | 0.40 |
| 10 | 0.16 | | | 10 | 0.03 | 5.860 | 19533 | 0.19 |
| 10 | 0.22 | 2.576 | 1171 | 10 | 0.05 | 8.280 | 16560 | 0.27 |
| 12 | 0.16 | 1.309 | 818 | 12 | 0.04 | 4.786 | 11965 | 0.20 |
| 12 | 0.16 | 1.275 | 797 | 12 | 0.02 | 4.126 | 20630 | 0.18 |
| 12 | 0.22 | 3.170 | 1441 | 12 | 0.05 | 7.290 | 14580 | 0.27 |
| 17 | 0.22 | 1.850 | 841 | 17 | 0.07 | 7.540 | 10771 | 0.29 |
| 17 | 0.29 | 3.421 | 1180 | 17 | 0.07 | 6.460 | 9229 | 0.36 |

**TABLE V-1:** Continued.

**C.** Day 3 – Incubation with cadmium for three days.

| Shoot | | | | Root | | | | Total dry mass (g) |
|---|---|---|---|---|---|---|---|---|
| Tobacco Line | g | mg/L Cd | µg Cd/g dry wt. | Tobacco Line | g | mg/L Cd | µg Cd/g dry wt. | |
| WT | 0.07 | 1.973 | 2819 | WT | 0.10 | 5.490 | 5490 | 0.17 |
| WT | 0.41 | 2.505 | 611 | WT | 0.13 | 7.110 | 5469 | 0.54 |
| WT | 0.24 | | | WT | 0.11 | 5.370 | 4881 | 0.35 |
| WT | 0.23 | | | WT | 0.04 | 3.280 | 8200 | 0.27 |
| WT | 0.34 | 1.094 | 322 | WT | 0.15 | 6.910 | 4607 | 0.49 |
| WT | 0.31 | 2.834 | 914 | WT | 0.08 | 9.360 | 11700 | 0.39 |
| WT | 0.18 | 1.618 | 899 | WT | 0.05 | 5.980 | 11960 | 0.23 |
| 10 | 0.77 | 7.460 | 969 | 10 | 0.16 | 14.360 | 8975 | 0.93 |
| 10 | 0.18 | 3.384 | 1880 | 10 | 0.04 | 8.140 | 20350 | 0.22 |
| 10 | 0.35 | 5.610 | 1603 | 10 | 0.08 | 12.110 | 15138 | 0.43 |
| 12 | 0.21 | | | 12 | 0.07 | 10.230 | 14614 | 0.28 |
| 12 | 0.17 | 0.976 | 574 | 12 | 0.02 | 4.792 | 23960 | 0.19 |
| 12 | 0.29 | 4.202 | 1450 | 12 | 0.11 | 11.400 | 10364 | 0.40 |
| 12 | 0.28 | 2.005 | 716 | 12 | 0.07 | 7.760 | 11086 | 0.35 |
| 12 | 0.22 | 10.440 | 4745 | 12 | 0.06 | 7.430 | 12383 | 0.28 |
| 17 | 0.20 | 2.646 | 1323 | 17 | 0.06 | 6.580 | 10967 | 0.26 |
| 17 | 0.21 | 3.691 | 1758 | 17 | 0.26 | 7.970 | 3065 | 0.47 |
| 17 | 0.43 | 2.635 | 618 | 17 | 0.13 | 16.080 | 12369 | 0.56 |

**TABLE V-2: ANOVA analysis of the concentration of cadmium in different tobacco plants.** The degree of freedom for each of the effects is listed. It calculated as n-1 for the main effects, where n is 4 for the effect of tobacco line and 2 for the effect of day. The effect of tobacco line includes all three transgenic lines and wild-type plants, and the effect of day includes day 1 and day 3. The degree of freedom for the interaction is calculated by multiplying the degree of freedom for tobacco and day together. The F value and the probability are given. A probability value less than 0.05 was considered significant, and the null hypothesis was rejected. The null hypothesis was one of the three hypotheses: there is no difference between the tobacco lines, there is no difference between the day, or there is no difference between the interaction between the two effects. **A.** ANOVA analysis of the shoot samples. Statistical difference is not detected in the effect of tobacco line, the effect of day, or the interaction of the two effects. **B.** ANOVA analysis of the root samples. Statistical difference is detected in the main effect of tobacco line in root samples.

**A.**

| Shoot | | | |
|---|---|---|---|
| Main effect or interaction | degree of freedom | F Value | P > F |
| Tobacco line | 3 | 0.48 | 0.70 |
| Day | 1 | 1.92 | 0.19 |
| Tobacco line x Day | 3 | 0.10 | 0.96 |

**B.**

| Root | | | |
|---|---|---|---|
| Main effect or interaction | degree of freedom | F Value | Pr > F |
| Tobacco line | 3 | 6.48 | 0.0024 |
| day | 1 | 0.14 | 0.72 |
| Tobacco line x day | 3 | 0.05 | 0.99 |

**TABLE V-3: Mean separation of tobacco line in roots and least square means.** The units are in μg Cd/g dry mass. **A.** Mean separation of the main effect of tobacco line in root samples. Similar letters in the letter group column showed similar means. The least squares mean and standard error were calculated using SAS and are averaged over both days. The means of tobacco lines T10 and T12 are significantly different from WT. **B.** Least square means and standard error of shoot samples. **C.** Least square means and standard error of root samples.

**A.** Root

| Tobacco Line | Least Square Mean | Standard Error | Letter Group |
|---|---|---|---|
| WT | 7459 | 1283 | C |
| 10 | 14855 | 1789 | AB |
| 12 | 15103 | 1600 | A |
| 17 | 9400 | 2000 | BC |

**B.** Shoot

| Tobacco | Day | Least Square Mean | Standard error |
|---|---|---|---|
| WT | 1 | 614 | 440 |
| WT | 3 | 1113 | 440 |
| T10 | 1 | 841 | 696 |
| T10 | 3 | 1484 | 568 |
| T12 | 1 | 1019 | 568 |
| T12 | 3 | 1871 | 492 |
| T17 | 1 | 1011 | 696 |
| T17 | 3 | 1233 | 568 |

**C.** Root

| Tobacco | Day | Least Square Mean | Standard error |
|---|---|---|---|
| WT | 1 | 7446 | 1959 |
| WT | 3 | 7472 | 1656 |
| T10 | 1 | 14888 | 2530 |
| T10 | 3 | 14821 | 2530 |
| T12 | 1 | 15725 | 2530 |
| T12 | 3 | 14481 | 1959 |
| T17 | 1 | 10000 | 3098 |
| T17 | 3 | 8800 | 2530 |

two means indicates that the means do not differ statistically while assignment of different letters indicates the means are statistically different (at the $p < 0.05$ level of significance). The maximum amount of cadmium in root tissue that was used to separate the means of the tobacco lines was 5550 μg Cd/g dry mass, which was the value calculated by SAS. The mean levels of cadmium in T10 and of T12 were statistically different from the mean level of cadmium in wild-type plants. The mean level of T17 was not statistically different from the mean of wild-type plants or of T10.

## D. CONCLUSIONS

### 1. T10 EXPRESSES AGNT84

Although T10 was the only tobacco line showing expression of AgNt84 RNA on a northern blot, T12 or T17 might still be expressing AgNt84. A possible reason for T12 and T17 not showing RNA expression might be because of the probe binding mainly to the pAgNt84 positive control that was by mistake overloaded on the gel. If the other transgenic lines contained weaker expression of AgNt84 than T10, the probe might not have bound at detectable levels. RNA degradation was evident in T17, and small RNA was not as visible in T12. It was possible that if these lines express AgNt84, AgNt84 RNA was degraded or was lost in the process of isolating RNA. Furthermore, a positional effect may prevent or lower the expression of AgNt84 in some types of tissue. Some of the plant tissue used for RNA extraction did not contain root tissue because of the small amount of tissue processed, so RNA isolation was not representative of all the tissue present for all the tobacco lines. Finally, the use of a small amount of tissue was not representative of the population of seeds. The tobacco seeds used for these experiments were T1 seeds, so some of the seeds did not contain the AgNt84 gene. The possibility exists that a tobacco plant not expressing AgNt84 might have grown on selection, and that plant might have been chosen for RNA analysis.

Another northern blot should be attempted where the root tissue and the shoot tissue of several plants are analyzed. Using several plants and isolating RNA from both roots and shoots would provide RNA that is more representative of all the tissue present.

Despite the need for another northern, one transgenic tobacco line did show expression of AgNt84, and that tobacco line was T10.

### 2. T10 AND T12 ACCUMULATED MORE CADMIUM

For many of the background samples, cadmium was below detection levels in tissues of all four tobacco lines. However, after one day of incubation in 200 μM $Cd(NO_3)_2$ there was an increase in the amount of cadmium present in the plants. The least square means for the effect of tobacco line over all three days in the root tissue were 7,459 μg Cd/g dry mass for wild-type tobacco, 14,855 μg Cd/g dry mass for T10, 15,103 μg Cd/g dry mass for T12, and 9,400 μg Cd/g dry mass for T17 (**Table V-3**). In increasing order, the amount of cadmium in the different tobacco lines is wild-type, T17, T10, and T12. The means for T10 and T12 were double that of wild-type tobacco, and T17 was roughly halfway between wild-type and the transgenic lines T10 and T12. The expression of AgNt84 has yet to be confirmed in T12 for several reasons already mentioned. If T12 does not express AgNt84, the cause for the increase in accumulation of cadmium in the transgenic lines T10 and T12 could not be attributed to AgNt84.

Although the differences were not found to be significant in the shoot tissue, the means for the transgenic tobacco were higher than that of wild-type tobacco. On day 3, the means of each tobacco line were 1,113 μg Cd/g dry mass for wild-type, 1,484 μg Cd/g dry mass for T10, 1,871 μg Cd/g dry mass for T12, and 1,233 μg Cd/g dry mass for T17. However, the standard error was great, and statistical differences were not evident. It may be that if more plants were tested, the presence of statistical difference would be detected because the standard error would be lessened, allowing the detection of potential differences between the tobacco lines.

In a short-term cadmium uptake experiment using tobacco hairy roots, Nedelkoska and Doran (2000b) report that the amount of cadmium taken up in the roots is around 1,000 μg Cd/g dry mass after a 9 hour incubation in MES buffer containing about 178 μM cadmium. When the plants are incubated in MS medium containing around 89 μM cadmium, the roots accumulate approximately 8,000 μg Cd/g dry mass within a week (Nedelkoska and Doran 2000b). The hairy roots Nedelkoska and Doran

(2000b) use are from wild-type tobacco plants transformed with an *A. rhizogenes* strain, and they do not contain a transgene. The amount of cadmium taken up by the root tissue of wild-type tobacco used in the experiments described in this thesis was similar to that reported by Nedelkoska and Doran's (2000b) for long-term uptake experiments. The T10 and T12 tobacco lines accumulated twice the amount reported by Nedelkoska and Doran. Nedelkoska and Doran (2000b) also report on cadmium uptake by *Thlaspi caerulescens*, and the amount of cadmium found in *Thlaspi* after incubation with cadmium for 30 days is between 10,000 and 11,000 μg Cd/g dry mass. The amount found after 9 hours is roughly 1700 μg Cd/g dry mass.

Dhankher et al. (2003) reports that expression of bacterial arsenate reductase in tobacco increased the amount of cadmium present in the shoot tissue from 300 μg Cd/g dry mass to 450 μg Cd/g dry mass and from 1,300 μg Cd/g dry mass to 1,750 μg Cd/g dry mass in the root tissue, and the transgenic plants were statistically different from the wild-type plants. The estimated means listed by Dhankher et al. (2003) are less than the means in the present experiment even though the plants in that experiment were grown for a week in media containing cadmium. The means of the transgenic plants containing AgNt84 were sometimes as high as ten times the amount reported by Dhankher et al. (2003). Wild-type plants were sometimes close to the estimated means Dhankher reports, but the highest means seen in the present experiment were five to ten times higher than those reported by Dhankher et al. (2003).

Although the transgenic plants would have different metal-binding characteristics based on the transgene that they expressed, the amount of cadmium present in wild-type plants used in this experiment and in Dhankher et al. (2003) should be similar. This leads to the possibility that errors in processing the tissue may have contributed to an increase in the amount of cadmium reported in this experiment. Dhankher et al. (2003) use Clark's medium in their experiments, and it is possible that the use of Clark's medium dramatically decreases cadmium uptake making it difficult to compare the results of the two studies. The conditions used in this experiment do not reflect field conditions. In a field study in the Alps, it is found that soils contaminated with cadmium contained 37 μg Cd/g of soil (Wenzel and Jockwer 1999). *Thlaspi rotuntifolium* in this soil accumulates

108 μg Cd/g dry mass in the shoot, and the roots accumulate 53.9 μg Cd/g dry mass (Wenzel and Jockwer 1999). In the field, cadmium would be competing for uptake and binding with other ions that have greater concentrations, and the concentration of bioavailable cadmium in the soil would be substantially less than that used in this experiment. The purpose of this experiment was to determine if the accumulation of cadmium increased in plants expressing AgNt84 and it appears to have done so.

### 3. Dithizone Staining of Tobacco Supports Statistical Results

The results of dithizone staining are consistent with those of ICP analysis. T10 and T12 tobacco lines were similar in color when stained with dithizone and both stained more intensly than T17 and wild-type, suggesting that they accumulated more cadmium. The staining of T17 was darker than wild-type tobacco but lighter than the other two tobacco lines. The means in μg cadmium/g dry mass of T10 and T12 roots were higher than the means of wild-type tobacco while the mean of T17 was similar to T10 and wild-type. The staining of day 1 and day 3 plants was similar, and there were no differences in the means of day 1 and day 3 in the statistical analysis of the ICP results. Not seeing a visual or statistical difference between the day 1 and day 3 might indicate that no more cadmium could be taken up by the plants after day 1 or that the amount of cadmium in the solution was limiting.

The use of dithizone as a histochemical marker for the location of cadmium in tobacco was not without problems. Dithizone did not readily stain the shoots of the plant and root tissue showed variation in staining pattern and intensity, probably due to the inability of dithizone to reach all the cells in the tissue. The use of a whole root created problems because several cell layers were out of the plane of focus when observing the roots in the microscope, and coloration could not be completely differentiated as to the plane of focus it belonged to. The variation in staining among cells was also problematic because a cell would stain darker than its neighboring cells for unknown reasons. Many of these problems would be solved if a single layer of cells, or the BY-2 cells expressing AgNt84 were used instead of whole tobacco plants.

### 4. Potential Reasons Why a Statistical Difference Was Detected in Root Tissue But Not in Shoot Tissue

It is reported that over three fourths of the cadmium taken up by tobacco plants is transferred to the shoot tissue (Dorlhac de Borne et al., 1998). Therefore, the effect of tobacco line in the analysis of shoot tissue should have shown statistical significance, but it did not. Dithizone staining of the trichomes reveals one possible explanation for lack of statistically significant differences in the amount of cadmium accumulated in shoot tissue. Choi et al. (2001) finds that tobacco produces cadmium-containing crystals that are excreted from the trichome. Therefore, it is likely that cadmium was being excreted from the heads of the trichomes as crystals, and the excretion would have reduced the amount of cadmium present in the leaf tissue. The crystals would have fallen off of the plant, and the cadmium in the crystals would not have been measured. The crystals may have fallen onto other leaves and dropped off the leaves as the plants were being rinsed. Some crystals may have fallen into the MES buffer containing cadmium. If the crystals could be dissolved, the plants could have taken up cadmium again. However, if the crystals were not soluble, the crystals would remain in the MES buffer. This would give an inaccurate representation of how much cadmium was moved to the shoot because the quantity measured would be much lower than what was actually moved.

Formation of cadmium crystals in the trichomes might have contributed to the changes in the amount of cadmium recorded, but this reason could not completely explain the results. In Dorlhac de Borne et al. (1998) tobacco plants expressing a metallothionein gene show a reduction in cadmium in the leaf tissue. The transgenic plants show a slight increase in cadmium in root tissue although the percentage of cadmium in roots remains below 25 percent (Dorlhac de Borne et al., 1998). Approximately half of the cadmium is located in the stem of the transgenic plants whereas wild-type plants contain the cadmium in the leaf tissue (Dorlhac de Borne et al., 1998). It is possible that the metallohistin protein AgNt84 may have accumulated more cadmium in the root tissue than in the shoot tissue like the metallothionein protein, preventing its movement to the leaves.

The shoot tissue in the plants used for this experment did not contain most of the cadmium that was taken up. In Table V-1, the last replicate of the T10 shoot and root

samples for day 1 can be used to illustrate this point. On day 1, the dry mass of this replicate was 0.22 g and 0.05 g for shoot and root tissue, respectively. The amount of cadmium taken up by the plant was 1,171 and 16,560 μg Cd/g dry mass in the shoot and root tissue. Therefore, the amount of cadmium from day 1 that was taken out of the hydroponic container was 258 μg for the shoot tissue, and the amount of cadmium from day 1 that was taken out of the hydroponic container was 828 μg for the root tissue. In total, 1,086 μg was taken up in the plants selected for day 1. If the shoot tissue contained approximately 75% of the cadmium as reported by Dorlhac de Borne et al., 1998), 828 μg of cadmium should be found in the shoot tissue instead of in the root tissue. There is a possible explanation for why in this case, so much lower levels than predicted were found in shoot tissue. In the hydroponic system designed for the cadmium uptake experiments, plants were grown in a closed container where humidity was high. Movement of cadmium from the roots to the shoots involves transport in the xylem and is heavily influenced by transpiration rates, which were greatly reduced in the humid containers. Had the tops of the plants been out of the containers, they would have transpired at a higher rate and uptake of cadmium to the shoot tissue would probably have increased.

Several factors might have contributed to the large standard errors found in the calculated cadmium concentrations. The dry mass of the root tissue was extremely low, increasing the difficulty of accurately measuring the mass and as a result these samples would have greater variation than would be the case with larger sample sizes. Second, processing the plant tissue through several rinses may have damaged some of the vasculature of the shoots and roots, preventing uniform uptake of cadmium.

Increasing replication would likely decrease the standard error of the means. Small differences in the accumulation of cadmium in shoot tissue of wild-type and transgenic tobacco might exist but might not have been shown as being statistically significant because of the large standard error. More samples would increase statistical power, allowing small differences to be detected.

However, the most important factor contributing to variation may be that the tobacco plants are of the T1 generation. Some plants were much larger in shoot mass than the other counterparts in a sample of four plants, yet these same plants would have a

similar mass in root tissue. How would a heterozygous plant differ from a homozygous plant with two genes for AgNt84, and how would this affect the shoot mass? Another question that remains is what effect does tobacco plant size have on the concentration of cadmium per gram of dry mass? Two plants may be accumulating cadmium at the same rate, but a bigger plant would have less cadmium for every gram of dry mass. In a hypothetical example, two plants accumulate cadmium at 5 μg Cd/min. After a minute, a plant with a dry mass of 0.10 g would contain 50 μg Cd/g dry mass whereas a plant with a dry mass of 0.20 g would contain 25 μg Cd/g dry mass. However, a larger plant may be able to accumulate cadmium at a different rate.

Besides these possible variables, two other questions were raised after the experiments were completed. What effect does plant number have on the availability of cadmium to each plant? Some of the replicates initially were able to fit more plants in the disk, but problems with growing the tobacco plants occurred. The germination rate of tobacco line T10 was much less than any of the other tobacco lines, and the three transgenic tobacco lines had problems with fungus on the seed coat reducing the number of seeds available for study. These problems limited the number of plants used in the cadmium uptake experiment. Another question raised was what effect does adding roots that are growing above the disk to the sample have on calculated cadmium concentration in the root? Tobacco easily grew adventitious roots on the stem of a plant lying horizontally, but the roots were not submerged as completely as the primary and secondary roots. Uptake of cadmium would most likely be at a different rate in the adventitous roots. The use of hairy root cultures could solve these potential problems.

### 5. Implications for the Role of AgNt84 in the Nodule of *Alnus*

AgNt84 has several possible functions in *Alnus glutinosa* nodules. The functions are sequestering metals from *Frankia* as a means to control growth, helping *Frankia* with symbiosis by giving the bacteria metals, or supporting the cell wall structurally. Two of the hypothetical functions are based on the metal-binding properties of AgNt84. The tobacco line expressing AgNt84 showed a significant increase in the amount of cadmium present in the root tissues, which may mean that one of the first two hypotheses is true. In

a review on the physiology of cadmium in higher plants, one of the processes that cadmium is thought to negatively affect is nitrogen metabolism (Seregin and Ivanov 2001). If cadmium does bind to AgNt84, another role of AgNt84 might be to protect the process of symbiosis by sequestering metals that could damage the enzymes involved in the process of symbiotic nitrogen fixation. These possibilities could only be tested in an actinorhizal plant.

## 6. Possible Future Experiments

An important experiment to repeat is the northern blot. If T12 does not express AgNt84, what could be the possible explanation for the significant increase in cadmium in this line? One possible explanation for the increase in cadmium in T12 is that AgNt84 inserted into a region that enhances the expression of another metal-binding protein and that AgNt84 itself was silenced.

More cadmium uptake experiments should be done. These experiments should include using plants from transgenic lines whose seeds would be homozygous for the AgNt84 gene to prevent problems with heterogeneity of the sample. In addition, there was no difference between day 1 and day 3 within the tobacco lines. This lack of difference might be because the plant could not take up more cadmium or because all of the cadmium was taken up by day 1. Concentrations of cadmium that are more than and less than 200 μM should be used to measure a difference in metal uptake in wild-type tobacco and T10 on day 3. More than three replicates should be done to reduce variation. In addition, experiments should be designed to differentiate cadmium-binding occurring outside the cell from cadmium sequestration inside the cell. Finally, these experiments should be tried using nutrient medium rather than MES buffer because the medium would be close to what is occurring in nature. Plants would have nutrients for growth, and the ions that would complex with cadmium would be present in the medium. Ift AgNt84 binds more cadmium in this environment, this information would be worthwhile finding out if AgNt84 can be used for phytoremediation.

A truncated form of the protein was found to bind other metals *in vitro*. Experiments involving other metals may show more of an increase in uptake in

transgenic tobacco than wild-type tobacco. Alternatively, metal uptake might be shown to be the same in transgenic and wild-type tobacco. These experiments may help in understanding the role of AgNt84.

Finally, the use of tobacco plants has several advantages in experiments designed to determine whether uptake of cadmium is increased in transgenic plants expressing AgNt84. Tobacco is a fast-growing plant that can be easily transformed despite the difficulties encountered in this work. If the results obtained in this work can be confirmed, in future experiments transforming high biomass producing, hyperaccumulating plants with AgNt84 should be a major goal that may lead to the development of improved phytoremediating systems.

# CHAPTER VI

# CONCLUSION

## A. SEVEN BINARY PLASMIDS

All seven binary plasmids were constructed and cloned into *E. coli* strain DH5α. The presence of the correct plasmid in *E. coli* was confirmed by restriction enzyme digestion of each of the seven plasmids, and the presence of BEAN1 was verified further by DNA sequencing of the AgNt84 gene. Substantiating by sequencing that the plasmid is correct was not done for any of the other six plasmids.

## B. *AGROBACTERIUM* AND PLANT TRANSFORMATION

Seven binary plasmids were used to transform LBA4404, AR10, and A4RS. No plasmids were transformed into A4RS. The transformation of the correct plasmid was verified by back transformation into *E. coli*. Colonies of *E. coli* did not grow when transformed with pBEAN1 or pBEAG1, and the plasmid isolated from the backtransformation of *E. coli* with pBCAN1 did not show the banding pattern of pBCAN1 digested with restriction enzymes. The pattern was that of pBCAG1 or pBCAGE1. The glycerol cultures from LBA4404 transformation with pBCAN1 were discarded. As for AR10, colonies grew when transformed with pBCAN1, pBCAG1, or pBCAGE1. *E. coli* was backtransformed with pBCAG1, and the correct banding pattern of pBCAG1 was present.

Particle bombardment of onion epidermal cells gave promising results. The expression of AgNt84 fusion protein was visualized for each of the four plasmids pBCAG1, pBCAGE1, pBEAG1, and pBHAG1. In addition, AgNt84-GFP was seen in the endoplasmic reticulum, which means that the protein might be targeted extracellularly. Proteins targeted to the extracellular matrix go through the endoplasmic reticulum and the Golgi complex before going to the plasma membrane. Expression of AgNt84-GFP was

not cytosolic. Transient transformation by agroinfiltration showed that AR10 (R24E7) could transform tobacco.

BY-2 cells were transformed with each of the seven binary plasmids, but expression of AgNt84 or AgNt84-GFP was not confirmed by RNA analysis. The expression of AgNt84-GFP was detected by fluorescence microscopy. In BY-2 cells transformed with AgNt84-GFP, the fusion protein could be clearly be detected within the cell, but was not seen in the cell wall. The absence of fluorescence in the cell wall did not exclude the possibility that the protein is targeted extracellularly since it was possible that GFP may not fluoresce outside the cell. It was also not known if AgNt84-GFP was located in the plasma membrane because of the refraction of light along the membrane. Proteins within the Golgi are thought to have two destinations, the vacuolar compartment or the plasma membrane. This suggests that AgNt84 might be targeted extracellularly because there is no evidence for its presence in vacuoles. However, as discussed by Tamura et al. (2003), conditions within plant vacuoles are not compatible with GFP fluorescence because GFP is rapidly degraded. Tamura et al. (2003) made a GFP mutant that is more stable in the vacuole, and using GFP mutants such this may help in visualizing the subcellular location of AgNt84.

Attempts at leaf disk transformation using *A. tumefaciens* were not successful because the leaf tissue died before transgenic plants could be regenerated. Expression of GFP was visualized by fluorescence microscopy in hairy roots transformed with AR10 pBCAG1. In addition, hairy roots were seen in tobacco plants transformed with 15834. The hairy roots arose from the leaves of wild-type tobacco and of the transgenic lines T10, T12, and T17. However, *Agrobacterium* overgrowth killed the tobacco roots, and the hairy roots were lost.

Using whole tobacco plants from the transgenic lines T10, T12, and T17 for cadmium uptake experiments was an alternative to the failed attempts at stable transformation of tobacco.

## C. CADMIUM UPTAKE EXPERIMENTS

Northern analysis of wild-type tobacco, T10, T12, and T17 showed that T10 was the only tobacco line in which mRNA for AgNt84 could be detected. Since concerns about the possible lack of expression in T12 and T17 exist, Northern analysis should be repeated to check for expression of AgNt84 in T12 and T17.

Plants hydroponically grown for 2 weeks were used for cadmium uptake experiments. The plants were from the three transgenic lines of tobacco as well as wild-type tobacco. The plants were incubated for up to 3 days in MES buffer with 200 μM $Cd(NO_3)_2 \cdot 4H_2O$. Samples were taken at day 0, 1, and 3. Samples were also separated into shoot tissue and root tissue. The cadmium concentration in plant tissue was analyzed by ICP analysis and visualized by dithizone staining. A completely randomized design factorial with two treatment factors was used to determine statistical differences in the cadmium concentrations as determined by ICP analysis. The two treatment factors were day and tobacco line.

In shoot tissue, no statistical differences were seen. In root tissue, statistical differences were evident in the effect that the tobacco line had on cadmium concentration. LSD mean separation was used to compare means for statistical difference. In root tissue, the statistical differences of tobacco line occurred in lines T10 and T12, and these lines showed an increase in cadmium uptake over T17 and wild-type tobacco. In the root tissue, the amount of cadmium in T10 and T12 was double the amount of cadmium in wild-type tobacco, and the amount of T17 was roughly halfway between that of wild-type tobacco and the other transgenic lines.

Dithizone staining of tobacco tissue was consistent with the results that were found by ICP analysis. T10 and T12 roots stained darker than T17 or wild-type roots. Also, day 1 roots were not drastically lighter in color than roots from day 3 plants.

Although further research needs to be done to support or reject the statistical difference found in this experiment, AgNt84 has the potential to be introduced into hyperaccumulators or high biomass plants to improve phytoremediation because a statistical difference was seen in tobacco roots expressing AgNt84. If AgNt84 does bind cadmium, it may bind other metals that have been shown to bind to AgNt84 *in vitro*.

Multiple binding of metals is a useful characteristic of a protein that may be exploited in phytoremediation because many contaminated sites do not contain only one metal. AgNt84 may be used in combination with other metal-binding proteins to optimize a plant's metal accumulating abilities.

## D. LESSONS LEARNED

The most important lessons learned were as a result of attempts to transform tobacco with *Agrobacterium rhizogenes*. *A. rhizogenes* is reported to be a fast means to obtain transgenic plant tissue, and this may be true in most systems, but it is not true for tobacco transformation. In tobacco, regeneration of plants following *A. tumefaciens* transformation takes around six weeks. In a plant system that has an easy and fast transformation system, a system that is not as widely used like *A. rhizogenes* transformation should be avoided unless absolutely necessary. The main problem encountered in this research when attempting to use *A. rhizogenes* for transformation was generating transformed roots, since untransformed roots can appear on the plant tissue faster than transformed roots and since antibiotics do not select against untransformed tissue as well as might be desired. Medina-Bolívar and Cramer (2004) even recommend against using *A. rhizogenes* to directly transform tissue because of selection problems. The knowledge gained from trying *A. rhizogenes* transformation allowed an understanding of what should and should not have been done. Transformation with *A. rhizogenes* should not have been attempted despite the benefits of using hairy roots in metal uptake experiments.

Further, a misunderstanding of what plant lines had been checked for expression by northern analysis created more work when setting up and running the cadmium uptake experiments. It had been understood that transgenic tobacco lines and *Arabidopsis* lines were both checked for expression of AgNt84 when in fact, expression had only been cohecked in *Arabidopsis*. As a result, lines that did not show expression of AgNt84 were used in cadmium uptake experiments when it was not necessary to include these lines in the uptake experiments. This misunderstanding cost a lot of time that could have been placed in other efforts. It would have been beneficial to study two transgenic lines

expressing AgNt84 to confirm that the metal uptake was not because of a positional effect. Instead, a line expressing AgNt84 (T10) and a line that might express AgNt84 (T12) showed an increase in cadmium uptake. With *Arabidopsis*, two expressing lines could have been chosen and studied in cadmium uptake experiments.

Finally, discussing the progress and problems of ongoing research with a broad range of persons can be helpful in many ways. Early problems with BY-2 transformation reinforced the importance of seeking help from people who are more experienced at a particular system or technique. In addition, the use of transient methods to check for GFP expression came from the recommendation of other people. The method of *A. rhizogenes* transformation that was finally successful came from contacting someone at another university who was working on hairy root transformation of tobacco.

## E. SUGGESTIONS FOR FUTURE EXPERIMENTS

Several goals could be considered for future experiments. These goals are:

- to sequence the binary plasmids used for transforming plant tissue
- to transform tobacco by leaf disk transformation using an *A. tumefaciens* strain
- to check expression of transformed BY-2 cells
- to use stable transgenic lines, such as BY-2 cells, in experiments that would support or reject extracellular targeting, such as preventing secretion of extracellular proteins to see if the AgNt84 protein fusion would accumulate in the cell
- to repeat agroinfiltration to gain more information about the subcellular localization of AgNt84 by using confocal microscopy
- to repeat the cadmium uptake experiments with homozygous transgenic tobacco lines expressing AgNt84
- to try other concentrations of cadmium to determine the metal uptake at a concentration that does not reach the limit of uptake after 1 day
- to try other metals to find out if these metals would show a difference in uptake in the root or shoot

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# APPENDICES

## A. BUFFERS AND SOLUTIONS

### 1. TE BUFFER

10 mM Tris-Cl (Trizma hydrochloride, Sigma), pH8.0

1 mM EDTA, pH 8

buffer autoclaved as with bacterial media (Appendix B)

(Maniatis et al., 1982)

### 2. 0.5 M EDTA

186.1 g of disodium EDTA dihydrate was added to 800 mL $H_2O$

Solution stirred vigorously while adjusting pH to8.0 with NaOH

Solution autoclaved as with bacterial media (Appendix B)

(Maniatis et al., 1982)

### 3. MINI-PREP SOLUTIONS

#### 3. A. SOLUTION W

50 mM Tris-HCl, pH 8.0

10 mM EDTA, pH 8.0

100 µg/mL of RNaseA (Sigma)

#### 3. B. SOLUTION P

0.2 N NaOH (Mallinckrodt)

1% SDS (Fisher)

#### 3. C. SOLUTION T

1.32 M potassium acetate ($KC_2H_3O_2$, Mallinckrodt)

pH adjusted to 4.8

4. TAE BUFFER

40 mM Tris-acetate

2 mM EDTA

A 50X stock solution was prepared with:

242 g/L Tris base (Trizma base, Sigma)

57.1 mL/L glacial acetic acid (Mallinckrodt)

100 mL/L 0.5 M EDTA

(Maniatis et al., 1982)

5. NORTHERN BLOT

5. A. 20X MOPS BUFFER

4.18g MOPS

0.65g sodium acetate

2mL 0.5 M EDTA

DEPC water to 50 mL

pH is approximately 7.0

5. B. SSC

20X SSC:

175 g NaCl

88.2 g sodium citrate, water to 1L

pH is approximately 7.0

6 X SSC:

300 mL 20X SSC

700 mL water

### 5. C. Denhardt's Solution

50 X Denhardt's solution:

1%Ficoll type 400

1% polyvinylpyrrolidone

1% bovine serum albumin

Filter sterilize and store at -20C.

### 5. D. Prehybridization Buffer

6 X SSC

0.5% SDS

5X Denhart's

100 µg/mL denatured fragmented salmon sperm DNA

### 5. E. Hybridization Buffer

6 X SSC

0.5% SDS

100 µg/mL denatured fragmented salmon sperm DNA

### 5. F. Wash Solutions

Wash solution 1:

1X SSC

0.1% SDS

Wash solution 2:

0.5 X SSC,

0.1% SDS

Wash solution 3:

0.1 X SSC,

0.1% SDS

Prewarm solutions to 65°C before use

## 6. Dithizone Solutions

### 6. a. Stain Solution

6 ml of acetone (Acros)

2 ml $ddH_2O$

2 drops of glacial acetic acid (Mallinckrodt)

4 mg dithizone (diphenyl thiocarbazone, Fisher)

Seregin et al. (2004)

### 6. b. Destain Solution

30 ml of acetone (Acros)

10 ml $ddH_2O$

10 drops of Glacial Acetic Acid (Mallinckrodt)

## 7. 5 mM MES Buffer

5 mM MES containing 200 µM $Cd(NO_3)_2 \cdot 4H_2O$ buffer was prepared as 200 mL:

195 mg MES (Fisher)

400 µg of 100 mM stock of $Cd(NO_3)_2 \cdot 4H_2O$ (Aldrich)

pH adjusted to 5.8

100 mM stock of $Cd(NO_3)_2 \cdot 4H_2O$ was equivalent to 30.847 g of $Cd(NO_3)_2 \cdot 4H_2O$ per liter of $ddH_2O$

For 5 mM MES buffer without cadmium, the cadmium had been omitted.

## B. Reactions and Protocols from Invitrogen and Products from Fisher

### 1. PCR Reaction

The products used in the PCR reaction came from Fisher products, with the exception of the primers. The primers were made by Integrated DNA Technology, Inc.

To make a 50-µL sample to use in PCR, the following was mixed in the following order: water, 10X ExTaq Buffer, dNTP, Primers, DNA, and ExTaq Enzyme. The amount of water used depended on the amount necessary to make the volume 50 µL. 5 µL of both the 10X ExTaq Buffer and the dNTP was added to the sample. The amount of primer added depended on the pmole range of the ends of the primer, and the range was 5 to 50 pmoles. Usually, the concentration of the primers was made as 5 µM, and 1 µL was added to the sample. The amount of DNA added ranged between 10 and 100 ng/µL, and 1 µL was added. 1 µL of ExTaq Enzyme was added to the reaction. The sample was mixed and briefly centrifuged. Mineral oil was added on top prior to placing the sample in the Thermocycler.

The following conditions were programmed in the Thermocycler for running the PCR:

| | | | |
|---|---|---|---|
| Initial Denaturation | | 94°C | 5 minutes |
| 30 Cycles: | Denaturation | 94°C | 1 minute |
| | Annealing | 65°C | 1 minute |
| | Extension | 72°C | 1 minute 30 seconds |
| Final Extension | | 72°C | 7 minutes |

Temperature held at 4°C until the samples were taken out.

The PCR samples were cleaned using the Quiagen PCR Clean-Up Kit.

### 2. The BP and LR Reactions

The vectors, enzymes, bacteria, reaction buffers, and protocol were from Invitrogen. The BP and LR reactions were the same, except for the solutions. In the BP reaction, the following was added to a microcentrifuge tube and mixed: 40-100 fmol of

the PCR product, 2 μL of 150-ng/μL pDONR/Zeo vector, and 4 μL of 5X BP Clonase reaction buffer. TE Buffer (pH 8.0) was added to make the total volume 20 μL. The BP Clonase enzyme was vortexed briefly for about 2 seconds and added to the above solution. For the LR reaction, the following was added to microcentrifuge tube at room temperature and mixed: 100-300 ng of the entry clone, 150 ng of the destination vector, and 4 μL of 5X LR Clonase reaction buffer. TE Buffer (pH 8.0) was added to make the total volume 20 μL. The LR Clonase enzyme was vortexed for 2 seconds, and 4 μL of the enzyme was added to the above microcentrifuge tube.

At this stage, the two reactions were treated similarly. The microcentrifuge tube was vortexed two times for 2 seconds each time. The tube remained on the lab bench for 1 hour, or the tube remained on the lab bench for up to 18 hours. To stop the reaction 2 μL of 2 μg/μL of Proteinase K solution was added to the microcentrifuge tube, and the tube was placed in a 37°C hot water bath for 10 minutes. *E. coli* strain DH5α was transformed with either the solution from the BP reaction of the solution from the LR reaction.

3. HEAT SHOCK

The cells and protocol were from Invitrogen. The competent cells were thawed on ice, and 50 μL of the competent cells were transferred into a microcentrifuge tube. 1 μL of DNA was added to the competent cells, and the tube was gently mixed by tapping the bottom and then placed on ice to incubate for 30 minutes. The microcentrifuge tube was then transferred to a 42°C water bath for 30 seconds to heat-shock the cell. The water bath was not shaking while the tube was being heat shocked. The tube was moved out of the water bath and placed on ice. 450 μL of room temperature SOC media was added to each microcentrifuge tube. The tube was parafilmed to prevent leakage and then taped to the bottom of the shaker. The tubes were incubated at 37°C for 1 hour while shaking at 225 rpm.

4. LIGATION REACTION

T4 DNA ligase and the protocol for the ligation reaction were from Invitrogen. The following were combined together:

4 µL 5X ligase reaction buffer

1 µL pAN59 digested with XbaI and BamHI

1 µL AgNt84 PCR product digested with XbaI and BamHI

13 µL filtered and autoclaved $ddH_2O$

1 µL T4 DNA ligase

The ligation incubated at room temperature for 1 hour, and the ligation products were transformed into DH5α as above.

5. RNA EXTRACTION

The RNA reagent and protocol were from Invitrogen. The tube was taken out of the liquid nitrogen, and 500 µL of cold Concert Plant RNA Reagent (Invitrogent) was added to each microfuge tube of plant tissue. Each tube was mixed for 10 seconds using the ivoclar machine and then placed horizontally on the lab bench for 5 minutes. The tubes were centrifuged at 12,000 x g for 2 minutes, and the supernatant was moved to a new microfuge tube. The pellet was discarded. 100 µL of 5 M NaCl was added to the supernatant, and each tube was mixed by tapping the bottom of the tube. 300 µL of chloroform was then added to each tube, and the tube was mixed by inverting the tube several times. All the microfuge tubes were loaded into a microcentrifuge, and the tubes were centrifuged at 12,000 x g for 10 minutes. The top phase of each sample was transferred to a new microfuge tube, and an equal volume of isopropanol was added to the microfuge tube. The tube was mixed and then kept on the lab bench for 10 minutes before centrifuging the samples for 10 minutes at 12,000 x g. The supernatant was discarded, and 1 mL of 75% ethanol was added to the microfuge tube. The microfuge tubes were placed in a centrifuge, and they were centrifuged at 12,000 x g for 1 mL. The ethanol was discarded, and the tubes were centrifuged for 30 seconds. The remaining ethanol was discarded. The pellet was resuspended in 30 µL of RNAase-free water.

## C. BACTERIAL MEDIA

### 1. COMPONENTS OF BACTERIAL MEDIA

#### 1 A. LB

10g/L of tryptone (Fisher)

5g/L of yeast extract (Fisher)

10g/L of NaCl (Fisher)

pH was adjusted to 7.5

15 g/L of agar (Sigma or Difco) was added to the medium to make plates.

#### 1. B. LOW SALT LB

10 g/L tryptone (Fisher)

5 g/L NaCl (Fisher)

5 g/L yeast extract (Fisher)

pH was adjusted to 7.5

15 g/L of agar (Difco) was added to the medium to make plates.

Low salt LB media was used to plate *E. coli* transformed with plasmid DNA containing zeocin as the antibiotic selection.

#### 1. C. L BROTH

10g/L tryptone (Fisher)

5 g/L yeast extract (Fisher)

5 g/L NaCl (Fisher)

1 g/L glucose (Fisher)

pH adjusted to 7.5

#### 1. D. SOC

2 ml of filter-sterilized 20% glucose (Fisher) was mixed into 10 mL of SOB media to make SOC medium.

### 1. E. SOB

SOB media was made using a mixture of three solutions.

20 g/L tryptone (Fisher)

5 g/L yeast (Fisher)

0.5 g/L NaCl (Fisher)

autoclave

To above solution, 10 ml/L of filter-sterilized 1 M $MgCl_2$ (Fisher) and 10 ml/L of filter-sterilized 1 M $MgSO_4$ (Baker) were added.

### 1. F. *AGROBACTERIUM* MEDIUM

0.5 g/L $K_2HPO_4$ (Fisher)

0.2 g/L $MgSO_4 \cdot 7H_2O$ (Baker)

0.1 g/L NaCl (Fisher)

5 g/L mannitol (Sigma)

1 g/L yeast extract (Fisher)

1 g/L casamino acids (Difco)

pH was adjusted to 7.2

15 g/L of agar (Sigma or Difco) was added to the medium to make plates.

The media used to grow *Agrobacterium rhizogenes* strains A4RS and AR10 was from Le et al. (1996).

### 1. G. NUTRIENT AGAR

The nutrient agar came from Sigma. 31 g of nutrient agar was added to 1 L of water and autoclaved.

### 1. H. NUTRIENT BROTH

The nutrient broth came from Sigma. 8 g of nutrient agar was added to 1 L of water and autoclaved.

2. CONDITIONS FOR AUTOCLAVING MEDIA

The media was autoclaved using the following settings: 121°C, 15 lb/in$^2$, and 20 minutes. The media was cooled in a water bath to 55°C before antibiotics were added, and plates were poured after thoroughly mixing in the antibiotics.

3. PREPARATION OF GLYCEROL STOCK CULTURES

A 15% glycerol solution was used for *E. coli* frozen stocks. To make the frozen stock culture, 850 µL of an overnight liquid culture of bacteria was added to 150 µL of autoclaved glycerol. The *Agrobacterium* glycerol cultures were made at 10% glycerol by adding 900 µL of an overnight liquid culture of *Agrobacterium* to 100 µL of autoclaved glycerol. The 15% or 10% glycerol cultures were mixed by vortexing. The vials were frozen using liquid nitrogen and stored at -80 °C.

## D. PLANT MEDIA

### 1. PREPARATION OF MS MEDIA AND B5 MEDIA

#### 1. A. MS MEDIA

The nutrients for the plant media Murashige & Skoog Media, known as MS media were used from the MS basal salts from Sigma or Agri-Bio. When organics were used, they were from a 1000X stock. The preparation of the organics is discussed later.

Unless otherwise noted, the following amount of Phytagel was added to the appropriate container. 75 mL of the MS media were added into Magenta boxes for germinating tobacco seedlings, and 0.188 g/L of Phytagel (Sigma) was added to make solid MS in each box. Petri plates containing MS were also made solid by adding 1.2 g/L of Phytagel to MS media. However, when square slant agar plates of media were made, 8 g/L of Phytagel was added to the media to make solid plates.

#### 1. B. B5 MEDIA

Gamborg's B5 media consisted of the following: 500 mg/L $KNO_3$ 250 mg/L $MgSO_4 \cdot 7H_2O$, 10.0 mg/L $MnSO_4 \cdot H_2O$, 2.0 mg/L $ZnSO_4 \cdot 7H_2O$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 150 mg/L $CaCl_2 \cdot 2H_2O$, 0.75 mg/L KI, 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 130.5 mg/L $NaH_2PO_4$, 3.0 mg/L $H_3BO_3$, 0.25 mg/L $NaMoO_4 \cdot 2H_2O$, 27.8 mg/L $FeSO_4 \cdot 7H_2O$, 37.3 mg/L $Na_2EDTA$, 100 mg/L myo-inositol, 1.0 mg/L nicotinic acid, 1.0 mg/L pyridoxine HCl, and 10.0 mg/L thiamine HCl.

The stocks were prepared as a 50X Nitrate stock, 50X Sulfate stock, 50X Halide stock, 50X P B Mo stock, and 50X NaFeEDTA stock. The stocks consisted of:

Nitrate stock:
125 g/L $KNO_3$ (Fisher)

Sulfate stock:
12.5 g/L $MgSO_4 \cdot 7H_2O$ (Baker)
500 mg/ L $MnSO_4 \cdot H_2O$ (Fisher)

100 mg/L $ZnSO_4 \cdot 7H_2O$ (Sigma)

1.25 mg/L $CuSO_4 \cdot 5H_2O$ (Fisher)

Halide stock:

7.5 g/L $CaCl_2 \cdot 2H_2O$ (Mallinckrodt)

37.5 mg/L KI (Sigma)

1.25 mg/L $CoCl_2 \cdot 6H_2O$ (Alfa Aesar)

P B Mo stock:

6.525 g/L $NaH_2PO_4 \cdot H_2O$ (Fisher)

150 mg/L $H_3BO_3$ (Mallinckrodt)

12.5 mg/L $NaMoO_4 \cdot 2H_2O$ (Fisher)

NaFeEDTA

1.39 g/L $FeSO_4 \cdot 7H_2O$ (Sigma)

1.865 g/L $Na_2EDTA$ (Sigma)

20 mL/L of each of the 50X stock solutions were mixed together. When 2 % sucrose was needed, 20 g/L of sucrose (Sigma) was added. The pH was adjusted to 5.7. 4 g/L of Phytagel was added to make solid plates.

### 1. c. Organics

The organics were prepared at 1000X. The organics were filter-sterilized, and they were added after autoclaving the media. The organics for MS consisted of 100 g/L myo-inositol (Sigma), 2g/L glycine (Sigma), 500 mg/L nicotinic acid (Eastman), 500 mg/L pyridoxine HCl (Sigma), and 100 mg/L thiamine HCl (Sigma). The organics for B5 were made with 100 g/L myo-inositol, 1 g/L nicotinic acid, 1 g/L pyridoxine HCl, and 10 g/L thiamine HCl.

### 1. D. ESTRADIOL STOCK

1.1 g/L 17-β-estradiol (Sigma) added to DMSO (Fisher) make 4 mM stock solution, and it was filter-sterilized. The stock was used to make 2 μM solution in MS plates.

### 2. MODIFIED HALF-STRENGTH MS MEDIUM

half-strength Murashige and Skoog salts (Agri-Bio)
B5 vitamins
2 g/L sucrose (Sigma)
0.4 g/L of MES (Sigma)
pH was adjusted to 5.8

### 3. DBI MEDIUM

MS medium containing Phytagel is prepared and autoclaved. When the medium has cooled to a temperature of 55 to 60$^0$C, 2 μg/mL kinetics and 1 μg/mL IAA were added. Round petri plates were poured.

### 4. INFILTRATION BUFFER

50 mM MES, pH 5.6
2 mM $Na_3PO_4$ (Fisher)
0.5% glucose (Fisher)
100 μM acetosyringone (Aldrich)
Stocks of above solutions were made as:
500 mM MES
20 mM $Na_3PO_4$
20 mM acetosyringone (in ethanol, AAPER)

### 5. BY-2 MEDIA

4.3 g/L of MS basal salts (Sigma or Agri-Bio)
10 ml/L of thiamine-HCl (0.1 g/L) and myo-inositol (10 g/L) (both from Sigma)

3.5 mL/L of Miller's (60 g/L $KH_2PO_4$ from Sigma)

20 µL/L of 2,4-D (10 mg/mL in ethanol from AAPER)

30 g/L of sucrose (Sigma)

pH was adjusted to 5.6 with KOH

To make solid plates, 1.2 g/L of Phytagel (Sigma) was added to the media.

### 6. Modified ¼ Strength Hoagland's Medium

1 liter of modified Hoagland's medium was made as follows:

5 mL/L 1 M $Ca(NO_3)$

5 mL/L 1 M $KNO_3$ (Fisher)

2 mL/L 1 M $MgSO_4 \cdot 7H_2O$ (Baker)

1 mL/L $KH_2PO_4$ (Fisher)

4 mL/L 1000X micronutrient stock

20 mL/L FeEDTA

Adjust pH to 6.85.

Three liters of water is used to dilute every liter of modified Hoagland's medium made before using.

The 1000X micronutrient stock mentioned above was made by mixing together:

2.86 g/L $H_3BO_3$ (Mallinckrodt)

1.81 g/L $MnCl_2 \cdot 4H_2O$

0.22 g/L $ZnSO_4 \cdot 5H_2O$

0.08 g/L $CuSO_4 \cdot 5H_2O$ (Fisher)

0.025 g/L $Na_2MoO_4$

The FeEDTA mentioned above was made by using the following:

5.56 g/L $FeSO_4 \cdot 7H_2O$ (Sigma)

7.45 g/L $Na_2EDTA$ (Sigma)

To mix the two chemicals together, the $Na_2EDTA$ was added to 200 mL $ddH_2O$, and the mixture was heated and stirred to dissolve. $FeSO_4 \cdot 7H_2O$ was added to 200 mL $ddH_2O$ and dissolved by mixing. $FeSO_4 \cdot 7H_2O$ was added to the hot $Na_2EDTA$, and the solution was cooled. The solution was brought up to volume.

### 7. Conditions for Autoclaving Media

The media was autoclaved using the following settings: 121°C, 15 lb/in$^2$, and 20 minutes. The media was cooled in a water bath to 55°C before antibiotics were added. Whenever applicable, plates were poured after thoroughly mixing in the antibiotics and allowed to become solid.

# VITA

Brook Kay Nelson was born in Knoxville, Tennessee and raised in Maryville, Tennessee. She began her college education at Pellissippi State Technical Community College where she earned an Associate of Science degree in 1999 and an Associate of Arts degree in 2000. She continued her undergraduate education at University of Tennessee, Knoxville. She earned a Bachelor of Science degree in Biology with a concentration in Honors Plant Biology in 2003. She remained at University of Tennessee, Knoxville for her graduate education. She completed her Master of Science degree in Botany in 2005, and she is pursuing a doctorate degree at University of Tennessee, Knoxville.