Expression of the murine ornithine decarboxylase gene in transgenic Nicotiana tabacum var xanthi

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Expression of the murine ornithine decarboxylase gene in transgenic *Nicotiana tabacum* var. *xanthi*

DeScenzo, Richard Anthony, Ph.D.
University of New Hampshire, 1991
EXPRESSION OF THE MURINE ORNITHINE DECARBOXYLASE GENE IN TRANSGENIC NICOTIANA TABACUM VAR. XANTHI

by

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B.S., University of Rhode Island, 1980
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A DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
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May, 1991
This dissertation has been examined and approved.

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To my friends, and there are too many to list without adding an additional volume to this dissertation, who shared the highs and lows of my graduate career, my eternal gratitude and friendship. To my family, words cannot express my thanks for your support and understanding during the last seven years. To my wife Jan, thanks for the love and understanding that you have given me over this seemingly unending quest. I could not have done this without you, thanks. To Julie and Jason, thanks for making dad smile even when I didn't feel like smiling. Jan, Julie, and Jason, you were my inspiration, love and thanks....
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ABSTRACT

EXPRESSION OF THE MURINE ORNITHINE DECARBOXYLASE GENE IN TRANSGENIC NICOTIANA TABACUM var. XANTHI.

by

Richard Anthony DeScenzo
University of New Hampshire, May, 1991

Ornithine decarboxylase (ODC), arginine decarboxylase (ADC), and S-adenosyl-methionine decarboxylase (SAMDC) are the three key regulatory enzymes for polyamine (putrescine, spermidine, and spermine) biosynthesis. In order to gain more insight into the relationship between polyamine metabolism and other physiological processes, research was undertaken to obtain increased putrescine biosynthesis in tobacco by overexpression of a murine ODC cDNA.

Both a full-length and a truncated murine ODC cDNA were cloned into a binary expression vector containing the Cauliflower Mosaic Virus (CaMV) 35S promoter. Using standard leaf-disc transformation procedures, transgenic tobacco plants containing either the full length or the truncated ODC cDNA were obtained. Presence of the murine ODC cDNA as well as transcription were confirmed via Southern and Northern blotting. Western blot analysis identified a polypeptide unique to the transformed plants which immunoreacted with anti-ODC antibody.
A series of enzyme assays were done to differentiate between native and murine ODC activity. Assays were run at the pH optima for native ODC (pH 8.2) and murine ODC (pH 6.8). At pH 6.8, there was very little activity in the control plants, but a significantly higher activity in the transformed plants. Difluoromethylornithine (DFMO), a specific irreversible inhibitor of ODC activity, completely inhibited ODC activity in the transformed plants at pH 6.8. However in the control plants at pH 6.8 and both the control and transformed plants at pH 8.2, DFMO only inhibited ODC activity by approximately 30-50%. Almost 100% inhibition of ODC activity by immunoprecipitation of ODC protein with anti-ODC antibody was observed in the transformed plants, at pH 6.8. The results show clearly that the activity of murine ODC can be detected and quantified even in the presence of the plant ODC. The transgenic plants containing the truncated ODC cDNA always had several fold higher activity than those containing the full-length cDNA.

Transgenic plants containing the truncated ODC cDNA contained 10-12 times the levels of putrescine than the control plants. Transgenic plants containing the full-length cDNA contained 4-5 times the level of putrescine as compared to the control. In addition to increased levels of putrescine, there was an amine-containing compound unique to the transformed plants with a retention time very similar to putrescine.
INTRODUCTION

The polyamines putrescine, spermidine, and spermine are naturally occurring compounds found in all living organisms. They are simple aliphatic amines (Fig. 1) which have been isolated in both free and conjugated forms as well as associated with various macromolecules. In the free form, the multiple amino groups provide a net positive charge to the molecule at physiological pH which allows them to interact with any biologically active molecule containing negatively charged realms. Due to their ubiquitous nature and capacity to interact with a host of biologically active molecules, many researchers have postulated various physiological roles for the polyamines (Kaur-Sawhney and Galston 1979, Galston 1983, Slocum et al. 1984, Davies 1987, Evans and Malmberg 1989, Robie and Minocha 1989, Hiatt 1989, Negrel 1989, Kaur-Sawhney et al. 1990).

For most groups of compounds which are involved in regulatory functions, much of the biological specificity is determined by the complex structure of the molecule. However, due to the simple structure of polyamines they are capable of interacting with a wide variety of cell components, exhibiting very little biological specificity in these interactions. As such, the polyamines had long been
$H_2N(CH_2)_4NH_2$

**PUTRESCINE**

$H_2N(CH_2)_4NH(CH_2)_3NH_2$

**SPERMIDINE**

$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

**SPERMINE**

Figure 1. Structure of the Polyamines
Putrescine, Spermidine and Spermine.
labeled as organic cations which offered a selective advantage over inorganic cations only in that intracellular synthesis was possible. Indeed, in many systems the effects of polyamines are not quantitatively different from those of divalent cations, particularly Mg²⁺. In a variety of in vitro systems the effects of polyamines follow a cationic progression, i.e., spermine is the most effective, followed by spermidine and then putrescine.

However, some studies indicate specific polyamines conform to precise structural demands required for interaction with biologically active molecules. Due to the fact that their positive charges are distributed at fixed lengths along a conformationally flexible carbon chain, polyamines are not point-localized charges as are the divalent cations. Therefore, polyamines are able to bridge critical distances between negatively charged groups within or between biologically active molecules. This feature allows specific interactions and functions which cannot be duplicated by metal cations (Harrison and Bode 1975, Schreier and Schimmel 1975, Schuber 1989).

Due to their simple structure, it would seem that many of the specific biological activities regulated by polyamines would be dependent on the subcellular location of polyamine biosynthesis and/or accumulation, as well as the timing of synthesis in conjunction with the regulated biological event. The association of high levels of ornithine
decarboxylase (ODC), a polyamine biosynthetic enzyme, with chromatin in germinating barley seeds is an example of subcellular location of polyamine synthesis during a process in which high levels of polyamines are required for transcription and translation (Koromilas and Kyriakidis, 1988). Similarly, ODC has been shown to be covalently linked to the cell membrane via inositol in T lymphocytes. Treatment of the lymphocytes with mitogenic ligands causes a release and rapid activation of the membrane bound ODC resulting in an increase in polyamines required for the initiation of the mitotic cell cycle (Mustelin et al., 1987).

The biosynthesis of polyamines is known to be a highly modulated process, and changes in the rates of polyamine production are known to be restricted to relatively narrow "time windows" during normal growth and development (Maudsley 1979). This in itself imposes limitations upon the range of potential biological activities that could be exerted by polyamine synthesis. However, polyamines covalently bound to other molecules, i.e., conjugated polyamines, could also be coordinately dissociated resulting in their being available to have a regulatory effect (Smith et al. 1983). Although the evidence for direct interaction between polyamines and biologically active molecules is somewhat limited, they have been implicated as playing a role in a large array of metabolic and developmental processes. Numerous studies indicate that polyamines associate closely
with DNA and RNA and are essential for transcription and translation. The rate of polyamine biosynthesis as well as the intracellular levels of polyamines have been shown to be important in stabilization of membranes, stimulation of protein synthesis and stabilization of cellular proteins. In addition, polyamine synthesis is essential for the completion of the mitotic cell cycle. Inhibitor-mediated blockage of polyamine synthesis results in the cells being locked into the G1 stage of the cell cycle (Rupniak and Paul 1978). Various mutants of *Escherichia coli* lacking in the synthesis of polyamines are not capable of undergoing normal cell growth and/or division unless exogenous polyamines are provided (Tabor et al. 1978).

Further evidence indicating that polyamines can exert a regulatory function involves the interaction between polyamines and hormones. When the hormone-induced increase in polyamine synthesis is blocked by the use of various inhibitors, the effects of the hormone are cancelled (for review see Russell 1989).

In plants also, polyamine biosynthesis and their intracellular concentrations can be modulated by various plant growth regulators including indoleacetic acid, naphthaleneacetic acid, benzylaminopurine, ethylene and abscisic acid (Palavan et al. 1984, Palazon et al. 1987, Roberts et al. 1984, Evans and Malmberg 1989). Similarly, modulating intracellular polyamine levels by addition of
polyamines or inhibitors of polyamine synthesis can affect the endogenous levels of plant growth regulators (Roberts et al. 1984). Indeed, many investigators feel that polyamines may be a second messenger required for the signal-response pathway for various plant growth regulators (Galston 1983, Kyriakidis 1983).

In plants, polyamines have been implicated in the regulation of light-induced growth responses, embryogenesis, organogenesis, fruit development, pollen formation, flower development, fertilization and senescence (Slocum et al. 1984, Evans and Malmberg 1989). In addition, a role for polyamines has been suggested in the response of plants to stress from both biotic and abiotic factors (Young and Galston 1983, Flores and Galston 1982, Wang 1987).

POLYAMINE BIOSYNTHESIS

The biosynthetic pathways for various polyamines have been intensively studied. Ornithine decarboxylase (ODC) (EC 4.1.1.17) is a key enzyme which catalyzes the decarboxylation of L-ornithine to form the diamine putrescine, which is the precursor for the formation of spermidine and spermine (Fig.2). In plants, ODC has been characterized from several sources (Table 1) including cultured tobacco cells, developing tomato ovaries (Heimer and Mizrahi 1982), germinating barley seeds (Koromilas and Kyriakidis 1988, Kyriakidis et al. 1983), and several other tissues (Altman
Figure 2. Putrescine Synthesis via Ornithine Decarboxylase
et al. 1982, Cohen et al. 1983, Aleksijevic et al. 1979 and, Escribano and Legaz 1983). The optimum pH for enzyme activity ranges from 7.0 - 8.5 depending on the species (Table 1). The enzyme is substrate specific for L-ornithine and is known to have an absolute requirement for pyridoxal-5'-phosphate in all species investigated. The enzyme is a homodimer with a subunit weight of approximately 55,000. The Km of the enzyme varies with the species and even within a species depending on the subcellular localization of the enzyme. These characteristics are similar to ODC purified from mammalian sources (Russell 1985, Isomaa et al. 1983, Seely et al. 1982 and, Kitana and Fujisawa 1983) (Table 2). Indeed, the two major differences between the mammalian and plant ODC enzymes characterized to date are the pH optima and the Km for L-ornithine. The decarboxylation of ornithine is the only de novo pathway to putrescine synthesis in animals, with the exception of the parasitic roundworm Ascaris (Smith, 1979).

In higher plants, as well as prokaryotes and some fungi, there is a second pathway for putrescine synthesis (Tabor and Tabor 1983, Galston 1983, Stevens 1981, Khan and Minocha 1989). In this pathway, arginine is decarboxylated in a reaction catalyzed by the enzyme arginine decarboxylase (EC 4.1.1.19) to yield agmatine (Fig.3). Agmatine is then converted to n-carbamoylputrescine via agmatine iminohydrolase (EC 3.5.3.12), which undergoes a hydrolytic
Table 1. Characterization of ODC from various plant and animal sources.

<table>
<thead>
<tr>
<th>Source</th>
<th>Subunit Mr (# subunits)</th>
<th>Km L-Orn (mM)</th>
<th>Optimum pH</th>
<th>Assay Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>54K (2)</td>
<td>0.14</td>
<td>8.0</td>
<td>25°C</td>
</tr>
<tr>
<td>Tobacco</td>
<td>54K (2)</td>
<td>0.14</td>
<td>8.0</td>
<td>25°C</td>
</tr>
<tr>
<td>Mung Bean</td>
<td>53K (2)</td>
<td>N.D.</td>
<td>8.0</td>
<td>37°C</td>
</tr>
<tr>
<td>Barley</td>
<td>55K (2)</td>
<td>0.36</td>
<td>8.5</td>
<td>37°C</td>
</tr>
<tr>
<td>Mouse</td>
<td>53K (2)</td>
<td>0.075</td>
<td>6.8-7.2</td>
<td>37°C</td>
</tr>
<tr>
<td>Rat Kidney</td>
<td>54K (2)</td>
<td>0.047</td>
<td>6.8-7.2</td>
<td>37°C</td>
</tr>
<tr>
<td>Human</td>
<td>52K (2)</td>
<td>0.054</td>
<td>6.8-7.2</td>
<td>37°C</td>
</tr>
<tr>
<td>Trypanosoma</td>
<td>45K (2)</td>
<td>0.280</td>
<td>7.2</td>
<td>37°C</td>
</tr>
<tr>
<td>Y Promptiates</td>
<td>52K (2)</td>
<td>N.D.</td>
<td>7.4</td>
<td>37°C</td>
</tr>
<tr>
<td>Neurospora</td>
<td>53K (2)</td>
<td>0.350</td>
<td>7.4</td>
<td>37°C</td>
</tr>
</tbody>
</table>

a- Heimer and Mizrahi, 1982  
b- Cohen et al., 1983  
c- Altman et al., 1982  
d- Koromilas and Kyriakidis, 1988  
e- Seely et al, 1982  
f- Kitani and Fujisawa  
g- Leinonen et al., 1987  
h- Phillips et al., 1988  
i- Fonzi and Sypherd, 1987  
j- DiGangi et al., 1987
Figure 3. Putrescine synthesis via the arginine decarboxylase pathway
Figure 4. Biosynthesis of Spermidine and Spermine
reaction catalyzed by n-carbamoylputrescine amidohydrolase (EC 3.5.1.13) yielding putrescine. The synthesis of spermidine from putrescine is catalyzed by the enzyme spermidine synthase. In this reaction an aminopropyl group derived from decarboxylated SAM is combined with putrescine to form spermidine (Fig. 4) (Torget et al. 1979, Cohen et al. 1981). S-adenosylmethionine (SAM) is a major donor of methyl groups for methylation reactions and is also a precursor for the plant growth regulator ethylene. Decarboxylation of SAM by S-adenosylmethionine decarboxylase (SAMDC) is non-reversible, committing SAM solely to the polyamine biosynthetic pathway. SAMDC is a pyruvate-dependent enzyme and is stimulated by putrescine (Maudsley 1979).

The synthesis of spermine from spermidine is catalyzed by the enzyme spermine synthase in a reaction similar to the formation of spermidine (Fig. 4). An aminopropyl group from decarboxylated SAM is added to spermidine resulting in the formation of spermine. Both spermidine synthase and spermine synthase are substrate specific aminopropyltransferases (Sindhu and Cohen 1984, Slocum et al. 1984). Not only are they expressed in a constitutive manner (Heby and Persson, 1990), the enzymes are generally present in higher concentrations and have much longer half-lives than the decarboxylases. This indicates that the spermidine and spermine synthase enzymes are not rate limiting steps in the synthesis of polyamines (Pegg and McCann 1982, Pegg 1986).
REGULATION OF POLYAMINE BIOSYNTHESIS AND CELLULAR POLYAMINE CONCENTRATIONS

In most instances, the rate limiting steps in the biosynthesis of polyamines are the activities of ADC, ODC and SAMDC, making them the target of several studies. The bulk of research on the regulation of polyamine biosynthesis has focused on ODC in mammalian systems and ODC and ADC in *E. coli*. Comparatively little research has been done on regulation of ODC or ADC activity in plant and fungal systems. However, due to the highly conserved nature of ODC-mediated polyamine biosynthesis in different organisms, it is likely that the regulation of ODC activity is similar between the two groups.

A wide variety of external stimuli which affect cell proliferation, cellular differentiation, organogenesis, stress response, etc., are known to induce both rapid and delayed changes in intracellular polyamines (for review see Maudsley 1979, Slocum et al. 1984, Pegg and McCann 1982, Russell 1989, Galston 1983, Evans and Malmberg 1989, Heby and Persson 1990). In mammalian systems, these responses have been well characterized. Stimulation of cell proliferation results in a large increase in ODC activity in all systems investigated including cell lines derived from mouse kidney (Janne et al. 1978, Russell 1989), rat liver (Janne and Raina 1969) and human myeloma cells (Leinonen et al. 1987). After induction there is a one hour lag period
followed by a large increase in enzyme activity for up to 4 hours. Ornithine decarboxylase activity then declines sharply and returns to the pre-induced levels after 8-10 hours (Maudsley 1979, Pegg 1988). Although similar patterns of response are observed in many different situations, the level at which the ODC activity is regulated varies. There is evidence that ODC synthesis and activity is controlled at transcriptional, translational, as well as post-translational levels.

Regulation of ODC synthesis and activity

Transcriptional Control: The use of transcription inhibitors and Northern Blot techniques have enabled researchers to document the transcriptional regulation of ODC activity in a variety of cell types and organisms. Fausto (1971) was the first to demonstrate that an induced increase in ODC activity was dependent on transcription. A large increase in ODC activity induced by injection of casein hydrolysate into rat liver was completely blocked by simultaneous addition of actinomycin D and puromycin. Addition of the inhibitors two hours after casein hydrolysate injection had no effect on the increase in ODC activity (Fausto 1971).

More recent experiments with cell lines pck12 from rat as well as BALB/cBT3 and kidney cells from mice demonstrate the control of ODC at the transcriptional level. Induction
of ODC activity in these cells with nerve growth factor (NGF), platelet derived growth factor (PDGF), serum, and androgens has been shown to be transcriptionally controlled (Einat 1985, Feinstein et al. 1985, Kahana and Nathans 1984, Kontula et al. 1984). In many ODC overproducing cell lines, e.g., murine D4.1 and L1210 (Persson et al. 1988, Brabant et al. 1988), Chinese Hamster ovary A7 (Pohjanpeltto et al. 1985) and human IgG-myeloma (Leinonen et al. 1987), gene amplification results in multiple copies of the ODC gene causing a high level of the transcript for ODC upon induction. These cell lines were utilized in the initial isolation of cDNAs which code for the ODC protein. In addition, transcription of Escherichia coli gene spec, which codes for ODC, is negatively regulated by cAMP and cAMP receptor protein complex (Wright et al. 1986).

**Translational Control**: Although induction of ODC activity by various stimuli is generally thought to be transcriptionally regulated, short term regulation of the enzyme appears to be controlled at the level of translation. An excess of polyamines, either added to or synthesized by the cells, resulted in a rapid cessation of ODC activity by feed-back inhibition (Persson et al. 1988). Likewise, inhibitor-induced depletion of cellular polyamine levels resulted in an increase in ODC activity. In both cases, this short term regulation of enzyme activity was achieved
without any change in the steady state level of ODC mRNA. Since the half-life of ODC is one of the shortest of any eukaryotic enzyme, changes in the rate of ODC synthesis can be quickly manifested in the amount of enzyme present and the rate of polyamine synthesis.

Similar results on translational control of ODC were observed in liver and prostate tissues from rats (Kameji and Pegg 1987). Quantities of ODC protein were determined by immunoprecipitation with antiserum specific to rat ODC. The alteration in ODC activity due to change in cellular polyamine levels was a direct function of cellular ODC concentrations. Addition of 0.8 mM spermidine or 0.15 mM spermine resulted in a 70% decrease in ODC synthesis.

Both the murine and rat ODC mRNAs contain an exceptionally long 5' non-coding leader sequence which is unusual in its base composition (Brabant et al., 1988). It contains an extremely high G+C content, particularly in the 5' portion, in which 85 of 102 bases are guanine or cytosine between positions -297 and -196. It has been theorized that an mRNA of such base composition could form one or more secondary structures which could play a regulatory role in translation of the mRNA. Indeed, it is conceivable that polyamines, which have a strong affinity for nucleic acids, could themselves affect the secondary structure of the ODC mRNA 5' leader in such a way as to inhibit initiation of translation. Although this has not been demonstrated in vivo, it
has been shown that removal of spermidine, a reaction buffer component, from \textit{in vitro} translation reactions with ODC mRNA results in higher levels of ODC being produced compared to reactions containing spermidine (Kameji and Pegg 1987, Persson et al. 1988). The 3' end of the ODC mRNA is also interesting in that it contains two polyadenylation sequences in most species which have been characterized, including mouse (Hickok et al. 1986, Coffino and Chen 1988), rat (Kranen et al. 1987) and hamster (Srinivasan et al. 1987).

\textbf{Post-Translational Control:} In most cases, the half-life of ODC is less than one hour. This rapid degradation of ODC plays an important role in its post-translational regulation and is being actively investigated. Post translational control of ODC activity has been demonstrated in a variety of \textit{in vivo} and \textit{in vitro} systems utilizing a number of regulatory mechanisms. As with translational regulation, the post translational control of ODC activity appears to be involved with short term, rapid changes in ODC activity.

In \textit{Neurospora sp.}, an increase in the cellular putrescine content initiates the inactivation of ODC (DiGangi et al. 1987). The inactivation is a result of the loss of protein, i.e., degradation. Putrescine induced inactivation of ODC via degradation has also been observed in several mammalian tissues (Seely and Pegg 1982, Holta and
Pohjanpelto 1986, Dirks et al. 1986), whereas in the lower eukaryotes such as Physarum polycephalum and Saccharomyces cerevisiae the inactivation occurs without protein degradation (Tyagi et al. 1981, Mitchell et al. 1978). Further studies on Neurospora indicate that putrescine has a direct effect on the half-life of ODC. In the presence of normal cellular levels of polyamines, ODC has a half-life of approximately 55 min. In cells where putrescine was depleted, the half-life of the enzyme was approximately 450 min (Barnett et al 1988). These results indicate putrescine can destabilize ODC acting in a feedback inhibition mode of post-translational control.

Rapid turnover of ODC due to degradation has been observed in a majority of the organisms investigated. The half-life of ODC varies from 10 min in mouse and rat liver (Kitani and Fujisawa 1983, Persson et al. 1988) to 55 min in Neurospora (DiGangi et al. 1987). One of the current theories on rapid turnover of enzymes due to degradation involves the PEST hypothesis (Rogers et al. 1986). This hypothesis postulates that proteins which contain regions of amino acid residues comprised predominantly of proline (P), glutamic acid (E), aspartic acid, serine (S), and threonine (T) are subject to rapid degradation. The murine ODC contains two regions which score high on the PEST test (Rogers et al. 1986). One of these PEST regions (amino acid residues 423-499) is located in the carboxyl terminus of the
enzyme which is a region shown to be unnecessary for biological activity (Ghoda et al. 1989). It was suggested that this PEST region plays a role in the rapid degradation of ODC. Using insertional mutation techniques, Ghoda et al. (1989) constructed expression vectors containing an ODC gene which contained a stop codon in place of amino acid number 425. The enzyme produced by cells transformed with these constructs lacked the carboxyl terminal PEST region, yet retained 100% of the biological activity as compared to controls transformed with the full length construct. In addition, control ODC had a half-life of approximately one hour in the presence of cyclohexamide as compared to four hours for truncated ODC. After four hours, cyclohexamide became toxic to the cells and could not be used to measure enzyme stability. Therefore, it is possible that the half-life of the truncated ODC could be extended much longer than four hours. These observations suggest that the carboxyl terminus PEST region is an important component in the post-translational regulation of enzyme activity involving rapid enzyme degradation.

Covalently Bound Intermediates: Another method of post-translational control involves covalently bound intermediates. Enzymes are covalently bound to another molecule resulting in a stable but inactive enzyme intermediate. ODC from both plants and animals has been
shown to form covalently bound intermediates.

In T-lymphocytes, treatment with mitogenic ligands results in a rapid activation of ODC. This rapid activation, within 1 minute, is independent of de novo protein synthesis but requires an intact cytoskeleton and energy metabolism (Scott et al. 1985). Mustelin et al. (1987) investigated this protein synthesis independent activation of pre-existing ODC during the transduction of mitogenic signal from the plasma membrane receptors to the cell interior. They determined that ODC was bound via phosphatidylinositol to the interior surface of the plasma membrane. The enzyme was in an inactive state when bound, but could be activated by the addition of a PI specific phospholipase C resulting in a several fold increase in enzyme activity.

In germinating Barley (Hordeum vulgare) seeds, ODC has been shown to be bound to chromatin in an inactive form. The exact mechanics of this binding have not been elucidated, but when ODC is removed from chromatin it becomes active (Koromilas and Kyriakidis 1988).

**Antizymes:** Antizymes (Az) are proteins which inhibit the activity of a specific enzyme and whose synthesis is usually induced by the end product of the enzyme which they inhibit. This novel mechanism of control of enzyme activity has been documented with ODC in both plant and animal systems (Panagiotidis and Kyriakidis 1985, Koromilas and Kyriakidis
Two antizymes of ODC have been isolated from germinating barley seeds, one of cytosolic origin with Mr 16000 and the other from chromatin with Mr of 9000. In both cases it has been determined that two molecules of antizyme bind to one molecule of ODC to form the inactive ODC-Az complex. Koromilas and Kyriakidis (1988) reported that ODC and antizyme exist as a complex both in cytosol and chromatin of germinating barley seeds. They believe that ODC antizyme is an important regulatory molecule in the post-translational regulation of ODC activity. Further work is being done to determine if the ODC-Az:free ODC ratio changes at different times during development and in response to stress.

To further complicate matters, the isolation of an ODC anti-antizyme protein (AAz) or activator, which binds to ODC-Az and liberates active ODC, has been demonstrated in rat liver (Fujita et al. 1982). A similar finding of ODC anti-antizyme in E. coli was reported by Huang et al. in 1984. However, the anti-antizyme was determined to be a small RNA molecule instead of a protein molecule. In rat liver, it has been suggested that inactive or cryptic forms of ODC (ODC-Az) and antizyme (Az-AAz) exist in subcellular structures or locations and that these can act as reservoirs from which their active forms can be released rapidly on demand (Canellakis et al. 1981). Although no anti-antizyme has been isolated from a plant source, it appears the
antizyme/anti-antizyme complex with ODC may be one more mechanism for post-translational regulation of this highly regulated enzyme which may be present in plants.

**Regulation of ADC synthesis and activity**

In comparison with ODC, research on the regulation of ADC synthesis is virtually non-existent. Although there is substantial information on regulation of the enzymatic activity of ADC, there is only one report on the regulation of synthesis of this enzyme. In response to red light, buds from *Pisum* exhibit a doubling of ADC specific activity within two hours. Likewise, epicotyls exposed to far-red light also exhibit an increase in ADC specific activity. These phytochrome mediated increases in ADC activity were completely inhibited by cyclohexamide. This suggests de novo protein synthesis, i.e. translational control is important in regulating ADC synthesis in response to phytochrome mediated increases in ADC activity (Galston et al. 1983).

**Regulation of intracellular polyamine levels**

Putrescine, spermidine and spermine are synthesized in the cell in a free form and then converted into other polyamines, conjugated with other molecules, and degraded via polyamine and diamine oxidases. The roles that these processes play in the regulation of cellular polyamine
levels and the mediation of their biological effects is being actively investigated.

Hydroxycinnamic acid amides of polyamines have been found throughout the plant kingdom. In a survey of 20 plant species representing 13 families, they occurred as the main phenolic constituents of the reproductive organs of all plants studied (Martin-Tanguy et al. 1978, Martin-Tanguy 1985, Malmberg and Rose 1987, Kaur-Sawhney et al. 1988). Polyamines conjugated with cinnamic acids form a variety of alkaloids. In addition, polyamines have been found conjugated to fatty acids, coumaric acid, caffeic acid and ferrulic acid (Smith, et al. 1983). Many of these conjugated polyamines have been implicated in specific functions, such as anti-pathogenic properties. There is some evidence indicating a requirement for caffeoyl-putrescine and other amides in the formation of fertile reproductive organs in tobacco (Martin-Tanguy, et al. 1987). Some researchers feel that the conjugated forms of polyamines, which can account for up to 90% of total cellular polyamines, act as a reservoir for free polyamines which can be rapidly released on demand (Torrigiani, et al. 1987). Although conjugation of polyamines may be a way of reducing the levels of free polyamines, it has yet to be shown that conjugated polyamines can be released to a free form in vivo. It is interesting to note that the polyamines involved in conjugation are apparently produced only through
the ADC pathway in maize (Berlin and Forche, 1981), and only through the ODC pathway in tobacco (Burtin et al. 1989).

In both plants and animals, degradation of polyamines occurs via oxidation reactions catalyzed by the enzymes diamine oxidase(s) (DAO) and polyamine oxidase(s) (PAO) (Kaur-Sawhney et al. 1981, Smith et al. 1983, Smith 1985). Diamine oxidases are capable of catalyzing oxidative degradation of a wide variety of amines including monoamines, diamines, polyamines and aromatic amines (Smith 1985, Slocum et al. 1984), whereas PAO are substrate specific for aliphatic polyamines. Although DAO have been observed in all plant families examined so far, PAO have been found mostly in the family Gramineae (Smith 1983) and recently in water hyacinth (Yanagisawa et al. 1987). This enzyme which is particularly active in oat (Kaur-Sawhney et al. 1981, Federico et al. 1989), has also been well characterized in barley (Smith and Bickley 1974), maize (Suzuki and Yanagisawa 1980, Federico et al. 1989), and millet (Hirasawa et al. 1986).

In plants the oxidation products of spermine are 1,3-diaminopropane, 1-(3-aminopropyl)-pyrroline and peroxide. Similarly, the oxidation products of spermidine are 1,3-diaminopropane, pyrroline and peroxide. Putrescine is oxidized to yield pyrroline, peroxide and NH₃.

There is no evidence of a linear degradation, i.e., spermine->spermidine->putrescine in plants. However, in
animal systems, acetyltransferases have been identified which are capable of acetylating spermine and spermidine. Oxidation of acetylated spermine by PAO yields spermidine and oxidation of acetylated spermidine yields putrescine (Pegg 1986). Due to the scarcity of PAO in the plant kingdom, it has been suggested that degradation of conjugated polyamines may be the only pathway for interconversion of polyamine moieties available in plant systems (Slocum et al 1984).

INHIBITORS OF POLYAMINE BIOSYNTHESIS

A number of compounds are available which can inhibit the enzymes involved in polyamine biosynthesis (Table 2). These compounds have been useful in investigating the interactions between polyamine synthesis and accumulation, with various physiological processes such as cell division, growth and differentiation, as well as embryogenesis, organogenesis etc. (for review see Pegg et al. 1982, Galston 1983, Slocum et al. 1984, Minocha 1988, Evans and Malmberg 1989).

Two of the more important inhibitors of polyamine biosynthesis are alpha-difluoromethylornithine (DFMO) and alpha-difluoromethylarginine (DFMA) which inhibit ODC and ADC, respectively. These compounds belong to a group of inhibitors known as suicide inhibitors. Suicide inhibitors are substrate analogues which bind irreversibly to the catalytic or active site of the enzyme. This irreversible
Table 2. Inhibitors of Polyamine Biosynthesis (modified from Pegg 1986, Slocum et al. 1984, Pegg and Williams-Ashman 1987)

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>INHIBITOR</th>
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<tbody>
<tr>
<td>Ornithine decarboxylase (ODC)</td>
<td>DL-alpha-Difluoromethylornithine (DFMO)</td>
</tr>
<tr>
<td></td>
<td>alpha-Fluoromethyl-(E)-dehydroornithine (2R,5R)-6-Heptyne-2,5-diamine (MAP)</td>
</tr>
<tr>
<td></td>
<td>monofluoromethylornithine (MFMO)</td>
</tr>
<tr>
<td>Arginine decarboxylase (ADC)</td>
<td>DL-alpha-Difluoromethylarginine (DFMA)</td>
</tr>
<tr>
<td>S-Adenosylmethionine decarboxylase (SAMDC)</td>
<td>Methyglyoxal bis(guanylydrazone) (MGBG)</td>
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<tr>
<td></td>
<td>Ethylglyoxal bis(guanilhydrazone) EGBG)</td>
</tr>
<tr>
<td></td>
<td>Dimethylglyoxalbis (guanylydrazone)</td>
</tr>
<tr>
<td></td>
<td>1,1-[(methylethanediylidene)-dinitri] bis (3-aminoguanidine) (MBAG)</td>
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<tr>
<td></td>
<td>1-aminoxy-3-aminopropane</td>
</tr>
<tr>
<td>Spermidine synthase</td>
<td>S-Adenosyl-1,8-diamino-3-thio-octane (AdoDATO)</td>
</tr>
<tr>
<td></td>
<td>Cyclohexylammonium phosphate (CHAP)</td>
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<td></td>
<td>5-[(3-aminopropyl)amino]-5-deoxyadenosine</td>
</tr>
<tr>
<td>Spermine synthase</td>
<td>S-Methyl-5-methylthio-adenosine (MMTA)</td>
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<tr>
<td></td>
<td>5-methyltubericidin</td>
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covalent binding results in deactivation of the enzyme (Sjoerdsma 1981).

Another widely used inhibitor of polyamine biosynthesis is methylglyoxal bis(guanylhydrazone) (MGBG). MGBG is a reversible, competitive inhibitor of SAM decarboxylase (Williams-Ashman and Schenone 1972). The inhibition of SAM decarboxylase results in the inhibition of spermidine and spermine synthesis.

Although many researchers are using inhibitors to investigate polyamine biosynthesis both in vivo and in vitro, there are problems associated with the use of inhibitors and the results must be interpreted with caution. Most inhibitors exhibit a great deal of variation in their effects among different organisms and even between in vivo and in vitro studies with the same organism. In in vitro studies, DFMO reduces animal ODC activity by 90-100% (Sjoedsma 1981), whereas in a variety of plants it appears to have less of an inhibitory effect (<50%) on ODC activity (Galston 1983, Flores and Galston 1984, Slocum et al. 1984, Slocum and Galston 1985) and may actually promote ADC activity (Robie and Minocha 1989). In addition, DFMO has little effect on E. coli ODC in vitro, yet appears to inhibit ODC activity in vivo (Kallio et al. 1981). DFMA has been a more effective inhibitor in vitro of ADC activity in plant tissues than DFMO is on ODC activity. However, DFMA had no inhibitory effect on ADC activity in potato tubers.
and amaranth leaves (Flores and Galston 1984).

The standard method used to determine ODC and ADC activity involves recovering \(^{14}\)CO\(_2\) produced from enzymatic decarboxylation of \(^{14}\)C-labeled ornithine and arginine. Birecka et al. (1985a,b), using recovery of \(^{14}\)C-labeled agmatine and putrescine synthesized from \(^{14}\)C-labeled arginine and ornithine, demonstrated the inhibition of ODC and ADC activity with DFMO and DFMA, respectively, even though the standard method indicated no inhibition. They suggested the possibility of a side reaction occurring, which caused the release of \(^{14}\)CO\(_2\) by a mechanism other than ODC or ADC decarboxylation of the labeled substrate. This side reaction was masking the inhibition of enzyme activity in cases where actual inhibition was occurring. In another series of experiments, Birecka et al. (1985b) tested the inhibitory effects of DFMO and DFMA on ODC and ADC activity in a wide variety of plants. Using the labeled products of the enzymatic decarboxylation, i.e., \(^{14}\)C-labeled agmatine and putrescine, they observed inhibition of ODC and ADC activity by DFMO and DFMA, respectively, in all plants tested.

Another problem associated with the use of inhibitors is their lack of specificity. Although an inhibitor is expected to exert its action on a specific target enzyme, there is no guarantee that it is the sole site of inhibitor action. The situation is further complicated by the fact
that most inhibitors can be metabolized by the cell to produce new compounds. In the case of DFMA, it was discovered that arginase can convert DFMA to DFMO in some plant tissues (Slocum and Galston 1985). In addition, ornithine and arginine are involved in a myriad of biochemical processes besides polyamine biosynthesis. It is conceivable that DFMO and DFMA could have effects on these reactions which in turn could influence various physiological processes. A practical problem associated with inhibitors of polyamine biosynthesis, is that most of the inhibitors have been synthesized only in small quantities and are, therefore, not commercially available for wide scale experimental use.

PHYSIOLOGICAL ROLE OF POLYAMINES

A great deal of literature has been published linking polyamine biosynthesis and their intracellular levels with various physiological processes. The following is a brief review of the literature in this area. Additional information can be obtained from Cohen (1971), Smith (1975 1985), Galston (1983), Slocum et al. (1984), Pegg and McCann (1982), Pegg (1986), Janne et al. (1978), Heby and Persson (1990).

Exogenous polyamines have been used as growth factors in both animal and plant cell cultures for many years (Herbst and Snell 1948, Bertossi et al. 1965). From early on, a
correlation was seen between rapidly proliferating cells and high levels of polyamine biosynthesis or accumulation. This was in contrast to dormant or non-meristematic tissues which exhibited low levels of polyamine biosynthesis and accumulation (Speranza and Bagni 1977, Heimer et al. 1979, Kaur-Sawhney et al. 1982, Serrafini-Fracassini et al. 1984, Cohen et al. 1983, Teitel 1983, Schwartz et al. 1983). DFMO inhibition of ODC in rapidly dividing cells resulted in a cessation of cell growth and division. This effect has been attributed to one or more of the following; DNA stabilization, protein synthesis and interaction with and stabilization of plasma membranes. In addition, polyamines have been implicated in hormone function, enzyme regulation, cell division, growth, differentiation, embryogenesis, organogenesis, senescence as well as stress tolerance and disease resistance. In short, polyamines are seemingly involved in almost every major physiological process from the individual cell to the intact organism.

**Stabilization of Nucleic Acids**

Polyamines have been shown to bind with and stabilize both DNA and RNA (Liquori et al. 1967, Seiler and Schmidt-Glenewinkel 1975, Igarashi et al. 1982). Using X-Ray analysis of spermine bound to DNA, Liquori (1967) suggested that two consecutive amine groups from spermine were bound to two phosphate groups on one strand of the DNA double-
helix. The (CH₂)₄ group was long enough to span the distance between the two strands and the remaining two amine groups were bound with two phosphates on the second strand. Polyamines can also form secondary hydrogen bonds with nucleic acid bases (Bertoluzzi et al. 1979, Feuerstein and Marton 1989). The stabilization of the secondary structure of nucleic acids protects them from various nucleases (Bachrach and Eilon 1969), thermal and/or X-Ray induced denaturation (Mahler and Mehrotra 1962, Brown 1964, Andreev and Kabeov 1982) and methylation (Rajalakshimi et al. 1978). Interaction of polyamines with DNA has also been suggested as essential for packaging of viral DNA and RNA into their protein coats (Gosule and Schellman 1976, Cohen and Greenberg 1981).

In addition to DNA stabilization, DNA synthesis can also be affected by polyamines. In rat brain cells, DNA polymerase is stimulated by stabilization of the polymerase:protein cofactor complex with polyamines (Chiu and Sung 1972). Nishiguchi et al. (1986) reported that addition of interferon to mouse liver cells resulted in the cessation of ODC activity and DNA synthesis. Addition of exogenous polyamines in the presence of interferon allowed DNA synthesis to proceed normally.

Polyamines also stabilize other double-helical structures, such as stems and loops in rRNA and mRNA (Cohen and Lichienstein 1960, Bagni et al. 1973). In addition they
can stabilize tRNA conformation via binding to specific sites. In fact, the binding of spermine to specific cooperative sites on the yeast tRNA is essential for the proper tertiary structure to form (Schreier and Schimiel 1975). Further work by Quigley et al. (1978) determined that two spermidine molecules were necessary for the proper conformation of yeast tRNA.

Although less work has been done on the interactions between polyamines and RNA synthesis, it has been shown that certain types of RNA synthesis increases with elevations in polyamine levels. RNA polymerase activity is stimulated by spermidine in preparations from maize seedlings, Helianthus tuberosus tuber tissue and soybean hypocotyls (Stout and Mans 1967, Guilfoyle and Hanson 1973, Bagni 1981, Smith 1985). In Physarum, rRNA synthesis was stimulated when ODC was phosphorylated (Kuehn et al. 1983). Spermidine and spermine stimulated a protein kinase activity which catalyzed the phosphorylation of ODC. Although the phosphorylated ODC lost its ability to decarboxylate ornithine, it promoted the activity of RNA polymerase I.

Protein Synthesis

The importance of polyamines in protein synthesis in in vitro translation systems was first suggested by Hershko et al. (1961). Since then, various studies have implicated the necessity of polyamines in all steps of protein synthesis,

The use of mutant strains of E. coli deficient in polyamine biosynthesis have provided more evidence for the absolute requirement of polyamines in protein synthesis. When these mutants were grown on media lacking polyamines they survived, but exhibited no growth (Hafner et al. 1979, Tabor et al. 1980). Transduction of the rpsL (streptomycin resistance) alleles into these mutants created an absolute requirement for polyamines to maintain cell viability (Tabor et al. 1981). Polyamine biosynthetic deficient mutants of E. coli which carry the rpsL alleles cannot translate mRNA, and thus survive, without the addition of polyamines to the growth media. These data suggest a possible role for polyamines in stabilization of the ribosomal complex by
interaction with a particular protein component.

**Influence of Polyamines on Membrane Structure and Function**

The negative charges of phospholipids and other membrane associated molecules provide yet another site of interaction for polyamines. A constant flow of membrane components, from incoming endocytic vesicles fusing with lysozymes to outgoing Golgi vesicles fusing with the plasma membrane, is required for cell growth. Cellular polyamine concentrations are sufficient to modulate membrane fusion by reducing the repulsive forces between negatively charged vesicles (Hong et al. 1983). Although Ca$^{2+}$ is capable of inducing membrane fusion in the absence of polyamines *in vitro*, Gilkey et al. (1978) determined intracellular levels of Ca$^{2+}$ were too low to explain vesicle exocytosis *in vivo*. They suggested the threshold Ca$^{2+}$ level necessary for membrane fusion was reduced in the presence of polyamines, which facilitated bringing the negatively charged vesicles close together. This interaction of polyamines with membranes was further substantiated by Harada et al. (1981) who observed a reduction in cell growth and simultaneous accumulation of intracellular vacuoles upon inhibition of ODC. Grimes et al. (1986) reported similar findings in their investigation of carrot protoplast fusion.

In addition to maintenance and synthesis of the plasma membrane, polyamine interactions with the membrane can
affect its stability (Chapel et al. 1984) and permeability (Srivastava and Smith 1982). Protoplasts of E. coli (Tabor 1962), Micrococcus lysodeikticus (Grossowicz and Ariel 1963) and oat leaf tissue (Altman et al. 1977) were resistant to the lytic activity of lysozyme in the presence of exogenous polyamines. Submillimolar concentrations of polyamines stabilize protoplasts against osmotic shock in E. coli (Tabor 1962, Souzo 1986) and Avena sp. (Flores and Galston 1982). The loss of chlorophyll from thylakoid membranes in senescing barley leaf tissue was halted when membranes were stabilized with polyamines (Popovic et al. 1979, Altman 1982). Membrane stability may be enhanced by the inhibition of various phospholipases, which degrade membranes, in the presence of spermine (Sechi et al. 1978). Polyamines can also affect activity of membrane bound enzymes (Matto et al. 1977). Johnson and Nordlie (1980) reported a polyamine mediated change in the catalytic activity of rat endoplasmic reticulum bound glucose-6-phosphatase. They correlated the change in enzyme activity with a change in membrane fluidity. Dose dependent lipid immobilization was observed with the addition of spermidine. It was conjectured that this was due to bridging between integral proteins and membrane lipid binding sites. The lipid immobilization resulted in a dramatic decrease in the lateral movement of transmembrane glycoproteins (Schindler et al. 1980). Polyamines have also been implicated in interactions with
transmembrane transport of ions and metabolites (Schuber 1989). In addition, polyamines have been shown to be essential for many of the processes involved in transmembrane signal transduction (Koenig, et al. 1988, Berridge 1987, Tadolini and Hakim 1989). These interactions between polyamines, membranes and membrane-bound proteins are complex and difficult to elucidate. However, the importance of these interactions has incited a great deal of research into these processes.

In addition to regulation of the enzymes involved with their own biosynthesis, polyamines have been shown to exert a regulatory effect on a variety of enzymes. In a number of organisms spermine has been shown to stimulate cyclic-nucleotide independent kinase activity resulting in phosphorylation and activation of RNA Pol I (Rose et al. 1981, Goueli et al. 1985, Mezzetti et al. 1985) and biosynthesis of phosphotidylinositol-1,3-diphosphate (Schuber et al. 1983). Spermine can also stimulate choline kinase in rat liver (Fukuyamma and Yamashita 1976) and 1,3-B-Glucan synthase in soybean (Fink et al. 1987). Acyl-transferases involved in the biosynthesis of glycerolipids and phospholipids are also stimulated by spermine (Bates and Saggerson 1981). Polyamines can also influence enzyme activity by binding directly to negatively charged realms of the proteins and inducing changes in enzyme conformation. Indeed, spermine prevented the activation of fructose-1,6-
bisphosphatase in spinach chloroplasts by binding to the enzyme and rendering it inactive (Corley et al. 1983). In another case, spermine bound to a receptor on the plasma membrane prevents phospholipase C from attaching to the membrane and being activated (Schuber 1989). This inhibition of phospholipase C activity is important in membrane function and signal transduction. There is evidence of polyamines binding covalently to a variety of proteins via amide linkages to the gamma-carboxyl groups of protein glutaminy1 residues (Beninati and Folk 1988).

POLYAMINES IN PLANT GROWTH AND DEVELOPMENT

It has been postulated that in plants, polyamines and their derivatives may function as plant growth regulators or as hormonal second messengers (Davies 1987, Galston 1983, Galston and Kaur-Sawhney 1987, Altman et al. 1986). A prerequisite to classifying a regulatory chemical as a hormone is to demonstrate translocatability. As early researchers were unable to demonstrate translocation of polyamines, they attempted to classify them as secondary messengers in response to hormone action (Galston, 1983). In 1984, Bagni et al. demonstrated uptake and translocation of polyamines in isolated apple corymbs. Likewise, Altman et al. (1986) observed translocation of polyamines in cultured plant cells using labeled precursors. Once translocation was demonstrated, some workers suggested that polyamines
were a new class of plant hormones or growth regulators. Opponents of this idea cite the high levels of polyamines required for physiological effect to occur [mM] in comparison to other plant growth regulators [uM]. In addition, they argue that polyamines may have superficially interesting correlations with developmental processes, but only as a consequence of a primary event, rather than as a causal agent (Evans and Malmberg 1989). Whether or not polyamines are a new class of plant growth regulators or secondary messengers will no doubt continue to be debated. However, polyamines have some clear physiological and developmental effects on plants which will be reviewed at this point.

In plants, polyamine concentrations and biosynthesis are at their highest in regions of the plant where the highest rate of cell division occurs, i.e., meristematic regions and in developing fruit (Galston and Kaur-Sawhney 1982, Palavan and Galston 1982, Smith 1985, Nadeau et al. 1987). During the intensive phase of cell division following fertilization in tomato (Cohen et al. 1982), apples (Costa and Bagni 1983, Biasi et al. 1988), Mandarin (Nathanetal 1984) and tobacco (Slocum and Galston 1985), a significant increase in ODC activity and polyamine levels was seen. Post-fertilization treatment with DFMO inhibited fruit growth in tobacco and tomato indicating that ODC-mediated polyamine synthesis was necessary for the high rate of cell division required for
normal fruit development. Increases in polyamine synthesis also correlate with onset of rapid cell division in a variety of other plant tissues including avocado (Kushad et al. 1988), bean (Palavan and Galston 1982, Palavan and Unsal 1987), crown gall tumors (Speranza and Bagni 1977), carrot (Feirer et al. 1985) and Jerusalem artichoke (Phillips et al. 1987, Bagni et al. 1981, D'Orazi and Bagni 1987). Although DFMO inhibition of ODC reduced cell division in some instances, Bagni et al. (1981) identified ADC as the primary polyamine biosynthetic enzyme involved with cell division in Jerusalem artichoke. There is still a great deal of research necessary to elucidate the roles of ADC and ODC mediated polyamine biosynthesis in rapid cell division.

Interaction Between Polyamines and Plant Growth Regulators

The groups of compounds classified as plant growth regulators (PGR) act in conjunction to affect the growth and development of plant tissues. The coordination of PGR concentration and ratios within spatial and temporal parameters results in cell differentiation, production of shoot and root meristems, somatic embryogenesis, initiation of inflorescence, and fruit development. Because a role for polyamines has been implicated in many of these processes, it is important to determine the effects of polyamines on PGR, as well as the effect of PGR on polyamines.

An increase in polyamine biosynthesis and titer, in
specific tissues, accompanies growth induced by the PGR. Dai et al. (1982) observed an increase in both ADC activity and polyamine levels in conjunction with GA-induced internode elongation in dwarf mutants of Pisum sativum. Using the same system, Kaur-Sawhney et al. (1985) demonstrated a 70% inhibition of GA induced internode elongation in response to DFMA. A direct correlation between polyamine quantity and amount of internode elongation was observed using four different internode phenotype mutants of Pisum (Smith et al. 1985). The application of DFMO or DFMA to these mutants inhibited internode elongation as well as the increase in polyamine titers. In germinating barley seeds, an increase in ODC activity was observed in response to treatment with GA (Kyriakidis 1983).

Cytokinins have been observed to increase polyamine levels in cotyledons of lettuce (Cho 1983) and cucumber (Suresh et al. 1978). In pea, Palavan et al. (1984) observed an increase in ADC activity in response to BAP. Similarly, addition of low quantities of IAA [1 μM] to tobacco cell cultures resulted in an increase in putrescine levels (Palazon et al. 1987) and addition of 2,4-D to dormant tuber tissue of Helianthus tuberosus induced the biosynthesis of polyamines (Serafini-Fracassini et al. 1980, Bagni et al. 1981). High levels of IAA [10 μM] resulted in lower levels of putrescine in tobacco, and reduced ADC
activity in red-light stimulated terminal buds of pea (Palazon et al. 1987, Palavan et al. 1984, Pinol et al. 1987). In contrast, Kyriakidis (1983) observed a stimulation of ODC activity with 1mM IAA, and no stimulation at a 1uM concentration.

The PGR which has been the focus of a majority of work on the interaction with polyamines is ethylene. Ethylene is produced by all plant tissues and is regulated by many developmental and environmental stimuli (Yang and Hoffman 1984). The biosynthesis of ethylene occurs during germination, fruit maturation, leaf abscission and senescence. A variety of factors including wounding, stress, and chemical agents, i.e., specific PGRs, are known to stimulate ethylene biosynthesis. Because polyamines are antagonistic to a number of the physiological effects of ethylene, and biosynthesis of ethylene and polyamines share a common precursor (Fig. 5), a great deal of effort has been made to analyze interactions between these compounds and their effect on plant growth and development (Apelbaum et al. 1981, Roberts et al. 1984, Galston and Kaur-Sawhney 1987).

The most common belief is that polyamines and ethylene may regulate each other's biosynthesis, either directly or by metabolic competition for SAM. In addition, Miyazaki and Yang (1987) suggested that polyamines and ethylene biosynthesis must both allow for recycling of methylthio-
adenosine and that this process may be as significant as the competition for the propylamine group from SAM. Polyamines inhibit ethylene formation in a number of plant tissues including apple fruits, bean and tobacco leaf explants (Apelbaum et al. 1981), *Tradescantia* petals and mung bean hypocotyls (Suttle 1981). Likewise, ethylene has been shown to inhibit polyamine synthesis in pea apical meristems (Apelbaum et al. 1985). In response to ethylene, the levels of ADC activity decreased by 90% within 18 h. Reducing ethylene levels resulted in a 30-50% increase in ADC activity. In cut carnation flowers, inhibitors of polyamine biosynthesis induced an increase in ethylene synthesis and inhibition of ethylene synthesis by aminooxyacetic acid resulted in an increase in spermine levels (Roberts et al. 1984). A variety of treatments used to inhibit ethylene synthesis in orange peel discs shunted \(^{14}\text{C}-\text{propylamine}\) groups from labeled methionine to spermidine indicating a shared precursor pool (Even-Chen et al. 1982). Two alternative forms of interaction between polyamines and ethylene biosynthesis have been proposed recently. Drolet et al. (1986) demonstrated that polyamines could act as scavengers of free radicals and could inhibit the superoxide-dependent conversion of ACC to ethylene. Likewise, Bors et al. (1989) also observed the ability of putrescine conjugates to act as scavengers of free radicals. Although there is ample evidence that a connection exists
Figure 5. Ethylene Biosynthesis and Interaction with Polyamine Biosynthesis
between polyamine biosynthesis and ethylene synthesis, it is
by no means exclusively antagonistic. A study of chilling
effects on cucumber seedlings demonstrated that inhibition
of ACC induction by AOA did not cause an increase in
polyamine levels. Indeed, an increase in spermidine levels
occurred in conjunction with elevated ACC levels in response
to chilling stress (Wang 1987). Similar situations in which
the formation of ACC and polyamines increased
simultaneously have been observed in apple and cherry buds
(Wang et al. 1985a, Wang et al. 1985b). There are other
systems in which polyamine synthesis does not seem to
compete with ethylene synthesis (Cohen and Kende 1986,
Kushad et al. 1988) and these contradictions to the
suggestions of antagonistic competition between the two may
indicate a far more complex interaction. A careful analysis
of the synthesis of ethylene and polyamines along with
changes in precursor and degradation product pools is
needed to determine the level of this connection, if any.

Recently a spate of research has been carried out to
investigate the role of polyamines in embryogenesis in the
carrot suspension culture system (Montague et al. 1978, 1979;
Feinberg et al. 1984, Feirer et al. 1984, Fallon and
Phillips 1988, Minocha 1988, Mengoli et al. 1987 and 1989,
Robie and Minocha, 1989), in alfalfa (Meijer and Simmonds
1988) and in eggplant (Fobert and Webb 1988). In carrot cell
cultures, increased ADC activity occurred during
embryogenesis which resulted in increased cellular polyamine
levels (Montague et al. 1978, 1979, Robie and Minocha 1989).
ODC activity remained at a low level for the first 10-12
days of embryogenesis followed by a sharp rise which
coincided with the appearance of mature, green plantlets
(Robie and Minocha 1989). Addition of DFMA to 2,4-D-free
medium resulted in an inhibition of ADC activity, as well as
embryogenesis, whereas inhibition of ODC with DFMO had no
effect on embryogenesis in medium which lacked 2,4-D (Feirer
et al. 1984). Mengoli et al. (1987) reported increased
levels of putrescine and spermidine in response to DFMO and
2,4-D. Robie and Minocha (1989) also observed an increase
in ADC activity and polyamine biosynthesis in response to
DFMO. Even more interesting was their observation of
embryogenesis which occurred in the presence of 2,4-D in
response to addition of DFMO to the medium. This DFMO
induced reversal of the 2,4-D inhibition of embryogenesis is
the first report of normal embryogenesis in the presence of
2,4-D.

The working hypothesis on the mode of action of 2,4-D in
the inhibition of somatic embryogenesis involves the
induction of ethylene synthesis by auxins contained in the
growth medium (Minocha 1988). Ethylene has been observed to
inhibit embryogenesis in medium lacking 2,4-D (Tisserat and
Murashige 1977, Wochok and Wetherell 1971, Verma and Tarka
1985). Also, endogenous levels of ethylene in many plant
tissues are regulated by internal levels of auxin (Sisler and Yang, 1984). Exogenously applied auxin has also been shown to stimulate ethylene synthesis, via the conversion of SAM to ACC (Yang and Hoffman 1984). Although the exact mechanisms have not been established for inhibition of embryogenesis by auxin, an increase in ethylene synthesis appears to be a viable explanation at this point.

The hypothesis suggested by Robie and Minocha (1989) to explain the auxin effects on somatic embryogenesis in carrot and its reversal by DFMO has three postulates: (1) ethylene is a major suppressor of embryogenesis and its production is promoted by auxin; (2) continued polyamine biosynthesis is required for cell division and embryogenesis to proceed; and (3) the increased biosynthesis of polyamines, especially spermidine and spermine, induced by DFMO probably "steals away" SAM from ACC synthase causing a reduction in ethylene synthesis and inhibition of embryogenesis (Minocha 1988, Robie and Minocha, 1989). As discussed earlier, there exists a complex interaction between ethylene synthesis and polyamine synthesis. It is of considerable interest to determine if this interaction carries over to embryogenesis. Further research is being done to investigate the interactions between polyamine and ethylene biosynthesis and their effects on embryogenesis.
Polyamines and Organogenesis

In general, increased concentrations of putrescine, spermidine, and spermine occur prior to root primordia development both *in vivo* and in response to exogenous auxin *in vitro*. Inhibition of polyamine biosynthesis, in particular via MGBG inhibition of SAMDC and DFMA inhibition of ADC in *Phaseolus* (Jarvis et al. 1985, Kakkar and Rai 1987) and DFMO inhibition of ODC in *Malus* (Wang and Faust 1986), resulted in inhibition of both root primordia initiation and growth of existing roots. Further evidence linking polyamines with *in vitro* root development has been shown in *Phaseolus* (Palavan-Unsal), *Sideritus* (Sanchez-Gras and Segura 1988), and Mung bean (Jarvis et al. 1985). Application of spermine or spermidine to rooting plants resulted in an increase in the number of roots formed. Investigations in *in vitro* systems have shown that exogenous spermidine acted in a synergistic manner with applied auxins to enhance rooting in mung bean (Jarvis et al. 1985) and in *Sideritus* (Sanchez-Gras and Segura 1988). In tobacco, Tiburcio et al. (1987) observed root production to be inversely related to putrescine concentration. Treatment with DFMA reduced putrescine levels resulting in increased root formation.

Very little research has been reported on the role of polyamines in shoot formation. In *Nicotiana tabacum* var. Xanthi, Burtin et al. (1989) observed a DFMO induced
increase in shoot formation and fresh weight in callus grown on shooting media. In addition, DFMO also induced shoot formation on callus maintenance media.

The use of thin layer tissue culture technique (Tran Than Van, 1973) in tobacco to directly produce flower buds has yielded data which indicate a strong correlation between polyamines and/or polyamine conjugates and floral bud initiation (Torrigiani et al. 1987, Kaur-Sawhney et al. 1988). Kaur-Sawhney et al. (1988), observed a 4.5-fold increase in spermidine levels in explants cultured on floral bud medium (FBM) vs. vegetative bud medium (VBM). Based on their observations, they added 0.5-5 mM spermidine to explants on VBM resulting in a reduction in vegetative bud formation and shift towards floral bud formation. Treatment of explants on FBM with 10-20 mM Cyclohexylammonium phosphate (CHAP) resulted in a 75% reduction in spermidine concentration concurrent with a shift from floral to vegetative bud formation. This effect was reversible by addition of spermidine along with CHAP. Although the shift from floral to vegetative bud formation can be influenced by other factors, e.g., pH (Tran Than Van 1981), cytokinin type and concentration (Tran Than Van 1981) and oligosachharins (Tran Than Van et al. 1985), the shift due specifically to changes in spermidine levels is indicative of a regulatory role for polyamines in floral bud initiation.

Interference with normal polyamine metabolism via the use
of inhibitors can also disrupt normal floral development. Heimer and Mizrahi (1982) observed high levels of ODC activity in developing tomato flowers. Treatment with DFMO blocked the normal development of the flowers. Likewise, Slocum and Galston (1985) observed an increase in ODC activity during tobacco ovary development. Inhibition of the ODC activity with DFMO interfered with normal ovary development. In addition, they observed that 90% of the total tobacco ovary polyamine content was found in the conjugated form.

Martin-Tanguy et al. (1982) observed a complete absence of polyamine conjugates in the anthers of male sterile plants of *Zea mays* as compared to high levels in control plants. It was of considerable interest that the lack of polyamine conjugates was restricted only to the anthers of the male-sterile plants and not to the rest of the plant organs.

Malmberg and his coworkers have done a great deal of research on mutant tobacco cell lines which are resistant to MGBG and DFMO (Malmberg 1980, Malmberg and McIndoo 1984, Hiatt et al. 1986, Malmberg and Rose 1987, Malmberg and Hiatt 1989, Evans and Malmberg 1989). Not all of the resistant cell lines are capable of producing flowers. Those which can, produce flowers with an abnormal floral morphology including petaloid anthers, stigmoid anthers, abnormal ovules, etc. Generally, these floral aberrations
are so severe that the plants are sterile.

Observations by Altman et al. (1977) on oat leaf protoplasts indicate that 0.1-1 mM spermidine stabilizes the protoplasts against progressive senescence which occurs after protoplast isolation. In a later study on oat leaf senescence (Kaur-Sawhney and Galston 1979), an immediate rise in ribonuclease activity was observed after leaf excision. This was followed by an increase in protease activity after 6 hours and the onset of chlorophyll breakdown after 24 hours. Each of these events could be completely inhibited by 0.1-1.0 mM polyamine with a correlation between increased polyamine charge and increased anti-senescence activity, i.e., spermine exhibited the highest activity and putrescine the lowest activity. Fuhrer et al. (1982) later demonstrated the ability of exogenously applied polyamines to inhibit the massive increase in ethylene synthesis associated with oat leaf senescence. At the time, they suggested that exogenous polyamines initially bind to the cell membrane and then inhibit ethylene synthesis.

Roberts et al. (1986) demonstrated that polyamines associate with the membrane lipids and substantially reduce membrane fluidity. Kaur-Sawhney and Galston (1979) observed that Ca²⁺ ions counteracted the ability of polyamines to stabilize chlorophyll levels in senescing leaves by competing for charged sites on the membrane lipids. Inhibition of ethylene synthesis could be due to the free radical
scavenging properties of polyamines (Drolet et al. 1986, Bors et al. 1989). In fact, the conversion of ACC to ethylene, via the membrane associated ethylene forming enzyme complex, is superoxide dependent and the free radical scavenging properties of polyamines can interfere with the formation of superoxides. Therefore, in theory, it would be feasible for polyamines to inhibit ethylene biosynthesis by binding to the membrane, preventing lipid peroxidation, and quenching the free radicals needed for the conversion of ACC to ethylene.

Dai and Galston (1981) were the first to observe the organ specific changes in ADC activity in response to red light. On Exposing etiolated peas to red light, they demonstrated a two-fold increase in ADC activity in buds which occurred simultaneously with a two-fold decrease in ADC activity in epicotyls. Goren et al. (1982a) observed an increase in polyamine levels in buds as well as a stimulation of bud growth in red light-illuminated peas. The red light treatment inhibited internodal growth and resulted in reduced levels of polyamines. In addition, Goren et al. (1982b) demonstrated that the polyamine titers did not change in response to cell growth, rather they changed in response to red light and stimulated cell growth. Illumination of the plants with far red light reversed the red light induced effects on polyamine synthesis and cell growth. Further work by Galston et al. (1983) corroborated
earlier findings that the effects of red light on polyamine synthesis were direct, and not in response to increased cell growth.

POLYAMINES AND PLANT STRESS

An increase in putrescine levels as a result of biotic and abiotic stress has been observed in many plants. Abiotic stress conditions such as low external pH (Smith and Sinclair 1967, Young and Galston 1983), NH$_4^+$ feeding (Kato 1980), high salinity (Stogonov 1973), osmotic shock (Flores and Galston 1982, 1984, Matsuda 1984), nutrient stress (Basso and Smith 1974) and low temperature (Wang 1987, Kramer and Wang 1990) all result in an increase in cellular putrescine levels. The increase in putrescine concentration results from de novo synthesis and activation of ADC in most cases (Young and Galston 1983, Flores and Galston 1984, Matsuda 1984). However, in response to low temperature, ODC appears to be the preferred pathway for putrescine synthesis (Wang 1987, Kramer and Wang 1990).

A number of possibilities have been suggested to account for the accumulation of putrescine in response to various abiotic stresses. In cases where stress affects the cells ionic balance, it has been suggested that intracellular putrescine concentrations modulate fluxes in H$^+$ levels (Smith 1971). In stress response where there is an accumulation of ammonia, arginine synthesis and conversion
to putrescine is a viable pathway for ammonia detoxification (Rabe and Lovatt 1986).

Biotic stress, or infection with a pathogen, has also been shown to cause altered polyamine metabolism. However, biotic stress can evoke a number of different changes in polyamine synthesis and accumulation. Infection by several different fungi, bacteria, and viruses results in increased polyamine levels. Protoplasts from Chinese cabbage leaves infected with Turnip Yellow Mosaic Virus exhibit 3-4 fold increase in spermidine levels as compared to controls (Cohen et al. 1981). In brown-rust infected barley plants, chlorophyll exists in localized areas, of otherwise chlorotic leaves, known as "green islands". Spermidine levels within these tissues were found to be 6-7 times higher than those found in control tissues (Greenland and Lewis 1984). In Scorzonera hispanica, tumors caused by infection with Agrobacterium tumefaciens contained 100 times the level of putrescine as compared to control tissues (Bagni et al. 1972). In addition to increases in free polyamine levels, increases in polyamine conjugates also occur in response to infection by a plant pathogen. In barley seedlings, the production of the antifungal hortadines, which are dimers of coumaroylagmatine, confers resistance to infection by Helminthosporium sativum (Smith et al. 1983). In rust resistant wheat cultivars, infection with the rust pathogen results in the accumulation of
hydroxycinnamic acid amide conjugates of 2-hydroxyputrescine (Samborski and Rohringer 1970). These putrescine conjugates are classified as phytoalexins and inhibit pathogenesis. The role of polyamines in disease resistance, or response to biotic stress has not been investigated thoroughly. However, the universal increase in polyamine levels in response to both abiotic and biotic stress suggests a conserved process for plant stress management.

GENETIC MANIPULATION OF POLYAMINE METABOLISM

It is obvious from the preceding sections that polyamines play an important role in a variety of metabolic and developmental processes in plants. However, most of the functions attributed to polyamines are mere correlations and, thus, only suggestive of an underlying causative role. The conflicting data and the difficulty in determining cause or effect relationships between polyamine biosynthesis and a particular plant response have understandably generated a great deal of confusion.

A part of the reason for this confusion is that most of this work is based upon the use of inhibitors of polyamine biosynthesis. Inhibition of enzyme activity, either substantially or partially, is then correlated with an observed physiological or morphological response. The problem with many of these studies is the inability to determine if the alterations observed in response to the
inhibitor are a direct result of the reduced polyamine synthesis, or if the reduced polyamine synthesis is a result of some confounding effect of the inhibitor. This confusion between cause and effect, along with the inability to experimentally promote the biosynthesis of a specific polyamine, independent of other variables, has made it difficult to determine polyamine function with certainty.

The techniques of molecular biology have recently become available for application toward the induction or suppression of specific genes in all organisms. These techniques are equally applicable to modulate the metabolic pathways and thus to alter specific reactions/pathways without altering other cellular metabolites. With this in mind, it would be theoretically possible to investigate the effect of increased ODC activity, via foreign gene transfer, on polyamine biosynthesis and accumulation in order to better understand the role of polyamines in plant developmental processes. The following is a brief review on the ODC coding genes available and the methodologies for inserting foreign genes into the plant genome.

The gene coding for ODC has been isolated and cloned from a number of organisms including mouse (McConlogue et al. 1984, Kahana and Nathans 1984, 1985, Gupta and Coffino 1985, Brabant et al. 1988, Coffino and Chen 1988), rat (Kranen et al. 1987), yeast (Fonzi and Sypherd 1987), trypanosomes (Phillips et al. 1987), hamster (Srinivasan et al. 1987),
human (Leinonen et al. 1987) and bacteria (Tabor and Tabor 1983). No report on the isolation and cloning of a plant ODC gene has been published yet. The following is a brief overview of the information currently available concerning the individual ODC genes which have been characterized.

**Murine ODC gene**

In 1984, four separate research groups reported the isolation of cDNAs which coded for murine ODC (McConlogue et al. 1984, Kahana and Nathans 1984, Kontula et al. 1984, and Berger et al. 1984). McConlogue et al. (1984) used a mutant cell line (S49 mouse lymphoma) which produced ODC as its major protein. Using cDNA cloning and mRNA hybrid selection followed by *in vitro* translation, two cDNAs (1.6 and 1.95 Kb) were isolated. The sequences in these cDNAs overlapped and between them they contained the entire coding region for ODC. Kontula et al. (1984) reported the isolation of a cDNA from kidney of androgen-treated mice. The cDNA directed cell-free synthesis of a polypeptide which immunoprecipitated with ODC antiserum. This polypeptide co-migrated on a SDS/polyacrylamide gel with purified ODC isolated from the mouse kidney. Later that year, Kahana and Nathans (1984) reported the isolation of several cDNAs corresponding to parts of the ODC mRNA from a mouse myeloma cell line with an amplified ODC gene which overproduced ODC mRNA. The following year, Kahana and Nathans (1985) as well as Gupta
and Coffino (1985) published the complete sequence for the murine ODC cDNA (Fig. 6).

The cDNA derived from ODC mRNA contains 2465 nucleotides consisting of a 737 nucleotide-long 5' noncoding segment, a coding segment of 1383 nucleotides terminated by a TAG triplet, and a 342-nucleotide 3' noncoding segment containing a polyadenylation site (AATAAA). Hickok et al. (1986) determined that ODC mRNA in mouse kidney was a mixture of two species with molecular sizes of approximately 2.2 Kb and 2.7 Kb. The cDNAs derived from the 2.7 Kb and 2.2 Kb mRNAs were identical in sequence except that the former contained an additional 429 nucleotides at the 3' end including a second polyadenylation site. It was suggested that a single gene could be responsible for the size heterogeneity in the mRNA. In 1988, Coffino and Chen published the complete sequence of the mouse ODC gene. The gene is approximately 6.2 Kb in length, contains 11 introns and two polyadenylation sites.

**Rat ODC gene**

Kranen et al. (1987) isolated the ODC cDNA from a cDNA library derived from testosterone induced rat kidneys. The rat cDNA contains 2442-nucleotides and consists of a 442-nucleotide 5' leader sequence, a 1383-nucleotide coding sequence terminated by a TAG triplet, and a 633-nucleotide 3' noncoding sequence containing two polyadenylation sites.
Figure 6. Sequence of nucleotides and corresponding amino acid residues of murine ODC cDNA (Kahana and Nathans, 1985)

AGC TTT ACT AAG GAC GAG TTT GAC TGC CAC ATC CTT GAT GAA GGC TTT
Ser Phe Thr Lys Asp Glu Phe Asp Cys His Ile Leu Asp Glu Gly Phe

ACT GCT AAG GAC ATT CTG GAC CAA AAA ATC ATT GAA GTC TCT TCC TCT
Thr Ala Lys Asp Ala Glu Ile Asn Leu Glu Val Ser Ser

GAC GAT AAG GAT GCG TTC TAT GTT GGC GAC CTC GGA GAC ATT CTA AAG
Asp Asp Ala Phe Tyr Val Ala Asp Leu Gly Asp Ile Leu Lys

CAT CTG AGG TGG CTA AAA GCT CTT CCC CGC GTC ACT CCC TTT TAC GCA
His Leu Arg Trp Leu Ala Leu Pro Arg Val Thr Phe Tyr Ala

GTC AAG TGT AAC GAT AGC AGA GCC ATA GAT AGC ACC CTA GCT GCC ATT
Val Lys Cys Asn Asp Ser Arg Ala Ile Val Ser Thr Leu Ala Ala Ile

GGG ACA GGA TTT GAC TGT GCA AGC AAG ACT GAA ATA CAG TGG GTG CAG
Gly Thr Gly Phe Asp Cys Ala Ser Lys Thr Glu Ile Gln Leu Val Gin

GGG CCT GGG GTT CCT GCA GAG AGC GTT ATC TAT GCA AAT CCT TGT AAG
Gly Leu Gly Val Pro Ala Glu Arg Val Ile Tyr Ala Asn Pro Cys Lys

CAA GTC TCT CAA ATC AAG TAT GCT GCC AGT AAC GGA GTC CAG ATG AGT
Gln Val Ser Ser Ala Tyr Ala Ala Ser Asn Gly Val Gln Met Met

ACT TTT GAC AGT GAA ATT GAA TCT AGT AAA GTC GCC AGA GCA CAT CCA
Thr Phe Asp Ser Glu Ile Glu Leu Met Lys Val Ala Arg Ala His Pro

AAG GCA AAG TTG GGT CTA CGG ATT GCC ACT GAT GAT TCC AAA GCT GTC
Lys Ala Lys Leu Arg Ile Ala Thr Asp Ser Lys Ala Val

TGT CGC CTC AGT GTT AAG TTT GGT GCC ACA CTC AAA ACC AGC AGG CTT
Cys Arg Leu Ser Val Lys Phe Gly Ala Thr Leu Lys Thr Ser Arg Leu

CTC TTG GAA CGG CAA AAA GAG CTA ATT GAC GTC ATT GGT GTG AGC
Leu Leu Glu Arg Ala Lys Glu Leu Asn Ile Asp Val Ile Gly Val Ser

TTC CAT GTG GCC AGT GGA TGT ACT GAT CCT GAT ACC TTC GTT CAG GCA
Phe His Val Gly Ser Thr Asp Pro Thr Phe Gly Val Ala

residues of murine ODC cDNA (Kahana and Nathans, 1985)
GTG TCG GAT GCC CGC TGT GTG TTT GAC ATG GCA ACA GAA GTT GGT TTC
Val Ser Asp Ala Arg Cys Val Phe Asp Met Ala Thr Glu Val Val Gly Phe

AGC ATG CAT CTG CTT GAT ATT GGT GGT GCC TTT CCT GGA TCT GAA GAT
Ser Met His Leu Leu Asp Ile Gly Gly Gly Phe Pro Gly Ser Glu Asp

ACA AAG CTT AAA TTT GAA GAG ATC ACC AGT GTA ATC AAC CCA GCT CTG
Thr Lys Leu Lys Phe Glu Glu Ile Thr Ser Val Ile Asn Pro Ala Leu

GAC AAG TAC TTC CCA TCA GAC TCT GGA GTG AGA ATC ATA GCT GAG CCA
Asp Lys Tyr Phe Pro Ser Asp Ser Gly Val Arg Ile Ile Ala Glu Pro

GCC AGA TAC TAT GTC GCA TCA GCT TTC ACG CTT GCA GTC AAC ATC ATT
Gly Arg Tyr Tyr Val Ala Ser Ala Phe Thr Leu Ala Val Asn Ile Ile

GCC AAA AAA ACC GTG TGG AAG GAG CAG CCC GGC TCT GAC GAT GAA GAT
Ala Lys Lys Thr Val Trp Val Tyr Tyr Val Asn Asp Asp Glu Asp

GAG TCA AAT GAA CAA ACC TTC ATG TAT TAT GTG AAT GAT GGA GTA TAT
Glu Ser Asn Glu Gln Thr Phe Met Tyr Tyr Val Asn Asp Gly Val Tyr

GGA TCA TTT AAC TGC ATT CTT TAT GAT CAT GCC CAT GTG AAG GCC CTG
Gly Ser Phe Asn Cys Ile Tyr Asp His Ala His Val Lys Ala Leu

CTG CAG AAG AGA CCC AAG CCA GAC GAG AAG TAT TAC TCA TCC AGC ATC
Leu Gin Lys Arg Pro Lys Pro Asp Glu Lys Tyr Tyr Ser Ser Ser Ile

TGG GGA CCA ACA TGT GAT GCC CTT GAT CCG ATC GTG GAG CGC TGT AAC
Trp Gly Pro Thr Cys Asp Gly Leu Asp Arg Ile Val Glu Arg Cys Asn

CTG CCT GAA ATG CAT GTG GGT GAT TGG ATG CTG TTT GAG AAC ATG GGT
Leu Pro Glu Met His Val Gly Asp Trp Met Leu Phe Glu Asn Met Gly

GCA TAC ACC GTT GCT GCT GCT TCT ACT TTC AAT GGG TTC CAG AGG CCA
Ala Tyr Thr Vak Ala Ala Ala Ser Thr Phe Asn Gly Phe Glu Arg Pro

AAC ATC TAC TAT GTA ATG TCA CGG CCA ATG TGG CAA CTC ATG AAA CAG
Asn Ile Tyr Tyr Met Ser Arg Pro Met Trp Gin Leu Met Lys Gin

ATC CAG AGC CAT GGC TTC CCG CAG GAG GTG GAG GAG CAG CAT GAT GCC
Ile Gin Ser His Phe Pro Pro Pro Glu Val Glu Glu Glu Asp Asp Gly

ACG CTG CCC ATG TCT TGT GCC CAG GAG AGC GGG ATG GAC CGT CAC CCT
Thr Leu Pro Met Ser Cys Ala Gin Glu Ser Gly Met Asp Arg His Pro

GCA GCC TGT GCT TCT GCT AGG ATC AAT GTG TAG ATGCCATTCTTGTAGCTCT
Ala Ala Cys Ala Ser Ala Ser Ala Ser Ala Asn Val *

CCTGCAAGTTTAGGGATTTAAGCGATTTTGGGAAAGCTACCATTTACTGCTAGTTTGGA
TGCTTTGTGAGATAGGTTGGCAGCAAAATGCAATGGAAGCGCTAGGATGGGGGTTCACA
CTTACTGTGTTCTATGAGCTTTATTTGTGACCCCATTTGTGAAATAAATATCTTTAAATAA
The rat-mouse cDNA homology is most pronounced in the 461 amino acid coding region, amounting to 94% and 97% at the DNA and protein levels, respectively.

**Trypanosomal ODC gene**

The ODC gene from the African trypanosome (*Trypanosoma brucei*) was isolated from a genomic library by probing with a murine ODC cDNA (Phillips et al. 1987). A 2.8 Kb segment containing the intact gene was partially sequenced and found to contain an open reading frame for 445 amino acids showing a 61.5% homology with the corresponding sequence in the mouse enzyme. The only major discrepancies between the two enzymes are the addition of a 20-amino acid N-terminal peptide and the deletion of a 36-amino acid C-terminal peptide in the *T. brucei* polypeptide. The deletion of the 36-amino acid N-terminal peptide, which contains a PEST sequence in the murine ODC, was suggested as an explanation for the slow turnover of the trypanosomal ODC.

**Yeast ODC gene**

The gene coding for ODC was isolated from a genomic library constructed from *Saccharomyces cerevisiae* (Fonzi and Sypherd 1987). Whereas the ODC mRNA is approximately 2 Kb in length, the open reading frame is only 1398 nucleotides long. This implies that the ODC mRNA contains about 600 nucleotides of flanking sequences which is larger than
normally found for other mRNAs in yeast. Further studies showed the 5'-noncoding region to contain approximately 225 nucleotides and the 3'-noncoding region to contain approximately 375 nucleotides. The predicted amino acid sequence of the yeast ODC indicates a 40% homology with the murine ODC. In addition, Chou-Fasman predictions of the secondary structure of these two enzymes suggest that the secondary structure is highly conserved.

**Hamster ODC gene**

The ODC gene was isolated from a hydroxyurea-resistant derivative of the Chinese hamster lung cell line V79/V6 (Srinivasan et al., 1987). A cDNA library was prepared from mRNA obtained from the resistant cell line. The cDNA contains an open reading frame of 461 amino acids and the nucleotide sequence of this coding region is approximately 92% homologous to the mouse cDNA. The high degree of homology extends into the 3' nontranslated region where the hamster cDNA differs from the mouse by only 21 nucleotides. Once again, there are two polyadenylation signals in the 3'-nontranslated region. The hamster cDNA isolated by these workers lacked the 5' end of the coding region which contains the start codon, therefore, it was not possible to determine sequence homology in the 5'-nontranslated region.
Bacterial ODC gene

The gene coding for ODC in *E. coli* was first isolated by Tabor et al. (1983). The coding region contains approximately 1400 nucleotides. Information on the 5' and 3' nontranslated regions is not available nor is any information available on sequence homology with various eukaryotic ODC genes.

Human ODC gene

The structure of the human ODC cDNA isolated from IgG-myeloma cells shows more than 90% homology with the murine ODC cDNA (Leinonen et al., 1987). The human mRNA is 2.3 Kb in length and unlike other mammalian ODC mRNAs, only appears as a single RNA species.

Heterologous expression of various ODC genes has been used to complement ODC deficient mutant cell lines and organisms (Ghoda et al. 1989, Brabant et al. 1988, Fonzi and Sypherd 1985). Ghoda et al. (1989) and Brabant et al. (1988) expressed a variety of murine ODC cDNAs and genomic clones in a CHO cell line (C55.7) devoid of endogenous ODC activity. Fonzi and Sypherd (1985) expressed the yeast ODC gene in an *E. coli* mutant lacking ODC activity. These systems are ideal because there is no endogenous ODC activity in the recipient cell lines/organisms prior to transformation. Therefore, detectable ODC activity is the result of expression of the transferred ODC gene. In plants, there
are no ODC deficient cell lines to use as recipients for a transferred ODC gene. Thus, verification of expression of a transferred ODC gene is a difficult task as one must differentiate between the native and the foreign gene product.

Hamill et al. (1989) produced transgenic *N. rustica* roots containing the yeast ODC gene via *A. rhizogenes* mediated transformation. The primary objective of their work was to modulate nicotine synthesis by increasing the cellular pool of putrescine. They detected elevated levels of ODC activity in 3 of the 6 root culture lines, resulting from independent transformation events, as compared to controls. As expected, increased ODC activity resulted in an elevation of nicotine levels in the root tissue. However, the increase in nicotine levels was less than the increase in ODC activity leading them to suggest that ODC was not the rate-limiting step in nicotine biosynthesis. As the focus of this research was on nicotine synthesis and not organogenesis, embryogenesis, etc., there was no attempt to regenerate plants from the root cultures. This research is the only reported instance of heterologous expression of an ODC gene in a plant system and has confirmed the feasibility of our approach to modulate polyamine metabolism in plants via foreign gene transfer.

TRANSFORMATION OF PLANTS

Successful transformation, i.e., transfer, integration
and expression of a foreign gene in plants requires the transferred gene to contain a promoter sequence recognized by the plant, an intact in-frame coding sequence, and a transcription termination sequence recognized by the plant. There are a number of promoters and transcription termination sequences available which are recognized by plants (Table 3). Utilizing these components a number of chimaeric genes have been constructed and successfully expressed in plants (For review see Weising et al. 1988, Kuhlemeier et al. 1987, Zambryski 1987). Currently, there are a variety of methods available to successfully transfer defined DNA segments into plant cells including; Agrobacterium-mediated transformation, direct uptake of DNA, electroporation, microinjection, liposomes, viral vectors, and microprojectiles. The method of choice most often depends on the species of interest. However, accessibility to specialized and often expensive equipment can be an additional factor.

**Agrobacterium-mediated transformation**  
*Agrobacterium tumefaciens* (Rhizobiaceae) is a gram negative soilborne, facultative, phytopathogen which is the causal agent of Crown Gall disease on most dicots and gymnosperms as well as a few monocots (De Cleene and De Lay 1976, Weising et al. 1988). These bacteria contain a large plasmid called Ti plasmid (Fig. 7) which is capable of inducing neoplastic
Table 3. Promoters and Termination Sequences Available for Construction of Chimeric Genes to be Expressed in Plants.

<table>
<thead>
<tr>
<th>Promoter</th>
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<td>PNOS*</td>
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<td>Herrera-Estrella et al. 1983</td>
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<td>PSSb</td>
<td>Pisum sativum</td>
<td>Herrera-Estrella et al. 1984</td>
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<tr>
<td>PCABc</td>
<td>Pisum sativum</td>
<td>Simpson et al. 1985</td>
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<td>P35Sd</td>
<td>CaMV</td>
<td>Odell et al. 1985</td>
</tr>
<tr>
<td>PCHS*</td>
<td>Phaseolus vulgaris</td>
<td>Lamb et al.</td>
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Terminator

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<tr>
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</tr>
<tr>
<td>tts5h</td>
<td>A. tumefaciens</td>
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</table>

a- Promoter for the nopaline synthase gene  
b- Promoter for the small subunit of RUBISCO gene  
c- Promoter for the chlorophyll a/b binding protein gene  
d- Promoter for the 35S RNA of CaMV  
e- Promoter for the Chalcone synthase gene  
f- Transcription termination region for nopaline synthase  
g- Transcription termination region for Octopine synthase  
h- Transcription termination region for gene 5 of pTiA6
growth resulting in the so-called crown gall tumors. The Ti plasmid is approximately 200 Kb long and contains four major regions: 1) the T-DNA, 2) the replication region, 3) the opine catabolizing region, and 4) the virulence region. The molecular mechanism of crown gall formation is the transfer and stable integration of a well-defined segment of the Ti plasmid called T-DNA (transferred DNA) into the plant genome. The T-DNA is delineated by two border sequences, TR and TL, which contain 25 bp repeat sequences analogous to transposable element border sequences (Geilan et al. 1984). As far as we know, the T-DNA is randomly inserted into the plant nuclear genome and induces rapid, uncontrolled plant cell divisions, i.e., tumor-like growth. This occurs via the expression of genes, located in the T-DNA region, which are directly involved in auxin and cytokinin biosynthesis. The T-DNA genes which are responsible for tumor formation are \texttt{tms1}, \texttt{tms2} and \texttt{tmr} (Klee et al. 1987, Melchers and Hooykaas, 1987). In addition to producing excessive amounts of auxin and cytokinin, the crown gall tumor cells are distinguished by the T-DNA-directed synthesis of a class of compounds known as opines (Nester and Kosuge 1981). Opines are derivatives of the amino acid arginine and are not normally found in the plant cells. It is believed that the opines serve as a carbon-nitrogen source for the invading bacteria (Bomhoff et al. 1976, Montoya et al. 1977).

In vivo, the infection process requires wounding of the
Figure 7. Functionally characterized regions of a nopaline type Ti plasmid. NOC, nopaline catabolism; REP, plasmid replication; VIR, virulence region; LB, left border sequence; RB, right border sequence; T-DNA, DNA sequence which is replicated and transferred into the plant cell. Genes within the T-DNA: AUX, genes involved with auxin synthesis (tms1 and tms2); CYT, gene involved with cytokinin synthesis (tmr); NOS, gene involved with nopaline synthesis (nopaline synthase).
plant tissue (Kahl 1982). The virulence (vir) region of the Ti plasmid is responsible for recognizing chemical signals, e.g., acetosyringone and hydroxyacetosyringone, released by susceptible, wounded plant cells (Okker et al. 1984, Stachel et al. 1985, 1986). Recognition of the inducer molecules (called signal molecules) activates transcription of the vir operon which initiates the T-DNA transfer process (Fig. 8). After attachment to the cell walls of wound-activated plant cells, Agrobacterium transfers part of its Ti plasmid, the T-DNA, into the host plant cell, where it becomes stably integrated in the nuclear DNA (Chilton et al. 1977, Zambryski 1988, Weising et al. 1988). Several gene loci on the bacterial chromosome (Thomashow et al. 1987, Douglas et al. 1985) in conjunction with the vir genes located on the Ti plasmid (Stachel and Nester 1986) code for functions involved in plant cell recognition and attachment as well as for the excision, transfer and probably the integration of the T-DNA into the target genome (Weising et al. 1988, Zambryski 1988, Kuhlemeier et al. 1987).

Although many researchers recognized the potential usefulness of Agrobacterium-mediated plant transformation, there remained the problem of the tumorous nature of the transformed tissue. It was apparent that the transformed tissue must be able to differentiate in a normal fashion rather than continue growing in an undifferentiated, i.e.,
tumorous fashion. An intensive analysis of the T-DNA region was undertaken by a number of laboratories to identify the nucleotide sequences which coded for the genes responsible for the tumor like growth (Holsters et al., 1980; Garfinkel and Nester, 1980; Garfinkel et al., 1981; Ooms et al., 1980, 1981; De Greve et al., 1981; Leemans et al., 1982; Willmitzer et al., 1982, 1983; Joos et al., 1983; Ream et al., 1983). The results of these studies led to the discovery of three oncogenic genes: \texttt{tms1}, \texttt{tms2}, and \texttt{tmr} which code for tryptophan monooxygenase (\texttt{iaaM}), indole-acetamide hydrolase (\texttt{iaaH}) and isopentenyl transferase (\texttt{ipt}), respectively. The tryptophan monooxygenase and indoleacetamide hydrolase act in conjunction to synthesize the auxin indolacetic acid. The cytokinin, isopentenyl-adenine, is synthesized by isopentenyl transferase. The T-DNA directed synthesis of these plant growth regulators is thus responsible for the tumorous growth of the transformed tissue. Based on the above information, Zambryski et al. (1983) constructed a T-DNA deletion mutant of wild type Ti plasmid C58 which contained only the border regions, TR and TL, and the nopaline synthase gene which codes for opine synthesis. In addition, they inserted a pBR322 sequence between the borders of the mutant T-DNA which made this new Ti plasmid, called pGV3850, a versatile cloning vector (Fig. 9). Using homologous recombination, any DNA sequence cloned in pBR322 could be inserted into the T-DNA region of
Figure 8. T-DNA Transfer Process. The virA gene constitutively expresses a protein product that functions as a transmembrane chemoreceptor for plant wound exudates such as acetosyringone (AS). After the virA regulatory protein detects the presence of AS, it transduces a signal to the constitutively expressed virG protein which results in activation of the virG protein. Activated virG protein initiates transcription of the inducible vir loci. The plant inducible virD encodes for a T-DNA border specific endonuclease. The T-DNA is transcribed from the right border nick to the left border nick (5' to 3') and generates a single stranded, linear T-DNA molecule. The T-DNA strand is transferred and integrated into the plant genome. (Stachel and Zambryski, 1986)
pGV3850 and the cointegrate plasmid could be used for transformation of plants. Following the construction of pGV3850, Agrobacterium-mediated transformation of plant tissues became a routine exercise. Several reports using this method of foreign gene transfer appeared in quick succession (for reviews see Weising et al. 1988, Zambryski 1988).

Although pGV3850 was an effective vector for plant transformation, its large size (200Kb) and the necessity of using homologous recombination to insert the DNA sequences of choice into the T-DNA region, made it a less than ideal vector. In 1984, Bevan et al. constructed the first binary vector for plant transformation. Shortly after that a number of laboratories successfully used a variety of binary vectors for genetic transformation of plants (An et al. 1985; An, 1986a, 1986b, 1987; Klee et al., 1985) Binary vectors contain only cis-acting elements required for plant transformation, including the T-DNA borders, a selectable marker expressible in plants (Kanamycin resistance), cloning sites (e.g., pUC-19 polylinker), and a wide host range replicon (Fig. 13). They are usually about 10Kb in size which makes them easy to manipulate. Other necessary functions, i.e., vir gene products etc., for transformation are provided in trans by a helper Ti plasmid. A DNA sequence of interest can be directly inserted between the T-DNA borders and any cloning or recombinant work can be done
in E. coli. Another benefit to the use of binary vectors is the ability to use a number of different helper Ti plasmids depending upon the plant to be transformed, as the host range of Ti plasmids varies significantly.

In addition to A. tumefaciens, A. rhizogenes has also been used to transform plant tissues (Tepfer, 1984; Hamill et al., 1987; Shahin et al., 1986; Simpson et al., 1986; Stougaard et al., 1987). A. rhizogenes is very similar to A. tumefaciens in that it is a facultative phytopathogen which transfers T-DNA into the host cell. However, instead of forming a tumor, plants infected with A. rhizogenes form numerous hairy roots, hence the term "hairy root disease". A. rhizogenes contains a large plasmid known as the Ri (root inducing) plasmid (Chilton et al, 1982). The Ri plasmid is almost identical to Ti plasmid and in fact, the vir region of either plasmid can act in trans on the other plasmid initiating transfer of the T-DNA. The difference lies in the genes within the T-DNA coding for plant growth regulators. Using binary vectors in combination with the Ri plasmid, A. rhizogenes has been used to successfully transform a number of plants (Hamill et al. 1987). A. rhizogenes, in conjunction with a binary vector system can be a useful tool for plant transformation in view of the extension of the host range which can be transformed with Agrobacterium.

The use of both binary and cointegrative systems for
Agrobacterium-mediated plant transformation is currently the most reliable and widely used method of inserting foreign genes into plants. The major drawback of this approach is the limited host range. Many of the more economically important plant species are monocots and have not been successfully transformed with Agrobacterium. However, recent reports indicate that it may be possible to use A. tumefaciens for the transformation of a number of monocot species (Usami et al. 1988, Weising et al. 1988). Another difficulty associated with this method of transformation is the necessity of eliminating the bacteria once transformation has occurred at which time the bacteria become a contaminant.

Viral vectors

Several plant DNA viruses have received attention as possible vectors for the introduction of foreign genes into plants (Brisson and Hohn, 1986; Buck and Coutts, 1983; Gronenborn et al., 1981; Howell, 1982). In 1984, Brisson et al. demonstrated the transfer and expression of a bacterial marker gene by transformation with cauliflower mosaic virus (CaMV). The CaMV contains 8 Kb of double stranded DNA (Al Ani et al., 1979). However, there are only two open reading frames which can be dispensed with while still retaining viral activity (Howell et al., 1981). This feature limits the size of the DNA which can be packaged into the virus.
Although there are advantages to using viral vectors including, systemic infection and high copy number of genes inserted per cell, there are many disadvantages. As mentioned before, the size of the transferred DNA is limited to the amount of DNA that can be deleted from the virus without inhibiting viral activity and the DNA is not necessarily integrated into the plant genome. In addition, most of the plant viruses are pathogens of the plants in their host range and the secondary effects associated with infection may be deleterious.

**Direct uptake by protoplasts**

Attempts to introduce DNA directly into plants, mainly via pollen or protoplasts, have been made for nearly twenty years. Conclusive evidence for successful plant transformation as documented by Southern blot analysis was first reported in 1982 (Draper et al., 1982; Krens et al., 1982). In both cases, protoplasts were incubated with isolated Ti plasmid DNA either in the presence of PEG and poly-L-ornithine or by using a modified Ca-phosphate co-precipitation technique. Transformants were selected for phytohormone autotrophy and screened for opine synthase activity. Some hormone-independent, opine positive calli were detected but the observed transformation frequencies were very low. A great deal of work has been done in the last eight years to optimize the preparation of competent
protoplasts (Hain et al., 1985; Junker et al., 1987; Lorz et al., 1985; Negrutiu et al., 1987, Paszkowski et al., 1984; Potrykus et al., 1985).

A variation on direct DNA uptake by protoplasts uses an externally applied electrical pulse. This technique, called electroporation, temporarily disrupts the membrane resulting in the formation of transient "pores" that the DNA can pass through (Zimmerman et al., 1974; Langridge et al., 1985; Nishiguchi et al., 1986). This system has been used to transform a variety of plant species with high efficiency (Fromm et al., 1985; Jones et al., 1987; Cocking and Davey, 1987). Electroporation is relatively simple and there is a high degree of control and therefore reproducibility. However, there is the initial cost of the electroporation apparatus which can range from $2000-$4000. While the efficiency of direct uptake is comparatively low, it circumvents any host range limitations associated with viral or bacterial transformation systems. The major drawback of using direct uptake or electroporation is the necessity of being able to regenerate a plant from isolated protoplasts, which is not possible for many desired plant species.

**Microinjection**

Microinjection is one of the most precise techniques for delivery of macromolecules into specific intracellular compartments and has been successfully applied to animal
cells (Wagner et al., 1981). Early attempts at plant cell microinjection were either hampered by the rigid cell wall or the fragility of protoplasts. Although there has been little success in transforming intact plant cells with this method, microinjection has been used to successfully transform microspore-derived embryoids (Neuhaus et al., 1987). The majority of work using microinjection has been done on isolated protoplasts (Crossway et al., 1986; Goodman et al., 1987). Although the transformation efficiency using microinjection is very high and it can be used on any species, the number of cells that can be handled at a given time is quite low. Furthermore, the best success is with protoplasts, and once again, it is a prerequisite to regenerate a mature plant from the protoplasts. In addition, there is the high cost of instrumentation involved in this technique. It has also been shown that plasmid DNA often breaks during the microinjection process as it passes through the submicron injection pipette resulting in integration of plasmid fragments (Steinbiss et al., 1983).

**Liposome-mediated transformation**

Another system that can be used in plant transformation involves encapsulating the plasmid or linear DNA into artificial membrane bound vesicles called liposomes. These artificial "protoplasts" are then fused with plant protoplasts using PEG to induce membrane fusion (Fraley and
Horsch, 1983; Deshayes et al., 1985). Once again it is necessary to use a totipotent protoplast in order to regenerate a mature plant.

Transformation by microprojectile bombardment

A novel technique of DNA delivery has been developed for transforming intact cells, tissues or even embryos by the bombardment of cells with small (1.2 um diameter), DNA coated gold or tungsten particles under slight vacuum. Using chimeric plasmids carrying appropriate reporter genes, successful transformation of Zea mays (Klein et al., 1988a, 1988b, Gordon-Kamm et al. 1990) and of epidermal tissue of Allium cepa (Klein et al., 1987) has been demonstrated in that transient expression of the reporter genes was observed. A great deal of work has been done on attempting to transform various monocot species using this method. Although it appears to be a very efficient method of transforming plant tissue, it is very expensive to utilize and out of the reach of many labs at this time.

RESEARCH OBJECTIVES

The primary objective of my research was to apply the techniques of genetic transformation to modulate the metabolism of polyamines in plants. The immediate goals were: (1) to produce transgenic Nicotiana tabacum plants which exhibited high levels of expression of the murine ODC
cDNA; (2) To analyze the transgenic plants for foreign gene expression; and (3) to analyze the effects of murine ODC gene expression on cellular polyamine levels in tobacco.

When we initiated this project in 1986, there was only one full length ODC cDNA available for use. A murine ODC cDNA in the plasmid construct pSP65ODC (Fig. 12) was obtained from Dr. Nathan's laboratory. This construct was well suited for our purposes, as the ODC cDNA was cloned into the polylinker of pSP65 and easily excised. A series of constructs were produced which contained the ODC cDNA and either the PSS or CaMV 35S promoter. These constructs were used to transform tobacco and the resulting transgenic plants were assayed for the presence of the murine gene as well as for evidence of foreign gene expression.

The results of the study were expected to provide useful information on the regulation of polyamine metabolism in tobacco, as well as tools to experimentally alter that metabolism by genetic transformation. This would allow us in the future to test if polyamine metabolism is indeed important for a specific physiological or developmental response of plants as suggested by various workers based upon studies with inhibitors of polyamine metabolism.
MATERIALS AND METHODS

SOURCE OF MATERIALS

Plant Material

*Nicotiana tabacum* var. Xanthi; Ms. Cheryl Robie, Biotechnica International Inc. Cambridge, MA.

Bacteria

*Agrobacterium tumefaciens* C58C1; Dr. Michael P. Timko, Dept. of Biology, Univ. of Virginia, Charlottesville, VA 22903

*Agrobacterium tumefaciens* A136; Dr. Gynheung An, Institute of Biological Chemistry, Washington State University, Pullman, WA 99164.

*Escherichia coli* HB101; Steve Torosian, University of New Hampshire, Dept. of Microbiology, Durham, NH 03824.

Plasmids

pGV3850, pMH-973 neo, pNNPT, and pRK2013; Dr. Michael P. Timko, Dept. of Biology, University of Virginia, Charlottesville, VA 22903

pAL4404 and pGA643; Dr. Gynheung An, Institute of Biological Chemistry, Washington State University, Pullman, WA 99164.

pUC-19; Dr. Robert Zsigray, University of New Hampshire,
Dept. of Microbiology, Durham, NH 03824.
pSP65ODC; Dr. Daniel Nathans, Johns Hopkins University
School of Medicine, Baltimore, MD 21205.

pRK2073; Dr. Tom Lee, St Anselms College, Manchester, NH
03819

Anti-murine ODC Antibodies
Antibody 1 (AB1), Dr. Lo Persson, Dept. of Physiology,
University of Lund, S-22362 Lund, Sweden.

Antibody 2 (AB2), Dr. Phillip Coffino, Departments of
Microbiology and Immunology and Medicine, University of
California, San Francisco, San Francisco, CA 94143

Antibody 3 (AB3), Dr. Mari Haddox, Univ. of Texas, Health
Science Center, Dept. of Pharmacology, Houston TX 77225

DESCRIPTION OF MATERIALS

Plant Material:
Nicotiana tabacum var. Xanthi was used for all studies on
transformation, tissue culture, and subsequent expression of
the ODC cDNA.

Bacteria:
C58C1- Agrobacterium tumefaciens containing Ti plasmid
A136- *Agrobacterium tumefaciens* containing Ti plasmid pAL4404

**Plasmids:**

**pGV3850** (Fig. 9) - This plasmid is a disarmed derivative of the wild type Ti plasmid C58 (Zambryski et al., 1983). The oncogenic functions of the T-DNA have been deleted and a pBR322 (Bolivar et al. 1977) sequence containing the ampicillin resistance gene has been substituted into the T-DNA region. It is a co-integrative vector into which DNA sequences containing chimeric genes can be inserted into the T-DNA region via homologous recombination with pBR322 sequences. The plasmid also carries a rifampicin resistance gene.

**pAL4404** - This plasmid is a disarmed derivative of the wild type plasmid pTiB6. The oncogenic functions of the T-DNA have been deleted (Hoekema et al., 1983). It is used to supply the vir functions in trans for mobilization of T-DNA from binary vectors. The plasmid carries a rifampicin resistance gene.

**pMH973-neo** (Fig. 10) - This plasmid is a derivative of pBR322 containing two chimeric genes. The first gene consists of the *A. tumefaciens* nopaline synthase promoter...
(PNOS) fused with the coding sequence of a bacterial neomycin phosphotransferase (NPT II) gene. The second gene contains the promoter for the small subunit of Rubisco (PSS) from *Pisum sativum* fused with the coding sequence for chloramphenicol acetyltransferase (CAT) (Timko et al., 1985). Both chimeric genes also carry the nopaline synthase 3' polyadenylation sequences. It was designed to be used in conjunction with a co-integrative vector system, such as pGV3850 (Zambryski et al. 1983), for the light inducible expression of a foreign gene in transformed plants.

**pUC-19** (Fig. 11) - This plasmid is a derivative of pBR322 and contains a synthetic polylinker with 10 unique restriction sites inserted 3' to the lac Z'(B-galactosidase gene) promoter. This plasmid carries the gene for ampicillin resistance and is a widely used cloning vector (Norrander et al. 1983).

**pSP65ODC** (Fig. 12) - This plasmid is a derivative of pSP65 (Promega Biotec, Madison Wisconsin) and contains a 1692 bp fragment of the murine ODC cDNA. The 5' terminus of the ODC cDNA is inserted in the Eco RI site of the polylinker and the 3' terminus is ligated to the polylinkers Bam HI site (Kahana and Nathans 1985).

**pGA643** (Fig. 13) - This plasmid is a binary expression
vector designed for plant transformation. The plasmid contains the wide host range origin of replication from an RK2 derivative which allows for replication in *E. coli* as well as *A. tumefaciens*. In addition, there are cis acting factors for conjugal transfer and a gene coding for tetracycline resistance which facilitate cloning and transfer of the plasmid. The right border of the T-DNA region containing the overdrive sequence, the left T-DNA border and a PNOS-NPT-TNOS chimeric gene conferring kanamycin resistance to transformed cells enables transfer of a foreign gene into a plant cell and the selection of transformed cells. The CaMV 35S promoter ensures high levels of transcription of any foreign gene inserted into the polylinker which contains 9 unique cloning sites immediately 3' to the CaMV 35S promoter (An et al. 1987).

**pRK2013** - This is a helper plasmid which provides, in trans, the RK2 transfer functions and the ColEl mob protein which act at the bom site of pBR322 derived co-integrative vectors and initiate mobilization into *A. tumefaciens*. The plasmid carries a kanamycin resistance gene (Ditta et al. 1980).

**pRK2073** - This plasmid functions identically to pRK2013 except that it carries a gene for spectinomycin resistance instead of kanamycin resistance (Clonetech Palo Alto CA).
Figure 9. Ti Plasmid Cointegrative Vector pGV3850
Functionally characterized regions of the nopaline type Ti plasmid pGV3850. NOC, nopaline catabolism; REP, plasmid replication; VIR, virulence region; LB, left border sequence; RB, right border sequence; T-DNA, DNA sequence which is replicated and transferred into the plant cell. Genes within the T-DNA: NOS, gene involved with nopaline synthesis; AMP, pBR322 gene encoding ampicillin resistance; pBR322, remainder of pBR322 sequence integrated into the T-DNA region.
Figure 10. Plasmid pMH-973-neo. PSS, RUBISCO small subunit promoter; NOS, nopaline synthase promoter; CAT chloramphenicol acetyltransferase gene; NPT II neomycin phosphotransferase gene; NOS3', nopaline synthase polyadenylation sequence; OCS3', octopine synthase polyadenylation sequence; Amp', ampicillin resistance gene.
Figure 11. Plasmid pUC-19. Amp°, ampicillin resistance gene; lac Z', B-galactosidase gene; pUC-19 polylinker, synthesized polylinker containing 13 unique restriction sites.
Figure 12. Plasmid pSP650DC. Amp', ampicillin resistance gene; ODC cDNA, 1800 bp murine ornithine decarboxylase cDNA.
Figure 13. The binary vector pGA643 contains the following functional regions; BR, right T-DNA border; NOS-NPT, the chimeric construct consisting of the nopaline synthase promoter combined with the neomycin phosphotransferase gene; P35S, the 35S promoter from Cauliflower Mosaic Virus; TTR, the transcription termination region of gene 5 of pTiA6; BL, the left T-DNA border; Ori T, pRK2 origin of conjugative transfer; trfA, trans-acting replication factor of pRK2; Tet, tetracycline resistance gene; and Ori V the pRK2 origin of replication.
Miscellaneous Materials

- All restriction endonucleases and DNA modification enzymes (ligases, Klenow, etc.) were purchased from Promega Biotec Inc., Madison, WI.
- Random Prime DNA Labelling kits were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN.
- Molecular biology grade chemicals were purchased from Fisher Scientific Inc. Boston MA, or VWR Scientific Inc., Boston MA, unless otherwise noted.
- Premixed media formulations for plant tissue culture were purchased from Sigma Chemical Co. St. Louis, MO.
- All other reagent grade chemicals and media constituents were purchased from Sigma Chemical Co. St. Louis, MO.
- Water used in all the experiments was purified to >15 megaohm using a Corning MP-190 MEGA-PURE system (Corning Science Products, Corning, NY) and sterilized by autoclaving at 15-20 psi for 20-30 min.

METHODS

Plant Tissue Culture

Aseptic plantlets of *Nicotiana tabacum* var. Xanthi were grown on basal Murashige and Skoog (1962) medium (Table 4). Leaf segments were aseptically excised and placed onto shooting medium (SM) (Table 5) in 100 x 15 mm plastic petri plates. The petri plates were wrapped with Parafilm and placed in a growth chamber at 25+1°C with a 12 h
photoperiod (80 uE m$^{-2}$ s$^{-1}$). After three weeks, small shoots (2-5 mm) were excised and placed on fresh SM. Three weeks following transfer, shoots were large enough to transfer to a rooting medium (RM)(Table 5). Root formation occurred approximately 2-3 weeks post-transfer. Plantlets were transferred to Magenta boxes (Sigma Chemical Co. St. Louis MO) containing 50 ml of basal MS medium. After 2 weeks, sufficient growth had occurred to use the plants as a source of leaf segments for transformation experiments.

**Plant Transformation**

*Agrobacterium tumefaciens* were grown overnight in YEB medium containing appropriate selection antibiotics (Table 6) to a concentration of $10^6$-$10^8$ bacteria/ml. The bacterial suspension was centrifuged for 5 min at 1500 X g in sterile polypropylene centrifuge tubes. The medium was poured off and the bacterial pellet was resuspended in 15 ml of sterile 0.85% NaCl solution. Acetosyringone (Aldrich Chemical Co., St. Louis) was added to a final concentration of 50 uM and the bacterial suspension was poured into a sterile 100 x 15 mm petri plate.

Leaf segments (approximately one cm$^2$) were cut from aseptically grown tobacco leaves using a sterile scalpel. The leaf segments were placed in the petri plates containing the bacterial suspension and co-incubated for 20-30 min at room temperature. The leaf sections were rinsed twice with
sterile distilled water, and blotted dry on sterilized filter paper (Whatman 3MM). Leaf segments were placed on the SM medium (20 ml/ petri plate), the plates were wrapped with Parafilm and placed in the growth chamber. After two days, leaf sections were transferred onto fresh SMCK medium (SM medium containing 50-100 mg/l kanamycin, and 250-400 mg/l cefotaxime). Leaf sections were transferred to fresh SMCK at two week intervals until shoot formation occurred. Shoots formed in the presence of kanamycin were excised and transferred to fresh SMCK. When the shoots had grown to 0.5-1 cm tall (usually 2-3 weeks), they were transferred to RMCK medium (RM medium containing 50-100 mg/l kanamycin and 250-400 mg/l cefotaxime). Shoots were transferred to fresh RMCK every two weeks. After 2-3 wks, when 4-10 roots had developed from each shoot, plantlets were removed from culture and the medium was gently washed from the roots under tap water. It is important to minimize root system damage prior to transfer to soil. The plantlets were transferred to 5" pots containing a mixture of peat:perlite:vermiculite (50:25:25) soaked with basal MS medium (w/o sucrose and agarose). The pots were covered with plastic wrap and a small hole was poked in the plastic. The pots were placed at 25° C under fluorescent light with a 12 h photoperiod. The pots were placed in a 40" x 20" x 8" plastic tray containing 500 ml of water which was replenished every four days.
Table 4. The constituents of Murashige and Skoog (1962) Medium. Premixed powder from Sigma Chemical Co. # M5524

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</table>

Sucrose 3%
Agarose 4.0 g/l
Adjust pH to 5.8 prior to autoclaving
Table 5. Shooting Media (SM) and Rooting media (RM) Constituents. SM and RM use MS inorganic mixture obtained as a premixed powder from Sigma Chemical Co. # 5524.

Shooting Medium (SM)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>10.0</td>
</tr>
<tr>
<td>myo-inositol</td>
<td>100.0</td>
</tr>
<tr>
<td>Indole-3-Acetic acid (IAA)</td>
<td>0.3</td>
</tr>
<tr>
<td>Isopentenyl Adenine (IPA)</td>
<td>10.0</td>
</tr>
<tr>
<td>MES 3.0 mM</td>
<td></td>
</tr>
<tr>
<td>Sucrose 3.0%</td>
<td></td>
</tr>
<tr>
<td>Agarose 4.0 g/l</td>
<td></td>
</tr>
</tbody>
</table>

Adjust pH to 5.8 prior to autoclaving

Rooting Medium (RM)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
</tr>
<tr>
<td>myo-inositol</td>
<td>100.0</td>
</tr>
<tr>
<td>Naphthaleneacetic Acid (NAA)</td>
<td>0.1</td>
</tr>
<tr>
<td>MES 3.0 mM</td>
<td></td>
</tr>
<tr>
<td>Sucrose 3.0%</td>
<td></td>
</tr>
<tr>
<td>Agarose 4.0 g/l</td>
<td></td>
</tr>
</tbody>
</table>

Adjust pH to 5.8 prior to autoclaving
After two weeks, 200 ml of basal MS medium w/o sucrose was added to the tray along with 300 ml of water.

Gradually, the plants were hardened off by removing the plastic and misting them twice daily with a spray bottle containing water. Alternatively, plants which had been in the soil mix for three weeks were placed on the mist bench in the greenhouse for a two week period. Plants were transferred to 8" pots containing standard greenhouse soil mixture and a slow release fertilizer (Agway Super Grow 5-5-10). The plants were maintained in the greenhouse under standard conditions.

**Bacterial Culture and Storage**

In most cases, bacterial cultures were shipped to us in agar stabs. Upon arrival they were streaked onto agar plates containing appropriate selection media. Agrobacterium tumefaciens strains were streaked onto YEB agar medium (Kao et al., 1982; Table 6) and grown at 25°C for 24-48 h. Individual colonies were picked and used to inoculate liquid YEB medium containing appropriate antibiotics. After 24 h at 25°C with shaking (150 RPM), 850 ul of bacterial suspension was added to 150 ul of sterile glycerol in a 1.5 ml microfuge tube and the tubes were vortexed. The bacteria were then stored at -70°C until needed for up to two years.
Table 6. Media used for bacterial cultures.

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto beef extract</td>
<td>5.0</td>
</tr>
<tr>
<td>Bacto yeast extract</td>
<td>1.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5.0</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.5</td>
</tr>
<tr>
<td>Agar (for solid medium)</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Medium adjusted to pH 7.0 prior to autoclaving

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto tryptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Bacto yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>10.0</td>
</tr>
<tr>
<td>Agar (for solid medium)</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Medium adjusted to pH 7.2 prior to autoclaving
Escherichia coli strains were streaked onto LB agar medium (Maniatis et al. 1982) (Table 6) containing appropriate antibiotics and grown overnight at 37° C. Single colonies were picked and used to inoculate liquid LB medium containing appropriate antibiotics. After 24 h of incubation at 37° C with shaking (150 RPM), bacteria were processed for storage as for A. tumefaciens.

Plasmid Isolation (Maniatis et al. 1982)

A single colony picked from a selection plate was used to inoculate 50 ml of LB medium containing the appropriate antibiotics. The culture was incubated on a shaker at 37° C for 16 h. The overnight cultures were used to inoculate 20 flasks of LB medium containing antibiotics (125 ml flasks with 50 ml per flask) with one ml of the overnight suspension per flask. The flasks were placed on a shaker (150 RPM) in an incubator at 37° C for 16 h. The bacterial cultures were poured into four 250 ml polypropylene centrifuge bottles and centrifuged at 2500 X g for 10 min at 4° C. The supernatant was discarded and the bacterial pellets in each bottle were resuspended in 6 ml of ice-cold TE (10 mM Tris pH 8.0, 1 mM EDTA). The resuspended bacterial pellets were then combined in one 30 ml Corex centrifuge tube and centrifuged at 2500 X g for 10 min.

The supernatant was discarded and the bacterial pellet was resuspended in 9 ml of ice cold Lysis Buffer A (18%
sucrose, 35 mM Tris pH 8.0, 100 mM EDTA) and transferred to a 50 ml polypropylene centrifuge tube. Three ml of freshly made lysozyme (10 mg/ml H₂O) were added and the tube was inverted gently to mix. The tube was placed on ice for 15 min and inverted occasionally.

Ten ml of ice cold Lysis Buffer B (1% Triton-X100, 15 mM EDTA, 50 mM Tris, pH 8.5) were added to the tube and it was inverted gently to mix. The tube was placed on ice for 15 min and inverted occasionally.

A small hole was made in the cap to release pressure and the tube was placed in 100°C water bath for 10 min with occasional mixing by inverting the tube. The contents of the tube were then poured into a 30 ml Corex centrifuge tube, placed on ice for 10 min, and then centrifuged at 25000 X g for 30 min at 4°C.

The supernatant containing plasmid (2-3 ml) was transferred to a 30 ml siliconized Corex centrifuge tube (Sigmacote, Sigma Chemical Co. Inc., St. Louis, MO) and treated with 15 ul of RNase (10 mg/ml H₂O) per ml of supernatant and incubated at 37°C for 30-60 min. An equal volume of water-equilibrated phenol was added to the solution and the mixture shaken gently to mix. The mixture was centrifuged at 25,000 X g for 5 min to separate the aqueous phase from the phenol. The aqueous layer (top layer containing the plasmid DNA) was removed and placed in a clean 30 ml siliconized Corex tube. A second extraction
was done using 1/2 volume of phenol and 1/2 volume of chloroform/isoamyl alcohol (24:1). The mixture was mixed gently and centrifuged at 25,000 X g for 5 min. The aqueous layer was transferred to a clean tube and an equal volume of chloroform/isoamyl alcohol mixture was added. The mixture was shaken gently and centrifuged at 25,000 X g for 5 min. The aqueous layer was removed to a clean tube and a half volume of 7.5 M ammonium acetate was added. The total volume was determined and 2 volumes of 100 % ice cold ethanol was added. The tube was shaken gently and placed at -70° C for 60 min. The plasmid preparation was centrifuged at 25,000 X g for 30 min at 4° C. The supernatant was poured off and the pellet was washed with 70 % ethanol. The pellet was dried in a Speed-Vac Concentrator (Savant Instruments Inc., Farmingdale NY) for 10 min and resuspended in one ml of H₂O. If desired, the plasmid solution can be treated with RNase at this stage to remove any contaminating RNA which might have carried over during the plasmid isolation. For this, 15 ul of RNase (10 mg/ml) (5'->3', Paoli, PA) per ml of plasmid solution was added and the mixture incubated at 37° C for one h. At this point the plasmid preparation was ready for purification on a CsCl gradient.

The plasmid solution was brought up to 25 ml in TE and 25 g of CsCl powder was added. The mixture was shaken gently until the CsCl was dissolved. Two ml of ethidium bromide
(10 mg/ml H₂O) were added and the mixture placed in a 30 ml Beckman polyallomar ultracentrifuge tube. The top of the tube was filled with mineral oil and sealed. The tubes were placed in a Beckman Ti60 rotor and centrifuged at 50,000 RPM for 24 h in a Beckman L-70M ultracentrifuge. After centrifugation, the tubes were illuminated with UV light and the band containing supercoiled plasmid DNA was identified and removed with a 10 ml syringe through an 18 gauge needle. This was done by piercing the side of the polyallomar tube with the syringe and gently aspirating off the band containing the plasmid (Maniatis et al. 1982). The ethidium bromide was removed from the plasmid by washing the plasmid solution 4X with isoamyl alcohol. The plasmid solution was then dialysed overnight in several changes of TE (10 mM Tris, pH 8.0, 1 mM EDTA) at 4°C. The plasmid was then ethanol precipitated with ammonium acetate as follows: one-half volume of 7.5 M ammonium acetate was added to the plasmid solution, followed by two volumes of ice cold ethanol. The mixture was placed at -70°C for one h and then centrifuged at 15,000 x g for 30 min. The pellet was washed with 70% ethanol and dried in the speed-vac. The pellet was then resuspended in 300 ul of TE. A second ethanol precipitation was carried out on the plasmid by repeating the above procedure.

The preceding plasmid isolation protocol was scaled down and used as a plasmid mini-prep method. Fifty ml of LB was
inoculated and grown overnight. The 50 ml of bacterial suspension was spun down and the pellet was resuspended in 1 ml of TE and transferred to a microfuge tube. The bacteria were spun for 2 min at 11000 X g and the pellet was resuspended in 0.45 ml of Lysis Buffer A. The microfuge tube was placed on ice and 150 ul of lysozyme (10 mg/ml) was added. The mixture was incubated as above followed by the addition of 0.5 ml of Lysis Buffer B. The mixture was incubated as above and the tube was then placed in the water bath for 5 min. The tube was then transferred to ice for ten min followed by centrifugation at 15000 X g for 30 min. The supernatant was transferred to a new microfuge tube and treated with RNase. The plasmid was then extracted with phenol and chloroform as above followed by ethanol precipitation.

Quantification of DNA

A260/A280 Method: Two ul of DNA suspension was added to 398 ul of H_2O in a microcentrifuge tube and vortexted for 10 sec. The dual beam spectrophotometer (Perkin Elmer Lambda 3B, Perkin Elmer, Oak Brook Instruments Division, Oak Brook, IL) was zeroed at 260 nm using H_2O in a quartz microcuvette. The diluted DNA solution was placed in a matching quartz microcuvette and the absorbance was measured at 260 nm (A_{260}). The above procedure was repeated at a wavelength of 280 nm to determine the absorbance at 280 nm (A_{280}). A clean
DNA solution, i.e., one with very little protein or RNA contamination, should have an $A_{260}/A_{280}$ ratio of approximately 1.8-1.9. Any DNA preparation which had lower $A_{260}/A_{280}$ ratio which indicated the presence of a contaminant was further purified via phenol:chloroform extraction and ethanol precipitation. The $A_{260}$ reading was used to calculate the DNA concentration (1 OD unit at $A_{260} = 50$ ug/ml DNA or 40 ug/ml RNA in the measured dilution).

**Fluorescence Method:** The fluorometer (Hoefer TKO-100, Hoefer Instruments Inc. San Francisco, CA) was calibrated according to the manufacturer's instructions with a standard solution containing known concentrations of DNA similar to what was being quantified, i.e., plasmid, restricted plasmid, genomic DNA, etc.. Two ul of DNA solution was added to 2 ml of TNE buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA) containing 0.1 ug/ml of Hoechst 33258 dye. The sample was excited at a wavelength of 360 nm and the emission were read at 465 nm. The emissions reading on the fluorometer was equal to ug/ml of DNA in solution.

**Restriction endonuclease digestion of DNA**

Restriction digests were done according to the methods described in Maniatis et al. (1982). All restriction reactions were done in microcentrifuge tubes. Approximately 5 units of enzyme were added per ug of DNA to be restricted. The 10X buffer and 10X BSA supplied by the manufacturer were
added at appropriate concentrations and the volume was adjusted with \( \text{H}_2\text{O} \). The reaction mixtures were incubated at 37° C for a minimum of one h and usually overnight. On occasion, one ul of RNase (10 mg/ml) was added to the reaction mixture if the DNA sample had not been previously treated with RNase. In addition, one ul of 100 mM spermidine was added to the reaction mixture to enhance restriction in DNA solutions containing contaminants which interfere with enzyme activity. After incubation, the reaction was stopped by adding one ul of 0.5 M EDTA to the reaction mixture. At this point the restricted DNA was ready for agarose gel electrophoresis.

**Agarose Gel Electrophoresis**

Horizontal gel electrophoresis was done according to protocols in Maniatis et al. (1982). Both TAE (40 mM Tris, 40 mM Acetic acid, 1 mM EDTA) and TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) buffer systems were used depending on the subsequent use of the DNA. TAE buffer was used in all gels where the DNA was to be eluted from the gel and used in ligation reactions. TBE buffer was used in all other gels used to separate DNA, e.g., genomic restrictions for Southern hybridizations. A high quality (molecular biology grade) agarose (IBI cat. #70040) was used in all agarose gel electrophoresis. Gel loading buffer (15% Ficoll type 400, 0.25% xylene cyanol, 0.25% bromophenol blue) was added
to the DNA samples (2 ul/10 ul of DNA) before loading DNA onto the gel. A molecular weight marker (lambda DNA restricted with Hind III, Promega Biotech, Madison WI) was included on all gels to estimate molecular weight of restriction fragments. The conditions of individual gel runs, i.e., time, voltage, temperature, gel size, etc., were determined based upon the desired result of the electrophoresis. The standard gel running conditions for isolating fragments to be electroeluted and used for ligation etc., consisted of using TAE buffer with a 0.8% agarose gel (14 x 18 cm, Hoefer HE 99). The standard gel running conditions for Southern blotting consisted of using TBE buffer with a 0.8% agarose gel (14 x 18 cm, Hoefer HE 99). The gels were run at 70 V for 5-6 h. After the gel runs were completed, the gels were stained for 5 min in ethidium bromide (100 ul of 10 mg/ml stock per 200 ml of running buffer) and then rinsed and destained in H₂O for one h. Bands of DNA were visualized on a U.V. transilluminator (Fotodyne Model 3-3000, Fotodyne Inc., New Berlin, WI) and photodocumentation of gels was done as in Maniatis et al. (1982) using a Polaroid MP-3 camera with both Type 55 and 52 Polaroid film.

**Electroelution of DNA Restriction Fragments**

Electroelution of DNA from agarose gels was done according to the method of Maniatis et al. (1982). Using a
new razor blade, the band containing DNA fragment of interest was excised from the gel while visualizing the gel on the U.V. transilluminator. The gel slice, containing the target band was placed into a piece of dialysis tubing (Spectrapore #1, Spectrum Medical Industries Inc., Los Angeles, CA) which was treated by boiling for 10 min in a large volume of 2% sodium bicarbonate containing 1 mM EDTA. The dialysis tube was then rinsed several times with H2O and autoclaved for 15 min in 1 mM EDTA. Before use, the dialysis tube was rinsed with electrophoresis buffer. The dialysis tube was filled with electrophoresis buffer and the gel slice was placed into the tube. Excess buffer as well as any air bubbles were squeezed out and the ends of the tube were closed with clamps. The tube was placed in the TAE filled electrophoresis unit and the tube was secured so that it could not move. The system was run at 60 volts for approximately 60 min depending upon the size/mobility of the fragment. The dialysis tube was checked on the U.V. transilluminator to make sure all of the DNA fragment was out of the gel slice. The gel slice was carefully removed and discarded. The tube was placed again in the original orientation in the electrophoresis chamber and the current was reversed for 30 sec. The walls of the dialysis tube were rinsed using the buffer in the tube and all of the buffer was transferred to a clean 15 ml polystyrene tube.

A minicolumn (Isolab QS-Q, Isolab Inc., Akron, OH) was
prepared by adding 0.5 ml of DEAE Sephacryl (Sigma Cat.# S-2403). The column was washed with 5 ml of TE (10 mM Tris, pH 8.0, 1 mM EDTA) followed by two 5 ml washes with high salt TE (10 mM Tris, pH 8.0, 1 mM EDTA, 4.0 M Ammonium acetate). The column was equilibrated with one wash of TE followed by a wash with low salt TE (10 mM Tris, pH 8.0, 1 mM EDTA, 0.25 M Ammonium acetate). The DNA + buffer from the dialysis tube were loaded onto the column and collected in a clean tube. The sample was run through the column a second time to ensure complete retention. The column was washed three times with 5 ml of low salt TE. The DNA was eluted from the column with one ml of high salt TE. The elutant was collected and added back to the column. The column was centrifuged in a benchtop clinical centrifuge at 2000 X g for 3 min. The elutant was collected in a clean tube and further purified by phenol:chloroform extraction and ethanol precipitation as described earlier.

**Ligation of DNA into Plasmid Vectors**

Ligation reactions were carried out following the protocol outlined in Ausabel et al. (1987). Blunt-end ligations were done using 500 ng of vector and insert in a total reaction volume of 50 ul. Five units of T4 DNA ligase was added to the reaction mixture along with the buffer supplied with the ligase. The reaction was run for 16 h at 15° C and terminated by heating to 65° C for 10 min.
Cohesive-end ligations were done by using a 1:2 (vector:insert) ratio with approximately 100 ng of vector and 200 ng of insert in a total reaction volume of 50 ul. Approximately 0.1 U of T4 DNA ligase was added to the reaction mixture along with the buffer supplied with the ligase. The reaction was run at 4° C for 16 h and terminated by heating at 65° C.

**Dephosphorylation of Vector**

Calf Intestinal Alkaline Phosphatase (CIAP) was used to dephosphorylate the restricted ends of plasmid vectors. The restricted plasmid was treated with CIAP (2 U/ ug DNA) (Boehringer Mannheim Biochemicals # 713023, BMB, Indianapolis, IN) using the buffer supplied by the manufacturer. The CIAP was added directly to the restriction reaction mixture after the restriction was complete. Restrictions generating 5'-protruding ends were incubated at 37° C for 30 min whereas, restrictions generating blunt-end fragments were incubated at 50° C for one h. At the conclusion of the incubation, 1/10 volume of 0.5 M EGTA (pH 8.0) was added to the reaction and it was incubated at 65° C for 45 min to inactivate the CIAP. The vector DNA was extracted with phenol/chloroform and ethanol precipitated.

**Isolation of Plant DNA**
DNA was isolated and purified from plant tissue by the procedure of Delaporta et al. (1983). Leaf tissue, 0.5 to 0.75 g fresh weight, was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Fifteen ml of Extraction Buffer (EB) [100 mM Tris pH 8.0, 50 mM Ethylene diaminetetraacetic acid, disodium salt (EDTA), 500 mM NaCl, 10 mM 2-mercaptoethanol] were added to the tissue and the mixture was placed in a 30 ml Corex centrifuge tube. For maximum DNA yields, the cells were further broken by homogenizing at 5000 RPM with a polytron (Brinkman Model PT3000, Kinematica, Littan, Switzerland). One ml of 20 % SDS (sodium dodecyl sulfate) was added and the sample was mixed thoroughly by gentle shaking and inversion. It was important to be gentle at this step so as to avoid shearing the DNA. After mixing, the sample was incubated at 65° C for 10 min. Five ml of 5 M potassium acetate were added and the sample was mixed gently and incubated at 0° C for 20 min. The sample was centrifuged at 25,000 X g for 20 min. The supernatant was filtered through Miracloth (Calbiochem-Behring Corp., La Jolla CA) into a clean 30 ml Corex centrifuge tube containing 10 ml ice cold isopropanol. The sample was mixed and incubated at -20° C for 30 min and then centrifuged at 20,000 X g for 15 min to pellet the DNA. The supernatant was poured off gently and the pellet was lightly dried by inverting the tube on paper towels for approximately 10 min. The DNA pellet was resuspended in 0.7
ml of 50 mM Tris, pH 8.0 + 10 mM EDTA. The DNA solution was transferred to a 1.5 ml microfuge tube and spun at maximum speed in a microfuge for 10 min to remove insoluble debris. The supernatant was transferred to a new 1.5 ml microfuge tube and 50 ul of 3 M sodium acetate and 100 ul of 1% CTAB (cetyl trimethylammonium bromide: Sigma Cat. No. H-5882) were added. The CTAB-DNA precipitate was pelleted by spinning for 30 seconds at maximum speed in the microfuge and the pellet was washed with 500 ul 70% ethanol. The pellet were resuspended in 400 ul of TE and 50 ul of 3 M sodium acetate were added. One ml of ice cold ethanol was added and the DNA was precipitated by incubation at -20°C for 20 min. The tubes were spun at maximum speed in the microfuge for 1 min. The supernatant was discarded and the pellet resuspended in 400 ul of TE. The sample was ethanol precipitated a second time and the pellet was resuspended in 200 ul of 10 mM Tris pH 8.0, 1 mM EDTA. Since residual CTAB can interfere with A260 readings, the DNA was quantified using the fluorometric method.

**Purification of DNA by Phenol Extraction**

In a sufficiently large, phenol:chloroform resistant centrifuge tube, equal volumes of DNA solution and water-saturated phenol were gently mixed until an emulsion was formed (when extracting plasmid DNA of less than 10 Kb in size, the solution can be vortexed). The sample was
centrifuged for 3 min at 11,000 X g and the aqueous (upper) layer was transferred to a new centrifuge tube using a large bore pipett. An equal volume of a 1:1 mixture of water-saturated phenol and chloroform:isoamyl alcohol (24:1) was added to the DNA suspension (aqueous layer from the previous step). The solutions were mixed and centrifuged as above. The aqueous layer was removed and transferred to a clean tube. An equal volume of chloroform:isoamyl alcohol was added to the DNA sample and mixed as above. The sample was centrifuged and the aqueous layer was transferred to a clean tube. The DNA was precipitated with ethanol as described below.

**Ethanol Precipitation to Recover DNA From a Solution**

Depending on the salt of choice, 1/10 volume of 3 M sodium acetate or 1/2 volume of 7.5 M ammonium acetate was added to the DNA suspension. The total volume of DNA+salt suspension was determined and two volumes of 100% ethanol at -20° C were added. The samples were mixed gently and placed at -70° C for 60 min. The samples were centrifuged at 18,000 X g for 30 min to pellet the DNA. The supernatant was poured off and the DNA pellet was rinsed with 1 ml of 70% ethanol. The 70% ethanol was discarded and the DNA pellet was dried in the Speed-Vac. The DNA pellet was resuspended in 100 ul of TE (10 mM Tris, pH 8.0, 1 mM EDTA) and quantified.
Isolation of Plant RNA

All glassware, plasticware and solutions were treated with 0.1% diethylpyrocarbonate (DEPC) (Sigma) as outlined in Maniatis et al. (1982). Although several methods were used to isolate total RNA, a modification of the method of Hughes and Galau (1988) yielded the highest quality RNA from our samples. One gram of plant material was placed in liquid nitrogen in a chilled mortar and ground to a powder. Ten ml of ice-cold homogenization buffer (200 mM Tris-HCl, pH 8.5; 1.5% lithium dodecylsulfate; 300 mM LiCl; 10 mM Na₂EDTA; 1% w/v sodium deoxycholate; 1% v/v NP-40; 5 mM thiourea; 1 mM aurantricarboxylic acid; and 10 mM dithiothreitol) were added and the slurry was transferred immediately to a 30 ml Corex centrifuge tube. The samples were placed at -80°C until frozen. After 30 min, the samples were placed in a 37°C water bath until just thawed and then placed on ice. The samples were mixed gently and 1/3 volume of 8.5 M potassium acetate, pH adjusted to 7.2 with acetate, was added. The samples were mixed by repeated inversion and allowed to incubate on ice for 15 min, then centrifuged at 5000 X g for 20 min at 4°C. The supernatant was transferred through one layer of Miracloth into a new 30 ml centrifuge tube and 1/9 volume of 3.3 M sodium acetate, pH 6.1, and 1/2 volume of ice cold isopropanol were added. The samples were mixed and stored at -20°C for at least 60 min. The samples were centrifuged for 30 min at 5000 X g. The
supernatant was discarded and the pellets were each resuspended in 800 ul of TE and transferred to 1.5 ml microfuge tubes. The samples were placed on ice and 1/4 volume of 10 M LiCl was added to each tube. Samples were incubated for 5-12 h on ice. The LiCl precipitate was collected by centrifugation at 10,000 X g for 30 min. The supernatant was discarded and the pellet resuspended in 200 ul of TE. The tubes were placed on ice and 1.5 volumes of 5 M potassium acetate were added. The samples were mixed by gentle inversion and incubated on ice for 3-5 h, then centrifuged at 10,000 X g for 30 min and the supernatant was discarded. The pellets were resuspended in 100 ul of TE and incubated on ice for 1 h, vortexing occasionally. The tubes were centrifuged at 10,000 X g for 30 min and the supernatant was removed to a new microfuge tube. The supernatant was mixed with 1/9 volume 3.3 M sodium acetate, pH 6.1, and two volumes of ethanol. The mixture was placed at -20° C for at least one h. The samples were centrifuged at 10,000 X g for 30 min and the supernatant discarded. The pellet was vacuum dried and resuspended in 25 ul of TE. The A_{260} was determined using 2 ul of the sample. Assume A_{260} = 1.0 for a solution containing 40 ug/ml of RNA.

Vacuum Transfer of DNA to GeneScreen membrane

For Southern blotting, a large (150 ml, 15x22 cm) 0.8% agarose gel (TBE buffer) was loaded with 5 ug of restricted,
genomic DNA/well. The gel was run at 40 volts for 12 h, stained with ethidium bromide and destained in H₂O as described earlier. After photographing the gel, the DNA was denatured by soaking the gel in 250 ml of 1.5 M NaCl+0.5M NaOH with gentle shaking for one h. The gel was then rinsed with H₂O and neutralized in 250 ml of 1.5 M NaCl+1.0 M Tris pH 8.0 with gentle shaking for one h. After neutralization, the gel was rinsed with H₂O and was ready for transfer. The denaturation and neutralization steps were not necessary if using 0.4N NaOH as the transfer buffer.

A 15x22 cm piece of GeneScreen Plus (Dupont NEN, Boston MA) nylon membrane was prepared according to the manufacturer's instructions, i.e., float the membrane on water for one min, followed by soaking in transfer buffer of choice for 20 min. The Vacu-Blot (Fisher Biotech, Fisher Scientific, Boston, MA) apparatus was assembled as described by the manufacturer and a 20x30 cm piece of Whatman 3MM paper, moistened with transfer buffer, was placed in the center of the porous platform. The membrane was centered on the filter paper, side B facing up, and the latex rubber mask (18x28 cm opening) was placed over the membrane. The membrane was adjusted so that the opening in the mask was centered on top of the membrane and overlapped the membrane on all sides.

The processed gel was placed over the opening in the mask, so that the gel overlapped the opening in the mask on
all sides. If the wells in the gel were over the mask opening, they were filled with melted agarose before blotting. Once the gel was adjusted, the top of the blotting device was fastened to the bottom half of the unit and the four holding screws were tightened. Approximately 50 ml of transfer buffer (10X SSC for high salt transfer buffer, 0.4N NaOH for alkaline transfer buffer) was poured on top of the gel and a vacuum of 40 mm Hg was applied to the system. Gentle pressure was applied around the edge of the gel to make a complete seal and then 500 ml of transfer buffer were gently poured into the buffer reservoir. It is imperative that the vacuum not exceed 40-45 mm of Hg or the gel would collapse and transfer would be very inefficient.

The vacuum was maintained for 2-3 h and then the excess buffer was poured off before stopping the vacuum. The apparatus was disassembled and the gel was removed (mark the gel orientation on the membrane). The membrane was removed and dried at room temperature. The membrane was then treated by soaking in 0.4 M NaOH for 30-60 sec, followed by 1-2 min in 0.2 M Tris, pH 8.0 + 2XSSC to ensure denaturation of all transferred DNA. When 0.4N NaOH was used as the transfer buffer (alkaline transfer) the GeneScreen was baked at 80° C for 2 h after drying at room temperature.

Prehybridization of GeneScreen Bound DNA

One-hundred ml of prehybridization buffer (PHB/50%
formamide) were prepared as follows:

50 ml Molecular Biology Grade Formamide
25 ml of 20XSSC
10 ml of 50X Denhardts Solution
5 ml of 1M K₂HPO₄
5 ml of 20% SDS
1 ml of Boiled Sheared Salmon Sperm DNA (10 mg/ml)
4 ml of H₂O

The solution was warmed to 45° C to ensure a homogenous mixture before initiating prehybridization. The membrane was soaked in 5XSSC for 5 min before loading into the Hybrid-Ease hybridization chamber (Hoefer Scientific Instruments Inc., San Francisco, CA) which was assembled according to the manufacturer's instructions. Approximately 50 ml of prehybridization solution were added to the Hybrid-Ease and the air bubbles were removed by pumping the prehybridization solution back and forth through the chamber as per manufacturer's instructions. The blot was prehybridized at 42° C for 3-6 h prior to adding the radiolabeled probe.

**Random-Prime Labeling of DNA**

Template DNA, 100-250 ng dissolved in 2-5 ul of H₂O, was added to a microfuge tube and placed in a water bath at 100° C for 10 min. The denatured DNA was then cooled on ice for 10 min prior to labeling. The labeling reaction was done
according to the instructions supplied with the Random Prime Labeling Kit (Boehringer Mannheim Cat. No. 1004760), the following reaction components were used:

2-5 ul of template DNA
5 ul of $^{32}$P-dATP (3000mCi/M, Cat. # 30004X, ICN inc.)
5 ul of $^{32}$P-dCTP (3000mCi/M, Cat. # 33007X, ICN inc.)
1 ul of dGTP 0.5 mM
1 ul of dTTP 0.5 mM
1 ul of Klenow (2 units)
2 ul of primer/buffer mix
H$_2$O to 20 ul

The reaction mixture was spun in a microfuge for 10 sec and placed at 37°C for 45 min. The reaction was stopped by adding 20 ul of 125 mM EDTA + 0.1% SDS. One ul of the reaction mixture was removed for counting. The reaction mixture was loaded onto a Sephadex-G25/80 microspin column and spun for 5 min at 1400 X g. Columns were prepared just prior to use by adding one ml of Sephadex G-25/80 (5 g/50 ml TE) to a microcolumn (Isolab QS-GS, Isolab Inc., Akron, OH). The column was washed by adding one ml of TE and spinning for 5 min. The column was then ready for use. One ul of recovered probe in the elutant was removed for counting. The probe was denatured by adding 0.1 volume of 6N NaOH and placing the tube in boiling water for 10 min. The probe was cooled on ice for 5 min and 10 volumes of 200 mM sodium acetate were added to neutralize the pH of the probe.
solution.

Southern Hybridisation and Washing

The probe was added to the Hybrid-Ease chamber according to manufacturer's instructions and incubated for a minimum of 14 h at 42° C. The blot was washed two times with 2X SSC + 0.1% SDS at 60° C for 15 min. The blot was then washed with 1X SSC at 60° C for 15 min followed by a wash with 0.1X SSC at 60° C for 15 min. The Hybrid-Ease was disassembled and the membrane was blotted on filter paper to remove excess liquid. Alternatively, the same prehybridization and hybridization protocols could be used with the membranes in a plastic box or heat sealable bags instead of the Hybrid-Ease. The membrane was wrapped in Saran wrap to avoid further drying. It is important to not let the membrane dry with the probe attached as this would interfere with any further washing. The membrane was now ready for autoradiography.

Northern Hybridisation and Washing

All solutions and apparatus used in the Northern hybridization analysis were treated with 0.1% diethylpyrocarbonate. A 100 ml 1.11% formaldehyde agarose denaturing gel was prepared as follows: 1.2 g of agarose was boiled in 87 ml of H₂O and the agarose solution was cooled to 60° C. Ten ml of 10X MOPS (0.4 M morpholinopropane-
sulfonic acid, 100 mM sodium acetate, 10 mM EDTA, pH 8.0) running buffer and 3 ml of 37% formaldehyde were added. The solution was mixed well and placed at 60° C until the gel was poured. The gel was poured and allowed to stand for 30-45 min to set. The gel was placed in the horizontal gel running apparatus and the buffer reservoir filled with 1X MOPS running buffer.

The RNA samples were prepared for electrophoresis by adding 10 ug of RNA (5.6 ul maximum volume) to the following mix: 2.5 ul 10X MOPS running buffer; 4.4 ul 37% formaldehyde; 12.5 ul formamide; H2O to 25 ul. The samples were vortexed briefly and incubated at 55° C for 15 min. Ten ul of formaldehyde loading buffer (1 mM EDTA, pH 8.0; 0.25% Bromphenol Blue; 0.25% xylene cyanol; 50% glycerol) were added to each sample and the samples were loaded onto the gel. The gel was run for 3 h at 70 V, removed from the electrophoresis apparatus and washed 3X with 250 ml H2O for 15 min per wash. The gel was then equilibrated for 45 min in 500 ml of 10X SSC with gentle shaking. The RNA was transferred to GeneScreen via vacuum transfer (40-45 mm Hg, 2-3 h) using 10X SSC as the transfer buffer. After transfer, the membrane was baked at 80° C for 2 h prior to prehybridization. The membrane was prehybridized for 3-4 h in PHB/50% formamide as described earlier for Southern Hybridization. The 32P-labeled probe was added to the prehybridization solution and the membrane was hybridized
overnight at 42° C. The next day, the hybridization mixture was removed and the membrane was washed twice with 1X SSC + 0.1% SDS at room temperature for 15 min per wash. This was followed by two washes with 0.25X SSC + 0.1% SDS at room temperature for 15 min per wash. The membrane was blotted on Whatman 3MM filter paper to remove excess moisture and wrapped in saran wrap. The membrane was now ready for autoradiography.

**Autoradiography**

The plastic-wrapped membrane was attached to a 8x10 inch piece of Whatman 3MM filter paper using tape. The membrane was placed inside a film holder with two intensifying screens and a piece of Kodak X-Omat film was placed between the two intensifying screens. The film holder was clamped between two 8 x 10 x 1/2 inch pieces of plexiglass and placed at -70° C for 3-5 days with AR type film and 7-10 days with RP type film. The film was developed using Kodak GBX developer and fixer according to manufacturer's instructions.

**Preparation of Competent Bacteria**

A single colony of HB101 was isolated from an overnight LB plate and used to inoculate 5 ml of LB media in a 125 ml flask. The flask was placed on a gyratory shaker (180 RPM) at 37° C for 2-3 h. Five ml of bacterial culture were then
used to inoculate 500 ml of prewarmed (37° C) LB medium in a 2 L flask. The culture was incubated with shaking at 37° C for 2-3 h or until OD_{595} was 0.3 to 0.5. The culture was distributed into sterile 250 ml centrifuge bottles and placed on ice for 10 min. The bacteria were pelleted by centrifugation at 1000 X g for 12 min at 4° C. The supernatant was discarded and each of the the pellets was resuspended in 133 ml of ice cold Transformation Buffer One (TB1) (Table 7) per 250 ml centrifuge bottle. The bacteria were incubated on ice for 90 min. The bacteria were then pelleted by centrifugation at 1000 X g for 12 min at 4° C. The pellets were resuspended in 8 ml Transformation Buffer Two (TB2) (Table 7) per centrifuge bottle, combined and distributed into prechilled microfuge tubes (200 ul/ tube). The tubes were flash frozen on dry ice and stored at -70° C.

**Transformation of Competent Bacteria with Plasmid DNA**

The microfuge tube containing competent HB101 was thawed at room temperature. The plasmid DNA (0.01-1 ug/100 ul) was added to the thawed bacterial suspension, mixed gently, and placed on ice for 30 min. The bacteria were heat-shocked at 42° C for 1-1.5 min and placed on ice for 3-5 min. Approximately 400 ul of LB media (37° C) were added to the bacteria and they were placed at 37° C for one h. The bacteria were then plated out (50-100 ul/plate) on a selection plate using a glass rod bent at a 90° angle.
Table 7. Constituents of Bacterial Transformation Buffers.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Constituents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB1</td>
<td>30 mM Potassium acetate</td>
<td>1.94 g/l</td>
</tr>
<tr>
<td></td>
<td>50 mM Manganese chloride</td>
<td>9.84 g/l</td>
</tr>
<tr>
<td></td>
<td>100 mM Potassium chloride</td>
<td>7.40 g/l</td>
</tr>
<tr>
<td></td>
<td>10 mM Calcium chloride</td>
<td>1.47 g/l</td>
</tr>
<tr>
<td></td>
<td>0.2 mM EDTA</td>
<td>0.06 g/l</td>
</tr>
<tr>
<td></td>
<td>15% Glycerol</td>
<td>150 ml</td>
</tr>
<tr>
<td></td>
<td>pH 6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Filter sterilize</td>
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</tr>
<tr>
<td></td>
<td>Store at 4°C</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Constituents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB2</td>
<td>10 mM Na-MOPS</td>
<td>2.09 g</td>
</tr>
<tr>
<td></td>
<td>75 mM Calcium chloride</td>
<td>11.02 g</td>
</tr>
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<td>10 mM Potassium chloride</td>
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<td>Store at 4°C</td>
<td></td>
</tr>
</tbody>
</table>
Transformation of Agrobacterium via Triparental Mating

Bacteria from a single overnight colony were used to inoculate 50 ml of LB medium containing selection antibiotics for each of the three strains used in the triparental mating experiment. The bacteria were grown overnight with shaking at 37°C for *E. coli* strains and 25°C for *A. tumefaciens*. One ml of each bacterial suspension (HB101:pRK2073, HB101:expression vector, Agrobacterium) were combined and spun at 2000 x g for 5 min. The resulting pellet was resuspended in 100 ul of LB and 50 ul was spotted on a LB medium plate. The plate was incubated overnight at 28°C during which conjugation and plasmid transfer occurred. After incubation, 5 ml of sterile 0.2 M MgSO₄ was added to the plate and the bacteria were resuspended by stirring with a sterile inoculating needle. The bacterial suspension was transferred to a sterile 15 ml centrifuge tube and spun at 2500 x g for 5 min. The resulting pellet was resuspended in 300 ul of sterile 0.2 M MgSO₄ and the bacterial suspension was spread onto LB plates containing appropriate antibiotics for selection. Colonies appearing on the plates after 3 days were transferred to fresh selection plates, incubated at 28°C and used for colony hybridization.

Colony Hybridization

Following transformation, bacteria were transferred to
fresh selection plates by manual transfer and grown for 12 h prior to replica plating. Sterilized Whatman 541 filter paper was placed in the petri plate and gently pressed down so the colonies could adhere to the paper. The orientation of the filter was marked asymmetrically using a syringe filled with india ink. The filters were left in place for 10 min to ensure binding of the bacteria. The filters were then placed for 10 min, colony side up, on a sheet of Whatman 3MM filter paper soaked with 0.5N NaOH. The filters were then transferred to a sheet of Whatman 3MM paper soaked with 2X SCP (50 mM disodium phosphate, 200 mM NaCl, 1 mM EDTA, pH 8.0) colony side up for 10 min. This step was repeated two more times with fresh paper and buffer to neutralize the filters. The filters were baked at 80° C for two h to fix the DNA to the filters. The filters were prehybridized for 2-3 h in a plastic box (5"x5") containing 40 ml of prehybridization mixture. Prehybridization mixture was prepared as follows: 22 ml H₂O; 2 ml of boiled sheared Salmon sperm DNA (10 mg/ml); and 4 ml of 10% Sarcosyl were mixed and boiled for 10 min followed by placing on ice for 10 min. Twelve ml of 20X SCP (500 mM disodium phosphate, 2 M NaCl, 10 mM EDTA) were added and the prehybridization solution was ready for use.

A radiolabeled probe was made using the Random Primer method described earlier and added to the prehybridization solution/filters in the box. The box was wrapped in plastic
and incubated for 12 h at 50°C with gentle shaking. The filters were washed twice with 2X SCP + 1% SDS for 20 min, using 250 ml/wash. This was followed by washing the filters twice with 2X SCP for 15 min, using 250 ml/wash. The filters were washed a final time with 250 ml 0.4X SCP for 15 min. The filters were blotted dry on 3MM paper and wrapped in plastic wrap. The filters were then exposed to AR type film for 4-6 h using standard autoradiography techniques.

**Protein Extraction and Western Blotting**

Total protein was extracted from tissue by grinding 0.5 g of tissue in liquid nitrogen and adding one ml of extraction buffer (100 mM MES, 15% ethylene glycol, 2% 2-mercaptoethanol and 100 mM sucrose, pH 6.8 at 4°C) plus 20 ul of 0.1 M phenyl methyl sulfonyl fluoride (PMSF made in isopropanol). Homogenate was then transferred to a microfuge tube and spun at 15,000 X g for 15 min. The supernatant was transferred to a clean tube and either quantified by the Bradford method (Bradford 1976) and used directly for electrophoresis or stored at -70°C. The protein samples were prepared for electrophoresis by mixing 100 ug of protein with an equal volume of 2X Maizel sample buffer (20% sucrose, 8% SDS, 67 mM Tris pH 6.8, 10% 2-mercaptoethanol). The samples were boiled for 4-5 min prior to loading onto the gel. Samples were run on 10% polyacrylamide gels in the presence of 0.1 % SDS (Hoefer Model
SE-400 Vertical Slab Apparatus). Gels were run 14-16 h at 10 mA constant current in a 4°C cold room.

Proteins were electrophoretically transferred to GeneScreen as follows. The gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) for 60 min (3 X 20 min) prior to transfer. GeneScreen was cut to the exact size of the gel and equilibrated for 20 min in transfer buffer. The GeneScreen was placed on top of two pieces of Whatman 3MM paper which were soaked with transfer buffer and any air bubbles between the paper and the GeneScreen were removed. The gel was placed on top of the GeneScreen and covered with two pieces of Whatman 3MM paper soaked in transfer buffer. After making sure all air bubbles in the transfer stack were removed, it was placed between two Scotch-Brite pads and placed in the electroblotter cassette. A Bio-Rad Trans Blot Cell (Bio Rad, Rockville Centre, NY) electroblotter was used and set up as per manufacturer's instructions. The protein transfer was done overnight (12-16 h) at 20 volts at 4°C. The GeneScreen was removed from the electroblotter and allowed to dry at room temperature.

**ELISA Procedure**

The blot was placed in a dish containing PBS (10 mM Potassium phosphate, 150 mM Sodium chloride) for two min to wet the membrane. The membrane was then placed in a heat-sealable bag with 50 ml of blocking solution (PBS with
either 1% Bovine Serum Albumin-BSA or 10% Nonfat Dry Milk-NFDM) and incubated for 8-16 h at 25°C at 150 RPM on a rotary shaker. The blocking solution was then removed from the bag and replaced with 50 ml of primary incubation solution (PBS, 0.5% BSA or 10% NFDM, 0.5% Tween-20, proper dilution of primary antibody). The membrane was incubated with the primary antibody for 14-18 h at 25°C with constant moderate agitation. The membrane was then removed from the bag and placed in a plastic tray containing 100 ml of wash solution (PBS, 0.1% Tween-20). The membrane was washed with moderate constant agitation at 25°C for 45 min. The wash step was repeated two more times and the membrane was incubated in a heat sealable bag with 50 ml of secondary antibody incubation solution (PBS, 1% BSA or 10% NFDM, proper dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase) for 2-4 h at 25°C with constant moderate agitation. The membrane was removed from the bag and placed in a plastic tray containing 100 ml of wash solution (PBS, 0.1% Tween-20) at 25°C with constant moderate agitation for five min. The wash step was repeated two more times. The blot was then washed twice in 100 ml of PBS under the same conditions as the first wash. The blot was then washed with 100 ml of 50 mM potassium phosphate, pH 7.4 using the same conditions as the first wash. The blot was then incubated in the chloronaphthol development solution (20 ml methanol containing 60 mg of 4-chloro-1-
naphthol added to 100 ml of 50 mM potassium phosphate containing 60 ul of 30% H₂O₂ just prior to use) until purple bands appeared.

**Enzyme Assay for ODC Activity**

Ornithine decarboxylase was extracted and assayed using the procedure of Robie and Minocha (1989). Tissue to be assayed for enzyme activity was homogenized in cold extraction buffer (2 ml/g fresh weight) on ice for one min using a Brinkman Polytron (23K RPM). The extraction buffer, also referred to as TAB, contained 50 mM Tris, 0.1 mM Na₂EDTA, 0.5 mM pyridoxal-5'-phosphate and, 0.05 mM dithiothreitol. The pH of the extraction buffer was adjusted as necessary using 1.0 N HCl and/or 1.0 N NaOH. In the experiments designed to analyze the effects of pH on ODC activity, the pH of the extraction buffer was adjusted to 6.5 and 8.5. The homogenate was centrifuged at 20,000 x g for 20 min at 4° C in 15 ml Corex glass centrifuge tubes. The supernatant was removed and placed in microfuge tubes on ice and protein content was determined using the Bradford method (1976). Dye reagent was purchased from Bio-Rad Laboratories and used according to the manufacturers instructions.

The activity of ODC was determined by measuring the amount of ¹⁴CO₂ released from L-[1-¹⁴C]-ornithine. The amount of enzyme extract used in the assay was 150 ul with the final
volume brought up to 200 ul using TAB. In various assays, addition of DFMO and/or anti-ODC antibody were added to the enzyme extract and the volume was brought up to 200 ul with TAB when necessary. In the experiments designed to investigate the inhibitory action of DFMO, 50 ul of 50 mM DFMO in extraction buffer, at the corresponding pH, was added to each reaction tube. Similarly, 5-10 ul of anti-ODC antibody was brought up to 50 ul with the appropriate extraction buffer and added to the reaction mixture. Reactions without DFMO or antibody had 50 ul of extraction buffer added to the reaction mixture. In the DFMO and antibody experiments, all reactions were preincubated for 30 min at 37°C prior to the addition of substrate. The substrate for the assay (2mM L-ornithine containing 0.1 uCi ¹⁴CO₂/50 ul) was added to the reaction mixture just prior to the start of the assay. The reactions were done in 15 ml disposable glass test tubes which were kept on ice until the reaction was started. Control tubes which contained 200 ul of TAB and 50 ul of substrate were run with each reaction. The tubes were capped with rubber stoppers from which plastic wells (Kontes Scientific Glassware/Instruments, Vineland, NJ, 08360) were suspended. Each plastic well contained a 1 cm² piece of Whatman 3MM filter paper soaked with 50 ul of Tissue Solubilizer (Beckman, B450). The reaction was started by removing the tubes from the ice bath and placing them in a 37°C waterbath immediately after
addition of the substrate. The reaction was allowed to proceed for 60 min at which time it was terminated by the injection of 0.2 ml of 0.5N H₂SO₄ through the rubber stopper. The tubes were placed back in the 37°C waterbath for an additional 60 min to completely adsorb the ^1⁴CO₂.

At this time the caps were removed and the filter papers were placed in glass scintillation vials (Wheaton Scientific Co.). Ten ml of OCS scintillation fluid (Amersham Co.) was added to each vial and the vials were counted on Program #3 in a Beckman 7000 LSC for 10 min each. The counts per min were used to calculate specific activity for ODC as follows:

\[
\frac{\text{CPM (treatment)-CPM (blank)}}{\text{LSC counting efficiency for } ^{14}\text{C}} = \text{DPM Recovered}
\]

\[
\frac{\text{DPM Recovered (}^{14}\text{CO₂)} \times \text{Final substrate}}{\text{DPM in reaction mixture concentration (0.1 uCi = 2.22x10⁵ DPM)}} = \text{nmol } \text{CO₂/hr}^{-1}
\]

\[
\frac{\text{nmol CO₂/h}^{-1}}{\text{mg protein/ 150 ul}} = \text{nmol CO₂ h}^{-1}\text{mg}^{-1}\text{ protein}
\]

Polyamine Extraction and Dansylation

Leaf and/or callus tissue was collected and placed on ice. Just prior to use it was weighed and cut into small pieces to facilitate homogenizing in the Polytron. The
tissue was homogenized (0.25 g fresh weight/ml 5% PCA) at 21,000 RPM in a Brinkman Polytron for 2 x 45 sec on ice. The homogenate was then incubated on ice for one h prior to centrifugation at 16,000 RPM for 15 min at 4° C. Dansylation of the polyamine extracts along with the internal standard heptanediamine was done at the same time as dansylation of the polyamine standards according to the procedure of Minocha et al. (1990). For dansylation, 30 ul of 20.84 uM heptanediamine was added to 120 ul of tissue extract. Fifty ul of the mixture was then placed in a reactivial along with 100 ul of saturated Na₂CO₃ and 100 ul of dansyl chloride (10 mg/ml acetone). The mixture was vortexed for 10 sec and incubated for one h at 60° C. At the end of the incubation, 50 ul of L-proline (100 mg/ml H₂O) was added to each vial and they were vortexed for 10 sec. The samples were then incubated at 60° C for 30 min followed by three min of vacuum drying in a Speed-Vac to remove the acetone. Four-hundred ul of Toluene (Photrex grade, Baker) were added to each sample and the mixtures were vortexed for 30 sec. After incubating for 5 min at 25° C, the reactivials were spun for one min in a low speed centrifuge (Savant Instr. Inc) to ensure separation of the two phases. After the centrifugation, 200 ul of the top layer was removed and placed in a microfuge tube. The samples were evaporated to dryness in a Speed-Vac. The dried samples were reconstituted in 1 ml of methanol and
vortexed for one min. The samples were then stored overnight at -20°C. The samples were vortexed briefly and centrifuged for 5 min at 12K RPM at 4°C prior to being quantified using HPLC. The polyamine standards were dansylated using the same method and consisted of a mixture which contained 20 pmole of each polyamine, as well as heptane diamine, per injection (6 ul).

Separation and Quantification of Polyamines by HPLC

The liquid chromatographic system consisted of a Perkin-Elmer series 400 pump, a Rheodyne injector valve fitted with a 6 ul loop, a Perkin-Elmer Pecosphere-3x3 CR C18, 33x4.6 mm I.D. cartridge column (3 um particle size), and a fluorescence detector (LS-1, Perkin-Elmer). The excitation and emission wavelengths were set at 340 and 510 nm, respectively. Peak areas were calculated using a LCI-100 integrator (Perkin-Elmer). The elution program used was modified from Minocha et al.(1990) (Table 8).

Neomycin Phosphotransferase (NPT II) Assay

Non-radioactive quantitation of NPT activity was done using a NPT ELISA kit from 5 Prime->3 Prime, Inc.. Protein was extracted from the plant tissue by grinding in a microfuge tube with a micropestle at 4°C (100 mg fresh weight tissue/ 100 ul ice cold extraction buffer). The
Table 8. Modified method for HPLC analysis of polyamines.

<table>
<thead>
<tr>
<th>STEP</th>
<th>TIME (min)</th>
<th>FLOW RATE (ml/min)</th>
<th>% ACN&lt;sup&gt;1&lt;/sup&gt;</th>
<th>% HSC&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1</td>
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<td>6</td>
<td>0</td>
<td>2.5</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

1- Acetonitrile
2- Heptane sulfonic acid
extraction buffer (EB) consisted of 62.5 mM Tris-HCl (pH 6.8), 5.0% 2-mercaptoethanol, 10.0% (v/v) glycerol and 0.1% (w/v) SDS (Nagy et al. 1988). The homogenate was centrifuged at 12,000 × g for 5 min at 4°C and the supernatant transferred to a prechilled microfuge tube and stored on ice. The protein content of the extract was determined by the Bradford method.

The quantitation of NPT activity was done by following the manufacturer's instructions. Briefly, a 1:900 dilution of the NPT Coating Antibody was prepared and 200 ul was added to each well in the microwell strip. The strips were covered with plastic wrap to prevent drying and incubated at 37°C for 2 h. The wells were emptied and each well was washed three times with 1X PBS. The wells were emptied of the last 1X PBS wash and 400 ul of 1X Dilution Buffer were added to each well. The strips were incubated at 25°C for 30 min which ensured blocking of any additional protein binding sites in the wells. The wells were emptied of the 1X Dilution buffer and washed 5X with 1X Washing buffer. After removing the last of the 1X Washing buffer, 200 ul of the plant cell extracts (100 ug of protein with volume brought up to 200 ul with EB) were added to each well. In addition, the control extracts (NPT) were added to the control wells and a blank well with just EB was also used. The strips were incubated at 25°C for 2 h. The wells were emptied and washed 5X with 1X Washing buffer.
removing the last of the 1X washing buffer, 200 ul of a 1:1250 dilution of Biotinylated antibody to NPT in 1X Dilution Buffer was added to each well. The strips were incubated for one h at 25° C. The wells were emptied and washed 5X with 1X Washing buffer. A 1:750 dilution of Streptavidin Conjugated Alkaline Phosphatase (SA/AP) in 1X Dilution buffer was added to each well (200 ul/well) and the strips were incubated for 30 min at 25° C. The wells were then washed 5X with 1X Washing buffer and 200 ul of the color development substrate solution (2 mg para-nitrophenyl phosphate/ ml diethanolamine) were added to each well. The strips were incubated at 25° C for 30 min in the dark. The reaction was stopped by adding 50 ul of stop solution to each well. The A₄₀₅ for the contents of each well was measured against the reagent blank. The amount of NPT protein was calculated using the standard curve determined with the NPT controls (pg NPT=A₄₀₅ x 194.37 - 10.41).
RESULTS

Construction of Expression Vector pCS-1

Twenty ug of plasmid pMH-973 neo (Fig. 10) were restricted overnight with 100 units of Eco RI and 100 units of Hind III (pMH973/RI+H3) using the Hind III buffer. The restricted plasmid was electrophoresed under standard conditions yielding three restriction fragments; 9.4 Kb, 1.8 Kb and 973 bp (Fig. 14, lane 7). After staining and destaining, the 9.4 Kb fragment was excised from the gel and electroeluted. Twenty ug of pUC-19 (Fig. 11) were restricted overnight with 100 units of Eco RI and Hind III using the Hind III buffer. The restricted plasmid was loaded (2 ug/well) onto a 2 % TAE agarose gel (16x22 cm, Hoefer HE 99) and electrophoresed at 70 V for 2 h yielding two restriction fragments; 2.65 Kb, and 56 bp. The 56 bp polylinker was excised from the gel and electroeluted. The polylinker (200 ng) was ligated, using standard cohesive end ligation techniques, to the 9.4 Kb fragment (100 ng) from pMH973/RI+H3 and the resulting plasmid was called pCL-1 (Fig. 15).

Competent E. coli HB101 were transformed with the ligation product and plated on LBamp. After incubating overnight at 37° C, ten colonies were chosen and plasmid was isolated from each using the mini-prep method. The plasmids were restricted with Xba I to determine if the polylinker
Figure 14. Restriction analysis of plasmids. Lanes 1 and 8, lambda restricted with Hind III; lane 2, pMH-973 restricted with Eco RI and Hind III; lane 3, pCL-1 restricted with Xba I; Lane 4, pMH-973 restricted with Hind III; lane 5, pCS-1 restricted with Eco RI; lane 6, pCS-1 restricted with Hind III; lane 7, pCS-1 restricted with Pst I.
was present, as this restriction site was not present in the 9.4 Kb fragment from pMH973/RI+H3 (Fig. 14, lane 6). This plasmid was designated pCL-1 (Fig. 16).

Twenty ug of plasmid pCL-1 was restricted with 100 units of Hind III. The restricted plasmid was dephosphorylated by treatment with CIAP using the standard protocol for dephosphorylation of 5'-protruding ends described earlier. The pCL-1 (100 ng) was ligated, using the standard cohesive-end ligation protocol, with the 973 bp (PSS, Ribulose-1,5-bisphosphate decarboxylase/oxygenase promoter) fragment (Fig. 14, lane 5) from pMH-973 neo/H3 (200 ng) to form pCS-1 (Fig. 17). Competent *E. coli* HB101 were transformed with the ligation product and plated on LB selection plates. After incubating overnight at 37° C, the colonies were screened for the presence of PSS by colony hybridization using PSS as the probe. The orientation of the PSS was determined by restricting the plasmid with Eco RI. Plasmids with the promoter in the correct orientation yielded a 950 bp fragment. They were further characterized by restriction analysis with Hind III and Pst I (Fig. 14, lanes 4, 3, and 2) and one was chosen and designated as pCS-1 (Fig. 18).

**Construction of pUODC-1**

Twenty ug of pUC-19 was restricted with 100 units of Eco RI and 100 units of Bam HI using the Bam HI buffer. The restricted plasmid (pUC-19/RI+BHI) was electrophoresed under
Restrict with Eco RI and Hind III
Isolate pUC-19 polylinker and 9.4 Kb pMH-973 Fragment

Ligate polylinker into 9.4 Kb pMH973 fragment

Figure 15. Construction of pCL-1.
Figure 16. Plasmid pCL-1
Restrict with Hind III and isolate 973 bp PSS fragment

Restrict with Hind III and treat with CIAP

Ligate PSS into pCL-1

Figure 17. Construction of pCS-1.
Figure 18. Plasmid pCS-1
standard conditions yielding one visible fragment of approximately 2.65 Kb. The gel was stained and destained and the 2.65 Kb fragment was excised from the gel and electroeluted. Twenty ug of pSP65ODC were restricted with 100 units of Eco RI and 100 units of Bam HI using the Bam HI buffer. The restricted plasmid (pSP65ODC/RI+BHI) was electrophoresed using the standard conditions and yielded two restriction fragments; 2.90 and 1.8 Kb (Fig. 19, lane 7). The gel was stained and destained and the 1.8 Kb fragment, containing the ODC cDNA, was excised and electroeluted. The ODC cDNA fragment (200 ng) was ligated, using the standard cohesive-end ligation protocol, into pUC19/RI+BHI (100 ng) and the resulting plasmid called pUO-1 (Fig. 20). Competent E. coli HB101 were transformed with the ligation product and plated on LBamp. After incubating overnight at 37° C, the colonies were plated onto fresh LBamp plates and grown overnight. The plates were screened via colony hybridization using the ODC cDNA as a probe. Plasmids from ten positive colonies were isolated using the mini-prep method and screened by restricting with Eco RI and Xba I. A plasmid with the ODC cDNA inserted properly generated two fragments of 1.8 and 2.7 Kb (Fig. 19, lane 6). A single plasmid was chosen and designated as pUO-1 (Fig. 21). Plasmid pUODC-1 was constructed so that the ODC cDNA, containing a 5'-Eco RI site and 3'-Xba I site, could be cloned into both expression vectors. The presence of these
Figure 19. Restriction analysis of plasmids. lane 1, pSP65ODC restricted with Eco RI and Bam HI; lane 2, pUO-1 restricted with Eco RI and Xba I; lane 3, pCS-1 restricted with Xba I; lane 4, pSSODC-1 with ODC cDNA in wrong orientation restricted with Eco RI, lane 5, pSSODC-1 with ODC in correct orientation restricted with Eco RI; lane 6, pSSODC-1 restricted with Hind III; lane 7, pSSODC-1 restricted with Pst I.
Restrict with Eco RI and Bam HI

Ligate ODC cDNA into pUC-19

Figure 20. Construction of pUO-1.
Figure 21. Plasmid pUO-1
two restriction sites simplified determining the orientation of the ODC cDNA inserted into the expression vectors. The ODC cDNA used for cloning into the expression vectors pGA643 and pCS-1 in subsequent experiments was isolated from pUO-1.

Construction of pS80DC-1

Five ug of pCS-1 was restricted with Xba I (Fig. 19, lane 5), blunt-ended with Klenow and treated with CIAP (pCS-1/X1). Twenty ug of pUODC-1 was restricted with Eco RI and Xba I (pUO-1/RI+XI) and electrophoresed under standard conditions yielding two fragments; 2.6 and 1.8 Kb. The 1.8 Kb ODC cDNA fragment was excised, electroeluted and blunt-ended with Klenow. The ODC cDNA fragment (500 ng) was ligated, using the standard blunt-end ligation, into pCS-1/X1 (500 ng) (Fig. 22). Competent E. coli HB101 were transformed with the ligation product and plated on LBmp. The colonies were transferred to a fresh LBmp and incubated overnight at 37° C. The plates were screened via colony hybridization using the ODC cDNA as probe. Plasmids were isolated from ten positive colonies, using the mini-prep method, and restricted with Eco RI to determine orientation of the ODC cDNA. Plasmids containing the ODC cDNA in the correct (sense) orientation yielded a 1.8 Kb fragment and 950 bp fragment (Fig. 19, lane 3), whereas plasmids with the ODC cDNA in the antisense orientation yielded a 2.75 Kb
Restrict with Xba I and treat with CIAP

Restrict with Eco RI and Xba I
Isolate ODC cDNA

Ligate ODC cDNA into pCS-1

Figure 22. Construction of pSSODC-1.
Figure 23. Plasmid pSSODC-1.
fragment (Fig. 19, lane 4). A plasmid with the ODC cDNA in the correct orientation was further characterized by restriction analysis with Hind III and Pst I (Fig. 21, lanes 1 and 2), and used for all subsequent experiments. This plasmid was pSSODC-1 (Fig. 23)

Construction of p350DC

Five ug of pGA643 (Fig. 13) were restricted with 25 U of Xba I (Fig. 24, lane 8) followed by blunt-ending with Klenow (pGA643/XI). The blunt-ends were dephosphorylated with CIAP. The blunt-ended ODC cDNA was ligated into pGA643/XI using the standard blunt-end ligation protocol (Fig. 25). The ligation product was used to transform competent HB101 and the bacteria were selected on LBtet plates. Colonies were screened by colony hybridization using the ODC cDNA as a probe. Ten positive colonies were selected and plasmid was isolated from each colony using the mini-prep method. Plasmids were restricted with Eco RI to determine if the ODC cDNA was in the correct orientation. Plasmid with the insert in the correct orientation yielded a 2.5 Kb fragment (Fig. 24, lane 3), whereas those with the ODC cDNA in the antisense orientation yielded a 700 bp fragment. A single plasmid with the ODC cDNA in the correct orientation was further characterized by restriction analysis with Hind III (Fig. 24, lane 5) and designated p350DC for all subsequent experiments (Fig. 26).
Figure 24. Restriction analysis of plasmids. Lane 1 and 9, lambda restricted with Hind III; lane 2, pGA643 restricted with Xba I; lane 3, pUO-1 restricted with Eco RI and Xba I; lane 4, pUO-1 restricted with Eco RI and Nco I; lane 5, p350DC restricted with Hind III; lane 6, p350DC1270 restricted with Hind III; lane 7, p350DC restricted with Eco RI; lane 8, p350DC1270 restricted with Eco RI.
Restrict with Eco RI and Xba I
isolate ODC cDNA and blunt-end

Restrict with Xba I, blunt-end and
Treat with CIAP

Ligate ODC cDNA into pGA643

Figure 25. Construction of p35SODC.
Figure 26. Plasmid p35SODC.
Figure 27. Construction of plasmid p35SODC1270
Figure 28. Plasmid p35ODC1270
Construction of p35ODC1270

Five ug of pGA643 were restricted with 25 U of Xba I (pGA643/XI) (Fig. 24, lane 8) followed by blunt-ending with Klenow. The blunt-ends were dephosphorylated using CIAP. Twenty ug of pUO-1 was restricted with 100 units of Eco RI and 100 units of Nco I using the Nco I buffer. The restriction fragments were electrophoresed under standard conditions yielding two fragments; 3.2 and 1.3 Kb (Fig. 24, lane 6). The 1.3 Kb fragment contained the ODC cDNA which was truncated at bp 1273. The truncation removed sequences which coded for amino acids corresponding to a PEST region (Ghoda et al. 1989). The 1.3 Kb truncated, ODC fragment was blunt-ended with Klenow and ligated into the CIAP treated pGA643/XI using the standard blunt-end ligation protocol (Fig. 27). The ligation product was used to transform competent HB101 and the transformed bacteria were selected on LB_tet plates. Colonies were screened by colony hybridization using the ODC cDNA as a probe. Ten positive colonies were selected and plasmid was isolated from each colony using the mini-prep method. Plasmids were restricted with Eco RI to determine if the truncated ODC cDNA was in the correct orientation. Plasmids with the insert in the correct orientation yielded a 2.1 Kb fragment (Fig. 24, lane 2), whereas those with the insert in the antisense orientation yielded a 700 bp fragment. A plasmid with the truncated ODC in the correct orientation was further
characterized by restriction analysis with Hind III (Fig. 24, lane 4) and designated p350DC1270-1 for all subsequent experiments (Fig. 28).

**Construction of pUCCAT**

Twenty ug of pMH973 neo were restricted with 100 U of Bam HI and 100 U of Hind III using the Bam HI buffer. The restricted plasmid was electro-phoresed under standard conditions and yielded 5 fragments: 5.1, 2.3, 1.8, 1.5, and 0.973 Kb (Fig. 29, lane 6). The 1.8 Kb fragment containing the intact coding region for the chloramphenicol acetyl transferase (CAT) gene was excised from the gel and electroeluted. The CAT gene fragment had a 5'-Hind III site and a 3'-Bam HI site. Five ug of pUC-19 were restricted with 25 U of Bam HI and 25 U of Hind III. The restricted plasmid was electrophoresed under standard conditions and the 2.7 Kb fragment (pUC-19/H3+BHI) was excised and electroeluted (Fig. 29, lane 5). The CAT gene (200 ng) was ligated into pUC-19/H3+BHI (100 ng) using the standard cohesive-end ligation protocol (Fig. 30). Competent *E. coli* were transformed and the resulting colonies were screened by colony hybridization using the labeled CAT gene as the probe. Plasmids were isolated from ten colonies using the mini-prep method and restricted with Bam HI and Hind III. Plasmids containing the CAT gene yielded two fragments of 2.7 and 1.8 Kb (Fig. 29, lane 4).
Figure 29. Restriction analysis of plasmids. Lane 1, lambda restricted with Hind III; lane 2, pMH-973 restricted with Bam HI and Hind III; lane 3, pUC-19 restricted with Bam HI and Hind III; lane 4, pUCCAT restricted with Bam HI and Hind III; lane 5, pGA643 restricted with Hind III and Kpn I; lane 6, pGA643 restricted with Hind III and Bam HI; lane 7, pGACAT restricted with Hind III and Bgl II.
Restrict with Hind III and Bam HI
Isolate 1.6 Kb CAT gene

Ligate CAT gene into pUC-19

Figure 30. Construction of pUCCAT.
Figure 31. Plasmid pUCCAT.
Figure 32. Construction of plasmid pGACAT.
Figure 3. Plasmid pGACAT.
One plasmid which contained the CAT gene was designated pUC-CAT-1 (Fig. 31).

**Construction of p35CAT**

Ten ug of pGA643 were restricted with Hind III and Kpn I using the Kpn I buffer (pGA643/H3+KI) (Fig. 29, lane 3). Ten ug of pUC-CAT-1 were restricted with Hind III and Kpn I. The restricted plasmid was electrophoresed under standard conditions and the 1.8 Kb CAT gene was electroeluted. The 1.8 Kb CAT gene fragment (200 ng) was ligated into pGA643/H3+KI (100 ng) using the standard cohesive-end ligation protocol (Fig. 32). The ligation product was used to transform competent *E. coli* and the resulting colonies were screened by colony hybridization using labeled CAT as the probe. Plasmids were isolated from ten colonies using the mini-prep method and the plasmids were restricted with Hind III and Kpn I. A plasmid containing the CAT gene yielded two restriction fragments of 11.4 and 1.8 Kb (Fig. 29, lane 1). A plasmid with the CAT gene inserted correctly was designated as pGACAT-1 (Fig. 33).

**Transformation of Agrobacterium with pSSODC-1, p35ODC-1, p35ODC1270-1 and pGACAT-1**

The triparental mating protocol was used for *Agrobacterium* transformation (Zambryski et al. 1983). *Agrobacterium* A136 was transformed with p35ODC-1, p35ODC1270-1 and pGACAT-1.
Figure 34. Autoradiograph of Colony Hybridization of *Agrobacterium* constructs.
These transformants were selected by growing on LB plates containing 50 mg/L rifampicin and 15 mg/L tetracycline. *Agrobacterium* C58C1 was transformed with pSSODC-1 and the transformants were selected by growing on LB plates containing 50 mg/L rifampicin and 50 mg/L kanamycin. *Agrobacterium* selected on the appropriate antibiotic medium and representing the four transformation events were screened by colony hybridization using either labeled ODC cDNA or CAT as the probe (Fig. 34 a, b). *Agrobacterium* A136 containing the plasmids pAL4404:p350DC-1 (AT350DC), pAL4404:p350DC1270-1 (AT350DC1270), pAL4404:pGACAT-1 (AT35CAT) and *Agrobacterium* C58C1 containing the plasmid pGV3850:pSSODC-1 (ATSSODC) were stored at -70° C in selection media containing 15% glycerol.

**Transformation of Nicotiana tabacum var. Xanthi**

A series of *Agrobacterium*-mediated transformations were done on *Nicotiana tabacum* var. Xanthi. In the initial experiments, the emphasis was on obtaining transformants with AT350DC and AT350DC1270 due to the high level of expression expected with the CaMV 35S promoter. AT35CAT was used as a control for transformation and gene expression. Leaf segments subjected to the transformation process began to swell up and curl after 5-7 days on selection medium (Fig. 35a). The leaf began to turn chlorotic after 10-14 days with isolated "islands" of dark green tissue (Fig.
Shoot formation from the dark green tissue became evident after 17-24 days (Fig. 35c). Shoots, with their basal area still attached to a small portion of the leaf segment, were excised from the leaf sections when they reached approximately 0.5 cm in height and were transferred to fresh SMCK (Fig. 35d). Well-defined shoots consisting of 3-7 leaves were transferred to RMCK (Fig. 36a) and initial root formation was observed after 14-21 days (Fig. 36b). Plants with a well-defined root system were transferred to and maintained at the greenhouse using the procedures outlined in the methods section (Fig. 36c,d).

While most of the 15 transformed plants were indistinguishable from the control (untransformed) plants, three of the transformants exhibited gross morphological abnormalities. These two transformed plants, ODC-30 and 12701-31, exhibited an abnormal floral morphology consisting of short stamens (Fig. 37). Of the two plants, the flowers on 12701-31 had the shortest stamens and the flowers did not produce seed. The flowers on ODC-30 had stamens which were shorter than normal flowers but longer than 12701-31. Some of the flowers on ODC-30 were able to produce seed, but at reduced levels. At this time, I was unable to determine if the lack of seed production was due to the physical inability of the plants to pollinate themselves, or due to some physiological/developmental abnormalities. The transgenic plant 12701-31 exhibited a stunted growth habit with small
Figure 35. Transformation of tobacco: a, Leaves swelling; b, green "islands" in chlorotic leaves; c, initial shoot formation; d, Transfer of shoots to fresh media.
Figure 36. Transformation of tobacco: a, shoots placed onto rooting media; b, root formation; c, transfer to pots; d, plants in greenhouse.
Figure 37. Normal floral phenotype (a), abnormal floral phenotype in plants ODC-30 (b) and 12701-31 (c) and growth habit of 12701-31 (d).
leaves and an overall "unhealthy" appearance (Fig. 37). In addition, it did not form shoots readily when subcultured on SMCK medium.

**Southern Blotting Analysis**

Ten plants which were believed to be transformed with AT35ODC and seven plants which were believed to be transformed with AT35ODC1270 were screened by Southern hybridization using the $^{32}$P-labeled ODC cDNA as the probe. Ten ug of plant DNA was restricted with 50 U of Hind III and electrophoresed under standard conditions for Southern hybridization. The DNA was transferred from the gel onto GeneScreen Plus by vacuum blotting for 2 h using 10X SSC as the transfer buffer. The blot was prehybridized and hybridized using the protocol described in the Materials and Methods. The probe had a specific activity of approximately $2.8 \times 10^8$ CPM ug$^{-1}$ DNA.

After the initial screening it appeared that only two of the AT35ODC transformed plants, designated ODC12 and ODC30 contained the ODC cDNA. Five of the seven AT35ODC1270 transformed plants, designated 12701-2, 12701-10, 12701-31, 12704-30 and 12704-31 contained the ODC cDNA. In the seven plants which were confirmed to be transformed, two plants, ODC30 and 12701-31, were the ones which exhibited an altered floral phenotype. Two plants from each Agrobacterium construct, one each with normal flowers (ODC-12 and 12701-2)
and one each with abnormal flowers (ODC-30 and 12701-31), were chosen for further studies.

Southern hybridization studies of the four transformants and two untransformed control plants (CX-1, CX-2) were carried out as above except that the transfer buffer used was 0.4 N NaOH. The specific activity of the probes used for these studies were consistently in the range of 2.0-4.0 x 10^8 CPM ug-1. Ten ug of plant DNA was restricted with either 50 U of Eco RI or 50 U of Hind III. The hybridization of blots with the Eco RI restriction of the DNA should yield a 2.5 Kb fragment in ODC-12 and ODC-30 and a 2.1 Kb fragment in 12701-2 and 12701-31. The results from the Southern hybridization (Fig. 38a) correspond to what was expected except for a slight difference in size between the fragments in ODC-12 and ODC-30. Interestingly, there are two DNA sequences in both transformants and controls which hybridize with the ODC cDNA. Whether or not these sequences contain segments of the native ODC gene is not known at this time. Hind III restriction analysis of ODC-12 and ODC-30 should result in two bands of approximately 0.8 and 0.9 Kb whereas, only the 0.8 Kb band is generated in 12701-31 and 12701-2. In addition, there should be a variable band in 12701-2 and 12701-31 as a result of the 3' half of the ODC cDNA being attached to genomic DNA in different locations in the two plants. The results of the Southern hybridization correspond with the expected results except for ODC-30 which
Figure 38. Southern analysis of transformed plants: ODC cDNA probe; (a) Eco RI restriction, (b) Hind III restriction: CX-1, CX-2, and W38 are controls; ODC-12 and ODC-30 were transformed with the full-length ODC cDNA; and 12701-2 and 12701-31 were transformed with the truncated ODC cDNA.
has apparently undergone a deletion event resulting in the removal of the 3' Hind III site (Fig. 38b). As with the Eco RI restriction, there are sequences in both control and transformed plants which hybridize to the ODC cDNA.

Results of Northern Blot Analysis

Ten ug of RNA from the two controls and four transformants was electrophoresed and transferred to GeneScreen Plus according to the protocol described under Materials and Methods. The blot was prehybridized according to the protocol and hybridized overnight with 32P-labeled ODC cDNA as the probe (2.7 x 10^8 CPM ug^-1). The results of the Northern blots varied with the quality of the RNA used for analysis. In this Northern blot (Fig. 39), the probe hybridized strongly to 12701-31 and to a lesser degree with ODC-12, ODC-30, and 12701-2. It appears that the transcript in ODC-12 is slightly larger than in the other transformants.

Results of the Western Blot Analysis

Anti-ODC antibodies from three sources were used to determine the presence of the murine ODC protein in total protein extracts from the transformed plants. Antibody AB1 (Dr. Persson) reacted strongly with a single polypeptide in ODC-30, 12701-2, and 12701-31 (Fig. 40a). In addition, the relative strength of the signal indicates higher levels of
Figure 39. Northern Blot Analysis of Transformed Tobacco:
(a) lane 1, CX-1; lane 2, ODC-12; lane 3, ODC-30; lane 4, 12701-2; lane 5, 12701-31
immunoreactive protein in 12701-31. Antibody AB2 (Dr. Coffino) reacted with several polypeptides in the control and transformed plants (Fig. 40b). However, a polypeptide which was unique to the transformed plants ODC-30, 12701-2 and 12701-31 reacted strongly to antibody AB2. The third antibody, AB3 (Dr. Haddock), also reacted with polypeptides in the control as well as transformed plants. As with AB2, the AB3 reacted strongly to a polypeptide which was unique to ODC-30, 12701-2, and 12701-31 (Fig. 40c).

**Results of Neomycin Phophotransferase Analysis**

The non-radioactive ELISA based NPT II assay was used to measure NPT II levels in the 2 untransformed control plants, four plants transformed with ODC cDNA and a plant transformed with the p35CAT construct. The results presented in Table 9 show significantly higher levels of NPT present in all transformed plants as compared to controls.

**Ornithine Decarboxylase Activity**

The analysis of ODC activity in the two control and four transformed plants was designed to determine the levels of ODC activity at different pH, in the presence or absence of DFMO, and in the presence or absence of anti-ODC antibody. The optimum pH for murine ODC activity is pH 6.8-7.0 (Seely et al. 1982), and that for tobacco 8.0-8.5 (Heimer and Mizrahi 1982). Therefore, the ODC activity of the
Figure 40. Western blot analysis of transformed tobacco.
Lane 1, CX-1; lane 2, CX-2; lane 3, ODC-12; lane 4, ODC-30; lane 5, 12701-2; lane 6, 12701-31. (a) AB1, (b) AB2, (c) AB3
Table 9. Quantitation of Neomycin phosphotransferase in Control and Transformed plants. Units equal to pg NPT mg\(^{-1}\) Total Protein.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>W38</td>
<td>18.22</td>
<td>31.75</td>
<td>26.69</td>
</tr>
<tr>
<td>CX-1</td>
<td>16.10</td>
<td>21.90</td>
<td>17.57</td>
</tr>
<tr>
<td>ODC-12</td>
<td>2189.83</td>
<td>2094.89</td>
<td>1895.27</td>
</tr>
<tr>
<td>ODC-30</td>
<td>2097.46</td>
<td>1868.61</td>
<td>1756.76</td>
</tr>
<tr>
<td>12701-2</td>
<td>1598.31</td>
<td>1428.47</td>
<td>1305.41</td>
</tr>
<tr>
<td>12701-31</td>
<td>2381.36</td>
<td>2159.12</td>
<td>1872.30</td>
</tr>
<tr>
<td>T35CAT</td>
<td>2124.35</td>
<td>1879.62</td>
<td>1837.42</td>
</tr>
</tbody>
</table>

*results are from a single ELISA assay per plant per experiment.
transformed and control plants was assayed at both pH 6.8 and 8.2. The results (Table 10) indicate a great deal of variability in ODC activity, between experiments, in leaf tissue from control and transformed plants. However, the effects of treatment within the experiments were consistent. The level of ODC activity at pH 6.8 was significantly higher in all transformed plants as compared to the control plants as determined by analysis of variance p<0.01. At pH 8.2, ODC-12 and ODC-30 had significantly higher levels of ODC activity than the controls and 12701-31 had significantly lower levels of activity. At pH 6.8, the elevated levels of enzyme activity varied with individual constructs, which was expected in assaying plants derived from individual transformation events. Transformant 12701-31 consistently exhibited the highest levels of enzyme activity in comparison with the other plants.

The effects of DFMO on ODC activity in transformed and control plants are shown in Table 11. There was no significant difference between the effect of DFMO on ODC activity in control and transformed plants at pH 8.2. However, there was a significant increase in the level of inhibition of ODC activity at pH 6.8 in 12701-31 as compared to the control plant.

The effects of anti-mouse ODC antibody on levels of ODC activity are shown in Table 12. In enzyme extracts from leaf tissue, there was a significant inhibition of ODC
Table 10. The effect of pH on ODC activity in the leaves of control and transformed plants. Specific Activity is given in nmol mg\(^{-1}\) protein h\(^{-1}\). Data were transformed to natural log and pairwise comparisons between transformants and control were made by one-way ANOVA.

<table>
<thead>
<tr>
<th></th>
<th>pH 6.8</th>
<th>pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>mean</td>
</tr>
<tr>
<td>Control</td>
<td>0.79</td>
<td>23.52</td>
</tr>
<tr>
<td>35CAT</td>
<td>1.34</td>
<td>33.71</td>
</tr>
<tr>
<td>ODC-12</td>
<td>14.52*</td>
<td>63.37*</td>
</tr>
<tr>
<td>ODC-30</td>
<td>8.85*</td>
<td>53.32*</td>
</tr>
<tr>
<td>12701-2</td>
<td>11.89*</td>
<td>26.03</td>
</tr>
<tr>
<td>12701-31</td>
<td>433.01*</td>
<td>13.84*</td>
</tr>
</tbody>
</table>

* - means within pH significantly different from control at p < 0.01

Control: pH 6.8 n=10, pH 8.2 n=18
ODC-12: pH 6.8 n=4, pH 8.2 n=9
ODC-30: pH 6.8 n=5, pH 8.2 n=9
12701-2: pH 6.8 n=3, pH 8.2 n=10
12701-31: pH 6.8 n=8, pH 8.2 n=13
Table 11. The effect of DFMO on ODC activity in leaves of control and transformed plants at pH 6.8 and 8.2. Specific Activity is given in nmol mg\(^{-1}\) protein h\(^{-1}\).

<table>
<thead>
<tr>
<th>pH 6.8</th>
<th>pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFMO (mM)</td>
<td>%Inhib.</td>
</tr>
<tr>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Control</td>
<td>0.13</td>
</tr>
<tr>
<td>Exp. 1(^a)</td>
<td>0.26</td>
</tr>
<tr>
<td>Exp. 2(^b)</td>
<td>0.08</td>
</tr>
<tr>
<td>Exp. 3(^b)</td>
<td>0.12</td>
</tr>
<tr>
<td>12701-31</td>
<td>24.30</td>
</tr>
<tr>
<td>Exp. 1(^a)</td>
<td>57.98</td>
</tr>
<tr>
<td>Exp. 2(^b)</td>
<td>774.64</td>
</tr>
<tr>
<td>Exp. 4(^b)</td>
<td>416.31</td>
</tr>
</tbody>
</table>

\(^a\) - Average specific activity of two replicates from a single leaf extraction (n=2).

\(^b\) - Average specific activity of two replicates from two leaf extractions (n=4).
Table 12. Effect of anti-ODC antibody on ODC activity in Control (CX-1) and transformed plants (12701-31). Specific Activity in nmol mg⁻¹ protein h⁻¹. Control tissue and transformed tissue assayed at pH 8.2 and 6.8, respectively.

<table>
<thead>
<tr>
<th></th>
<th>CX-1</th>
<th>12701-31</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody % change</td>
<td>Antibody % change</td>
</tr>
<tr>
<td></td>
<td>(-)  (+)</td>
<td>(-)  (+)</td>
</tr>
<tr>
<td>Leaves</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 1⁺</td>
<td>20.56 14.61 - 8.95</td>
<td>231.95 34.97 - 84.92</td>
</tr>
<tr>
<td>Exp. 2ᵇ</td>
<td>14.97 18.54 + 23.85</td>
<td>416.31 54.44 - 86.92</td>
</tr>
<tr>
<td>Exp. 3ᶜ</td>
<td>19.47 20.26 + 4.07</td>
<td>243.03 17.58 - 92.77</td>
</tr>
<tr>
<td>Callus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 1⁺</td>
<td>3.49 2.29 - 34.38</td>
<td>31.72 8.06 - 74.06</td>
</tr>
<tr>
<td>Exp. 2ᵇ</td>
<td>1.23 1.21 - 1.63</td>
<td>13.06 3.21 - 75.43</td>
</tr>
</tbody>
</table>

Numbers represents the mean of two extractions with two replicates per extraction (n=4).

a - Anti-ODC antibody 1 from Dr. L. Persson
b - Anti-ODC antibody 2 from Dr. P. Coffino
c - Anti-ODC antibody 3 from Dr. M. Haddox

activity by the antibody from Dr. Persson's lab in the
transformed plant as compared to the control. Although the antibodies from Dr. Coffino and Dr. Haddox did not inhibit activity in the control, they inhibited activity in 12701-31 at levels that were not significantly different from the first experiment. With enzyme extracts from callus tissue the results are similar to those obtained from the leaf extracts, although the overall levels of enzyme activity are lower.

**HPLC Analysis of Cellular Polyamine Levels**

Analysis of polyamine levels was done on leaf material as well as callus (Table 13). Analysis of leaf tissue indicated a slight increase in putrescine levels in the transformed plants containing the truncated ODC cDNA. However, there was no significant difference in levels of spermidine and spermine in the control leaves and transformed leaves. There was a good deal of variability in polyamine levels in the transformed leaves. Due to the difficulty in obtaining sufficient leaf material of similar size, age, and location on the plant, polyamine levels were analysed in callus tissue derived from the control and transformed plants. The idea behind using callus was to eliminate variability due to environmental and/or anatomical differences. Although the results of analysis of polyamine levels in callus tissue were still variable, the levels of putrescine were significantly higher in the
transformed plants with 12701-2, and 12701-31 exhibiting a ten-fold increase in putrescine levels. The control plant transformed with p35CAT showed no increase in putrescine levels. There was too much variability in levels of spermidine and spermine to determine if expression of the murine ODC gene had an affect on these levels.

In doing HPLC analysis on these samples, an interesting observation was made in the transformed plant extracts. In all of the transformed plants analyzed for polyamine content, there was a peak coming off the column shortly after putrescine (Fig. 41) which was not observed in the control plants or plants transformed with the CAT gene. In experiment one, the peak is at 3.07 in relation to putrescine, which is at 2.79-2.80. In experiment two, the peak is at 3.08 as compared to putrescine at 2.80. As this peak represents an amine containing compound which is not found in detectable levels in control plants, attempts are being made to determine the nature of this compound.
Table 13. Cellular Levels of Polyamines in Control and Transformed Plants. Units are in nmol g\(^{-1}\) Fresh Weight. Data analyzed by one-way ANOVA.

<table>
<thead>
<tr>
<th></th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX-1</td>
<td>63.13</td>
<td>201.51</td>
<td>43.12</td>
</tr>
<tr>
<td>ODC-12</td>
<td>57.41</td>
<td>139.64</td>
<td>35.12</td>
</tr>
<tr>
<td>ODC-30</td>
<td>93.08</td>
<td>147.61</td>
<td>37.68</td>
</tr>
<tr>
<td>12701-2</td>
<td>131.15*</td>
<td>175.62</td>
<td>62.66</td>
</tr>
<tr>
<td>12701-31</td>
<td>137.23*</td>
<td>115.14</td>
<td>41.08</td>
</tr>
<tr>
<td><strong>Callus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX-1</td>
<td>67.25</td>
<td>54.83</td>
<td>16.18</td>
</tr>
<tr>
<td>ODC-12</td>
<td>248.47*</td>
<td>83.17</td>
<td>18.45</td>
</tr>
<tr>
<td>ODC-30</td>
<td>220.52*</td>
<td>28.97*</td>
<td>7.50*</td>
</tr>
<tr>
<td>12701-2</td>
<td>603.08*</td>
<td>36.82</td>
<td>11.02*</td>
</tr>
<tr>
<td>12701-31</td>
<td>765.18*</td>
<td>17.92*</td>
<td>6.12*</td>
</tr>
<tr>
<td>T35CAT</td>
<td>48.87</td>
<td>24.33*</td>
<td>10.87*</td>
</tr>
</tbody>
</table>

\* - significantly different from control at \(p<0.05\).

\(a\) - mean of two replicates from one extraction

\(b\) - mean of two replicates from four extractions

\(\) - significantly different from control at \(p<0.05\) in comparisons between control and transformed plants.
Figure 41. HPLC Chromatogram of transformed and control tobacco. Putrescine, 2.79-2.80; Heptane Diamine, 3.74-3.76; Spermidine, 4.71-4.72; Spermine, 6.02-6.04, unknown compound unique to transformed plants, 3.07-3.08.
DISCUSSION

Understanding the roles of polyamines in plant growth and development has been the objective of numerous studies over the last 15 years. Polyamines have been implicated in an overwhelming array of plant growth and developmental processes. In spite of the vast amount of data collected to this point, it is difficult to say explicitly how or where polyamines function in the higher plant physiology. Most studies have focused on correlations between changes in the cellular polyamine levels and the developmental phenomenon in question. In many cases inhibitors of certain steps in the pathway of polyamine biosynthesis have been used to modulate cellular polyamines and study its effects on the developmental phenomena. With inhibitors, however, one can only lower the concentration of a metabolite. In order to obtain pertinent information on the role of polyamines one must also be able to achieve an increase in biosynthesis. In theory, increasing putrescine levels in the cell solely through increased ODC levels, i.e., without an environmental or a chemical stimulus which could simultaneously affect other physiological processes, should provide information on the ability of polyamines to regulate a particular growth or developmental processes. The only way to achieve this would be to increase the expression of the native genes for a
particular enzyme, or introduce a foreign gene for ODC or ADC which would be expressed at high levels. The present research was initiated with this goal in mind. The primary objective of the research was to prepare expression vectors for the transfer of a murine ODC gene into plant cells and to study the effect of overexpression of this gene on growth and development in transgenic tobacco plants.

At the onset of this project in 1987, a murine ODC cDNA along with a number of promoters active in plants were available to our lab for the construction of a plant expression vector. Due to the fact that (1) no genomic DNA was available at that time, and (2) the expression of a genomic DNA with one or more introns could have posed the problem of proper processing of the mRNA, cDNA for ODC was selected for use. The murine ODC cDNA has since been demonstrated to behave as a functional gene in heterologous cellular environments (Ghoda et al. 1989, Brabant et al. 1988). The promoters available at that time were PSS, PNOS, and CaMV 35S (Table 3). Due to the high transcription efficiency of the 35S promoter as compared to the PNOS and PSS promoters, the efforts to produce transgenic tobacco focused on using the constructs containing the 35S promoter. In addition to using the 35S promoter with the full length ODC cDNA, a truncated version of the ODC cDNA was also cloned into the expression vector. The rationale behind using the truncated ODC cDNA involved removing an area of
amino acids which scored high in the "PEST" analysis test (Ghoda et al. 1989). The enzyme product of the truncated gene has been shown to have a significantly longer half-life during heterologous expression in *E. coli* as well as a Chinese hamster ovary cell line. The removal of 37 amino acid residues from the carboxyl terminus transformed the murine ODC from an enzyme with a short half-life into a stable protein (Ghoda et al. 1989). In the final analysis of vector construction, it appeared that the combination of 35S promoter fused to truncated ODC cDNA would maximize the potential for transgene expression.

Initial analysis of putative transformed plants by Southern blotting indicated that not only was the murine ODC cDNA present in the transformed plants, but there were also native DNA sequences which were homologous to the murine ODC cDNA probe. In previous experiments, this probe had shown homology with sequences in the genome of *Arabidopsis thaliana* and *Daucas carota* (R. DeScenzo, unpublished data). This finding was not unexpected, given the high degree of conservation in the sequence of this gene in most eukaryotes from which the gene has been isolated (Heby and Persson 1990, Coffino and Chen 1988, Fonzi and Sypherd 1987, Phillips et al. 1987). Unfortunately, no plant ODC genes have been cloned and characterized to make comparisons of the nucleotide sequences. In all transformed plants, however, the murine ODC DNA was present as an easy to recognize
separate band in the Southern hybridizations. The results of the Southern hybridization with DNA isolated from the transformed plants were indicative of the transfer and integration of the complete coding sequence except for the plant designated ODC-30. This plant was transformed with a p35ODC which contained the full length ODC cDNA. However, the Southern blot analysis of the Eco RI and Hind III restrictions indicates that there has been a deletion in the 3' end of the cDNA. It is difficult to determine the extent or location of this deletion, but the deletion has apparently removed the 3' Hind III site from the ODC cDNA. Rearrangements and deletions of T-DNA sequences have been observed to occur prior to or during insertion of the T-DNA into the host plant genome (Weising et al. 1988, Zambryski 1988, Wirtz et al. 1987, Czernilofsky et al. 1986). In addition, it is quite common for multiple copies of the T-DNA to be inserted into the host genome in a single transformation event (Zambryski 1988, Weising et al. 1988, Hamill et al, 1990). In the plants chosen for further analysis in this study, i.e., ODC-12, ODC-30, 12701-2 and 12701-31, it appears that ODC-30, 12701-2 and 12701-31 had multiple copies of the ODC gene.

Northern blot analysis of the four transformants and two controls confirmed the difficulty of working with RNA. Although the 35S promoter is considered to be a constitutive promoter, there is evidence that differential rates
of transcription can occur in different tissues of a transformed plant. Indeed, the accumulation of transcript for the foreign gene can vary both temporally and spatially in various tissues in tobacco plants derived from a single transformation event (Williamson et al. 1989). In addition, rate of transcription also is dependent on the site of insertion in the host genome (Hamill et al. 1990, Weising et al. 1988, Zambryski et al. 1988, Jones et al. 1985). In the plants transformed with the murine ODC cDNA, the level of transcript varied among plants originating from independent transformation events. Overall, 12701-31 had a greater accumulation of transcript as compared with the other transformed plants. While the level of transcript in the transformed plants varied with the RNA isolation and blotting in different experiments, the untransformed control plants never showed any transcript homologous to the murine cDNA under any condition. This may be due to: (1) The lack of transcript production from the DNA which is homologous to (as shown by Southern hybridization) the murine ODC cDNA, or (2) The amount of the transcript was too low to be detected.

Due to the short run-length of the gel, it was difficult to determine if the truncated gene transcript was appropriately smaller than the full length transcript. However, the homologous transcript in ODC-12 appears to be slightly larger than in ODC-30, 12701-2 and 12701-31 (Fig. 28c).

Assuming expression of the foreign gene, the major
difficulty encountered in these types of experiments is to distinguish between the native gene product and the foreign gene product. In order to distinguish between the two types of ODC in the transgenic plants, four different approaches were used, namely Western blot analysis using anti-murine ODC antibodies, observation of ODC activity in control and transgenic plants at pH optima for murine and tobacco enzyme, inhibition of enzyme activity by DFMO, and inhibition of enzyme activity by the anti-murine ODC antibodies. While any one of these approaches may not provide a direct proof for the presence of active murine ODC in the tobacco cells, the combined data leaves little doubt that the mouse gene was expressed and active enzyme was present in the transgenic plants.

The analysis of protein in the transformed plants by Western blotting was crucial in determining the expression of the murine ODC gene. Limited amounts of anti-ODC antibody were obtained from three different sources for use in both the Western blots and enzyme activity assays. As these antibodies were produced in different laboratories, variation in their specificity was expected. The initial Western blot was done using a partially purified polyclonal anti-ODC antibody obtained from L. Persson. In this blot, the only strong hybridization signal was to the single band which was present only in ODC-30, 12701-2, and 12701-31. The signal intensity was much greater in 12701-31 compared
to the others indicating a higher level of immunoreactive protein. The second and third Western blots were done using crude sera containing anti-ODC antibody. In both cases, a band that was unique to the transformants was present in ODC-30, 12701-2, and 12701-31 as with the first Western blot. In addition, there were other immunoreactive proteins including a band which had high signal strength in the controls and became relatively weaker as the signal intensity of the transformed plant unique band increased. In comparison to the polyclonal antibody used in the first blot, the immunoreactivity between the crude sera used in the second and third blots to proteins common to the control and transformants could be due to immune reaction with the plant ODC or a non-specific binding of antibodies to some other protein. Down-regulation of a native gene via expression of a foreign gene coding for the same enzyme has been shown in tobacco containing a heterologous phenylalanine ammonia-lyase (PAL) gene (Elkind et al. 1990) and in Petunia transformed with homologous chalcone synthase or dihydroflavonol-4-reductase genes (Napoli et al. 1990, van der Krol et al. 1990). In Petunia, over expression of the chalcone synthase gene resulted in the suppression of the endogenous gene. Similar results were obtained with the dihydroflavonol-4-reductase genes. Over expression of the Phaseolus PAL gene in tobacco resulted in the suppression of the endogenous PAL. The apparent decrease in signal
strength of the putative native ODC protein concurrent with
the increase in signal of the murine ODC protein suggests
that down-regulation may be occurring here. However, data
from these initial experiments are not sufficient to
determine the nature of this immuno-reaction.

While there was a great deal of variability in
measureable ODC activity between different experiments in
transformed and control plants, the trends within each of
the experiments were very similar. This observation is
consistent with measurement of ODC activity by others
(Robie, Minocha, Personal communications) and can be
attributed to environmental factors at the time of leaf
harvest, e.g., light, water, temperature, or other factors
such as relative age or size of the leaf tissue harvested
for individual experiments. Enzyme assays were done at the
pH optima for the murine and tobacco enzymes. The optimum
pH for murine ODC activity falls between 6.8-7.2 and that of
tobacco ODC is 8.0-8.2. In general, there is very little,
if any, detectable ODC activity in both transformed and
untransformed control plants at pH 6.8. However, all of the
transformed plants had significantly higher ODC activity at
this pH, which could be attributed to the murine enzyme.
Indeed, 12701-31 had an average activity that was over 500X
the control level. At pH 8.2, ODC activity was not
significantly different between the control plants and the
12701-2. However, there was a slight, but significant,
increase in ODC activity in ODC-12 and ODC-30. Whether or not this increase in activity is due to the difference in the foreign gene products, i.e., full-length vrs. truncated, activity at a wider range of pH is not known. In addition, 12701-31 showed significantly lower levels of ODC activity at this pH indicating that down-regulation of the endogenous ODC may be occurring. These findings indicate that there is a significant increase in ODC activity, at the pH optimum for the murine ODC, exhibited in the transformed plants and are in accordance with the anticipated results.

Difluoromethylornithine (DFMO), an irreversible inhibitor of ODC activity, is able to completely inhibit the activity of mammalian ODC in vitro (Snyder 1989, Pritchard et al. 1981). In plants, the effects of DFMO on ODC activity have varied with the plant, the tissue, and the researcher. Treatment with DFMO inhibited 69% of ODC activity in mung bean (Bagni et al. 1983), 85% in tomato ovaries (Cohen et al. 1983) and 50% in Helianthus. In carrot, DFMO had little, if any, inhibitory effect on ODC (Mengoli et al. 1989, Robie and Minocha 1989). The control plants and the transformant 12701-31 were used to investigate the inhibitory effect of DFMO on ODC activity (Table 8). As expected, there was virtually no difference between the inhibitory effect of DFMO on the control and the transgenic plants at pH 8.2 or the control plants at pH 6.8. However, at pH 6.8, the high ODC activity in 12701-31 was inhibited
in excess of 99%. The effect of DFMO on ODC activity in the transformed plant at pH 6.8 coincides with the level of inhibition associated with mammalian ODC activity. This observation is probably due to the fact that at pH 6.8, the enzyme activity is largely contributed by the murine ODC.

Due to the limited availability of anti-ODC antibody, a limited number of trials were done to determine the effects of antibody on ODC activity. The effect of antibody on ODC activity was investigated at the optimum pH for ODC activity in the control and transformed plant. In similar assays in mammalian systems, preincubation of enzyme extract with antibody caused a 80-85 % inhibition of ODC activity (Seely and Pegg 1983, Isomaa et al. 1983). There are no published reports on the inhibition of plant ODC activity by anti-murine ODC antibody. The ODC activity in the transformed plant at pH 6.8 was significantly inhibited as compared to ODC activity in the control at pH 8.2. The level of inhibition of ODC activity by anti-ODC antibody in the transformed plant was comparable to that found in mammalian systems.

Data resulting from the combined enzyme assay experiments, i.e., pH optima, DFMO inhibition, and anti-ODC antibody inhibition, strongly support the expression of the murine ODC cDNA. The ODC activity in the transformed plants at pH 6.8 responds to the treatment with DFMO and antibody in accordance with what was expected of the murine enzyme.
Analysis of polyamine levels in the transformed and control plants indicates an increase in putrescine levels in the transformed plants ODC-30, 12701-2 and 12701-31 as compared to the control in both callus and leaf material. However, there is no increase in spermidine or spermine levels in the transformed plants as compared to the controls. The increase in putrescine, but not spermidine or spermine is in agreement with the observations of Hamill et al. (1990) in transgenic tobacco roots overexpressing the yeast ODC gene. It has been suggested that in tobacco, a majority of putrescine synthesized via the ODC pathway is conjugated to cinnamic acid derivatives to form cinnamoyl amides (Burtin et al. 1989). Therefore, the putrescine is not available for conversion to spermidine and spermine. If the putrescine synthesized by the murine gene product is sequestered in the same cellular pool as that of the native ODC, an increase in levels of cinnamoyl amides would be expected. In order to identify and quantify cinnamoyl amides using HPLC analysis, it is necessary to obtain representative samples of each to use as standards. Unfortunately, such samples have not been available to our laboratory at this time. However, a major dansylamine peak was seen in the HPLC chromatograms, which is unique to the plants transformed with the murine ODC. As this compound has slightly longer retention times than putrescine and its levels seem to correlate with the putrescine levels, it is
possible that this compound is a putrescine-cinnamoyl conjugate. However the true identity of this compound still needs to be confirmed.

Several reports on the role of putrescine-cinnamoyl conjugates in the floral development in tobacco have been published (for reviews, see Cabanne et al. 1981, Martin-Tanguy et al. 1987, Evans and Malmberg 1989). In general, alterations in normal levels of putrescine-cinnamoyl conjugates result in abnormal floral morphology. The abnormalities range from floral parts turning into other floral parts, e.g., petaloid anthers, stigmoid anthers, stamenoid ovules etc., resulting in varying degrees of sterility, to vestigial or no flower formation (Malmberg and Hiatt 1989). Interestingly, two of the four transformants used in my analysis exhibit an abnormal floral phenotype consisting of reduced stamens (Fig. 26). This abnormality is more pronounced in 12701-31, which shows higher levels of enzyme activity, and produces no seed, than in ODC-30, which has lower levels of activity than 12701-31, and which produces some seed. The difference in phenotype could be attributed to a dosage effect of the enzyme, but without being able to determine levels of putrescine-cinnamoyl conjugates it will be difficult to correlate phenotype with putrescine conjugate levels. Although, two out of seven confirmed transformants exhibited this phenotype, there are other possible explanations for the altered phenotype
including somaclonal variation and insertional mutagenesis. The chances of two independent somaclonal variants or mutational insertions expressing a similar phenotype are remote. However, by establishing genetic linkage between the presence of the murine ODC gene, elevated ODC activity at pH 6.8, increased levels of putrescine, kanamycin resistance, etc., and the abnormal floral phenotype through several generations of crosses with normal plants, one could easily make a distinction between the different possible explanations. In conclusion, data from this series of experiments clearly show that expression of the murine ODC gene is occurring in the transgenic tobacco. Although the native ODC activity is tightly regulated at the transcriptional, translational, and post-translational levels, it is not known what, if any, effect these control mechanisms exert on expression of the foreign gene. The results from this work raise a number of questions as to the interaction between the two forms of ODC, their regulation, and the metabolic pathways in which their products act as substrate. In addition, the hypothetical link between the observed abnormal phenotype and the expression of the foreign gene is an area which merits future investigation. I hope that these questions will be answered in the near future as their answers will help elucidate some of the roles polyamines play in plant growth and development.
Literature Cited


