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I am submitting herewith a dissertation written by Wei Shao entitled "5-Azacytidine increases the activity of neomycin phosphotransferase II in transgenic Nicotiana tabacum: posttranslational mechanism might play an important role." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

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5-AZACYTIDINE INCREASES THE ACTIVITY OF NEOMYCIN PHOSPHOTRANSFERASE II IN TRANSGENIC *NICOTIANA TABACUM*: POSTTRANSLATIONAL MECHANISM MIGHT PLAY AN IMPORTANT ROLE

A Dissertation

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Wei Shao

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To MY PARENTS

献给我的父母

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ABSTRACT

In previous studies, tobacco protoplasts were transformed with the bacterial gene encoding neomycin phosphotransferase II (npt II). Transformed calli produced from transformed protoplast cultures lost NPT II activity after several subcultures. Activity of this enzyme, however, could be increased by treating the calli with 5-azacytidine. Present studies show that the effect of 5-azacytidine could not be blocked by the DNA replication inhibitor, hydroxyurea, nor by its analogue, cytidine. In addition, the level of mRNA of npt II was not increased by 5-azacytidine, indicating the effect of 5-azacytidine was not at the transcriptional level. Treatment with cycloheximide, a protein synthesis inhibitor, had no effect on 5-azacytidine-increased NPT II activity, indicating that current protein synthesis was not involved in increasing NPT II activity. These results suggested that the effect of 5-azacytidine might be a posttranslational one.

This posttranslational hypothesis was further supported by ELISA analysis which showed that there was no increase of NPT II protein caused by 5-azacytidine, whereas 5-azacytidine increased the activity of the enzyme. In contrast to 5-azacytidine, however, the auxin 2,4-D increased both the quantity and the activity of NPT II.

Furthermore, in vitro studies involving standard

bacterial NPT II enzyme and crude extracts from untreated and 5-azacytidine- or hydroxyurea-treated calli showed that the activity of NPT II added to the untreated extracts was lower than the activity of NPT II added to the extracts from calli treated with 5-azacytidine or hydroxyurea, indicating that there was an unknown factor(s) existing in the crude extracts. This factor affected the activity of NPT II and itself was affected by 5-azacytidine and hydroxyurea treatment.

5-azacytidine is a potent DNA demethylating agent. However, present studies indicate that the effect of 5-azacytidine on increasing NPT II activity was not necessarily due to its demethylation function, and that 5-azacytidine might affect the enzyme in a postranslational mechanism.

Assays for malate dehydrogenase demonstrated that the effect of 5-azacytidine and hydroxyurea on NPT II was not due to an overall effect on callus metabolism.

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CHAPTER I

INTRODUCTION

A. DNA METHYLATION

In recent years, numerous observations have suggested that gene expression is correlated with methylation of promoters or structural gene sequences (Razin et al., 1984; Weissbach et al., 1989). DNA methylation is common in almost all organisms with the exception of some insects like Drosophila (Doerfler, 1983). In higher plants, up to 40% of cytosine residues are methylated whereas in animals, 4 to 6% of cytosine residues are methylated (Adams and Bardon, 1985). Only 5-methylcytosine is found in eukaryotic organisms whereas both 5-methylcytosine and 6-methyladenine are found in prokaryotic DNA (Marinus, 1984).

DNA methylation occurs at specific sequences. In plants, it occurs at both CG and CXG sites, where X can be any of the four nucleotides, whereas in animals, it only occurs at CG sites (Gruenbaum, et al., 1981). It initially occurs at or near replication forks in the presence of S-adenosylmethionine which donates a methyl group (Marinus, 1984).

It has been reported that patterns of DNA methylation are stable and heritable, and that patterns can be changed during organism development (e.g., Banks and Fedoroff, 1989;

Holliday, 1990). Therefore, DNA methylation was proposed as a mechanism of epigenetic control (Holliday, 1990).

DNA methylation may also affect DNA replication, repair, recombination, restriction-modification, mutation, DNA packaging, chromatin structure, tissue differentiation and gene expression (Razin and Riggs, 1980). Numerous studies indicate that there is a strong correlation between DNA methylation and gene expression (Weissbach et al., 1989; Holliday, 1990).

In plants, there is evidence indicating a correlation between gene expression and methylation status for native genes (Bianchi and Viotti, 1988; Kobayashi et al., 1990), tumor cell lines (Gelvin et al., 1983; Hepburn et al., 1983; Amasino et al., 1990), transgenic sequences (Hershkovitz et al., 1990; Weber et al., 1990; Matzke and Matzke, 1991) and transposable elements (Banks and Fedoroff, 1989; Taylor et al., 1989). DNA methylation has been found to be related to plant development. Vergara et al. (1990) reported that there was a difference in DNA methylation patterns between the adult type and embryonic-type clones in carrot. Banks and Fedoroff (1989) reported that patterns of DNA methylation of the suppressor-mutator transposable element changed during the development of corn. Ngernprasirtsiri et al. (1988a, 1988b) indicated that there was a difference in the DNA methylation level of plastid DNA between fruits and leaves of tomato. It was also reported that the change of the DNA methylation

patterns of rDNA in some fungi accompany changes in organism development (MaGill and MaGill, 1989). The effects of DNA methylation on gene regulation have been more thoroughly studied in animal systems than in plant systems (Clawson et al., 1990).

Most pertinent to the studies presented here are those by Hepburn et al. (1983) who observed that 5-azacytidine increased nopaline synthase expression in a flax tumor line which contained 22-24 copies of the nos gene, and that 5-azacytidine-induced-demethylation paralleled an apparent increase in transcription of the gene as measured by an increase in nos mRNA levels.

A cause-effect relationship between DNA methylation and gene expression has not yet been firmly established. Recent reports contradict the hypothesis that DNA methylation is a primary mechanism for regulation of gene expression. One contradiction is that no detectable DNA methylation has been found in Drosophila (Holliday, 1990). Walling et al. (1986) reported that active and inactive seed protein genes of soybean have similar methylation patterns. Marano and Carrillo (1991) indicated that in contrast to reports by Ngernprasirtsiri et al. (1988a, 1988b), no DNA methylation change could be detected during the development of plastids in tomato, but this could be the result of the use of the different cultivars. Ohta et al. (1991) suggested that DNA methylation could not affect gene regulation in the

chloroplasts and amyloplasts of pea.

Conflicting observations have also been reported with animal systems. McKeon et al. (1982) found that the expression of the α-2 (type 1) collagen gene was independent of DNA methylation. Kunnath and Locker (1983) reported that rat albumin gene was actively transcribed when it was heavily methylated in 18-day fetal liver. The gene was also active in adult livers when it was unmethylated. They also reported that another gene, α-feto-protein was only active in fetal livers, not in adult livers, although it was less methylated in adult livers. The authors pointed out that their data did not support the hypothesis that DNA methylation turns the gene off.

One of the possible mechanisms by which DNA methylation might affect gene expression is by an affect on chromatin structure. It was found that there is a correlation between hypomethylation, DNAase I sensitivity and in vitro and in vivo transcription (Weisbrod, 1982). Groudine et al. (1981) reported that the transcriptionally active ev-3 and inactive ev-1 endogenous retrovirus loci in chicken cells were different in that the active ev-3 was hypomethylated, DNAase I sensitive, and contained nuclease hypersensitive sites. Keshet et al. (1986) conducted research in which methylated DNA was transferred into mouse L cells. It was found that the methylated DNA altered overall DNAase I sensitivity and restriction enzyme-hypersensitivity sites in transfected cells

and caused the formation of a structure similar to that in genes in an inactive state. The authors implied that DNA methylation might influence chromosome structure, which, in turn, affected expression.

However, not all data support the hypothesis that DNA methylation affects gene expression by affecting chromatin structure. Macleod and Bird (1982) disagreed with the point of view that DNA methylation directly affects chromatin structure based on their observation that hypermethylation of rDNA genes was accompanied with its hypersensitivity to DNAase I. In addition, if there is a correlation between DNA methylation and the structure of chromatin, it is unknown if DNA methylation is the cause of structural changes of chromatin or a result of it.

Another proposed mechanism for the effect of DNA methylation on gene regulation is that DNA methylation affects the binding of regulatory proteins. There are numerous studies supporting this hypothesis. Meehan et al. (1989) described a methyl-CpG binding protein that only bound to methylated DNA. Antequera et al. (1989) reported this protein might protect methylated CG against nuclease digestion. Interestingly, Bednarik et al. (1991) reported that binding of a protein (NPI) was reduced when a sequence in the HIV-1 long terminal repeat was methylated whereas the binding of another protein (NPII) was increased when the sequence was methylated. Ehlich et al. (1990) reported a methylated DNA-binding protein

that bound specifically to DNA sequences only when the DNA sequences were methylated at CG sites. This protein might be a transcription regulatory protein.

However, there are some studies which do not support the model that DNA methylation affects gene expression by affecting DNA and protein binding. For example, Weih et al. (1991) reported that DNA methylation was not sufficient to prevent proteins from binding to a tyrosine aminotransferase gene in vivo.

Several methods have been developed to detect DNA methylation. The one most commonly used is to use isoschizomer pairs of restriction endonucleases, e.g. Msp I and Hpa II to digest DNA to produce different patterns because the enzymes have different sensitivity to methylated sites. The limitation of this method is that, in many cases, there are no specific sites in the area of interest that can be recognized by restriction enzymes. Therefore, methylation of the sites of interest can not be detected by this procedure. The second method is by using high pressure liquid chromatography (HPLC) or other kinds of chromatography to separate methylated C and non-methylated C residues from DNA samples. The limitation of this method is that it can only detect the overall methylation level in whole DNA samples and is not able to provide information on a specific sequence. Recently, direct genomic sequencing was used to detect DNA methylation by Church and Gilbert (1984). This method was

modified by using PCR technique to amplify specific sequences (Saluz et al., 1990). This method takes the advantage that methylated cytosine reacts poorly with hydrazine. Therefore, the sites of unmethylated cytosine can be cleaved whereas the sites of methylated cytosine can not be cleaved. After amplifying by PCR, fragments with different lengths will be generated. The PCR amplified fragments will be then electrophoresed on a sequencing gel and gap will appear whenever there is a methylated cytosine. This makes it is possible to assay all the potential CG or CXG sites.

B. 5-AZACYTIDINE

5-azacytidine is a well known DNA demethylating agent. It was first synthesized for cancer chemotherapy, and later was isolated from Streptoverticillium (Jones, 1984). DNA demethylation is not the only effect 5-azacytidine exerts on organisms. It interferes with DNA replication (Bhagwat and Roberts, 1987), increases mutation rate (Zimmerman and Scheel, 1984; Call and Wessler, 1986; Amacher and Turner, 1987), increases recombination frequency (Zimmerman and Scheel, 1984; Osgood and Seward, 1984) and breaks chromosomes (Viegas-Pequignot and Dutrillaux, 1976; Osgood and Seward, 1984). The most interesting property of this chemical is that it induces expression of inactive genes. A large number of studies have suggested that there is a correlation between DNA

demethylation and activation of silent genes after treatment with 5-azacytidine (Jones, 1984).

The proposed mechanism of 5-azacytidine-activation of silent genes is that 5-azacytidine replaces cytosine residues during DNA replication. Once it is incorporated into the newly formed DNA, it irreversibly inhibits DNA methylase by binding the methylase and preventing the DNA methylase from methylating the other sites (Jones, 1984). This model is supported by several lines of evidence. For example, DNA extracted from 5-azacytidine-treated E. coli inhibits methylase from the same cells in in vitro reactions, and its inhibitory effect could be eliminated by DNAase (Jones, 1984). Hepburn et al. (1983) observed that the azacytidine-inducing effect could be blocked by co-treatment with hydroxyurea, a DNA replication inhibitor, and by addition of a 40-fold excess of cytidine, a competitive inhibitor of 5-azacytidine. Both hydroxyurea, which inhibits DNA replication, and cytidine, which competes with 5-azacytidine for the same sites during DNA replication, theoretically block the incorporation of 5-azacytidine into DNA and thus, presumably, the demethylating effect of 5-azacytidine since the incorporation of 5-azacytidine into DNA is suggested to be necessary for it to inhibit DNA methylase (Jones, 1984). Hepburn therefore concluded that the 5-azacytidine-inducing effect on nos was due to demethylation of the nos gene by 5-azacytidine. However, other mechanisms by which 5-azacytidine induces gene

expression might exist. Foster et al. (1991) reported that 5-azacytidine increased the expression of metallothionein (MT) genes in Hep G2 cells. The effect of 5-azacytidine on MT genes might be due to the elevated level of cellular copper caused by 5-azacytidine rather than the result of DNA demethylation.

C. NEOMYCIN PHOSPHOTRANSFERASE II (NPT II)

Npt II from the bacterial transposon Tn5 (Davies and Smith, 1978) encodes an enzyme, neomycin phosphotransferase II (NPT II) (or aph(3')-II, aminoglycoside 3'-phosphotransferase II) which inactivates several antibiotics such as neomycin, kanamycin and G418 (Cabanes-Bastos et al., 1989). It catalyses the reaction in which phosphate from ATP is transferred to the 3'-hydroxyl group of kanamycin (Matsuhashi et al., 1976).

NPT II was first described and partially purified by Doi et al. (1969) and purified more completely by Matsuhashi et al. (1976). It appears as a single band on gel electrophoresis and has a molecular weight of 25,000 (Davies and Smith, 1978).

NPT II has been reported to be regulated by several compounds. It was found that cAMP could increase NPT II activity two-fold (Tsukada et al., 1972), whereas some chemicals such as EDTA, KCN, formycin, ADP, AMP, and adenosine

inhibit NPT II activity (Doi et al., 1969). Strong inhibition of NPT II was found in the extracts of several mammalian cell lines (Colbère-Garapin et al., 1981), and also in plant cell extracts (Platt and Yang, 1987; Staebell, et al., 1990).

The use of *npt II* as a selectable marker in gene transformation studies in higher eukaryotic cells was first described by Colbère-Garapin et al. (1981). Its usage as a selectable marker fused with the *nopaline synthase* promoter (*pnos*) in plant cell transformation was described by Herrera-Estrella et al. (1983). It has been successfully used in transformation of several plants (Schell, 1987; Offringa et al. 1990; Gharti-Chhetri et al., 1990; Zhu et al., 1990).

Because of its importance as a selectable marker in gene transformation studies, several assays to detect NPT II have been developed or modified, which include those involving P81 phosphocellulose paper blotting after gel electrophoresis (Reiss et al., 1984), direct P81 paper blotting (McDonnell et al., 1987; Platt and Yang, 1987; Staebell et al., 1990; Ramesh and Osborne, 1991), and PEI-cellulose thin layer chromatography (Cabanes-Bastos et al., 1989). The only method which does not use P^{32} is an ELISA method which is available as a kit from 5 Prime - 3 Prime, Inc.

D. THE REGULATION OF NPT II ACTIVITY IN TRANSGENIC TOBACCO

The chimeric gene pnos-npt II was transformed into protoplasts of Nicotiana tabacum var. Xanthi by liposome-mediated gene transformation in this laboratory (Zhu et al., 1990). It was found that after several subcultures, the calli showed no detectable NPT II activity although they grew slowly on medium with kanamycin. Addition of the demethylating agent 5-azacytidine caused detectable levels of NPT II activity in calli and increased growth rates of the calli of all three transformed lines assayed , i.e., XL102, XL103 and XL201. It was also found that the addition of 5-azacytidine to medium increased the proportion of kanamycin-resistant cells recovered from protoplasts. In addition, NPT II-negative shoot tips regenerated from transformed calli could become NPT II-positive after treatment with 5-azacytidine (Zhu et al., 1991). It was suggested that one of the possible mechanisms for 5-azacytidine to induce NPT II activity of the transformed tobacco was that it demethylated pnos-npt II gene sequences (Zhu et al., 1991).

E. HYPOTHESIS FOR THE FUNCTION OF 5-AZACYTIDINE IN TRANSGENIC TOBACCO LINE KH221

In this study, a transformed line, KH221, was selected for further analysis. KH221 has the same origin as transformed

lines XL102, XL103, and XL201, but contains an additional hygromycin-resistant gene hpt, Preliminary experiments indicated that 5-azacytidine was able to increase NPT II activity in KH221, consistent with previous studies (Zhu et al., 1991). The purpose of this study was to determine the mechanism of the regulatory effect of 5-azacytidine on a transgenic line of tobacco KH221. Our initial hypothesis was that 5-azacytidine demethylated the pnos-npt II sequence and demethylation of the gene induced gene transcription, which caused higher activity of NPT II in KH221. However, our study indicates that 5-azacytidine may increase NPT II activity through a mechanism other than DNA demethylation.

CHAPTER II

MATERIALS AND METHODS

A. PLANT MATERIALS

The callus line used in this research was KH221 which was derived from the protoplasts of Nicotiana tabacum var. Xanthi cotransformed with the plasmid pLGVneo23 (Fig. 1) containing npt II (or aph(3')II) (Beck et al., 1982; Depicker et al., 1982; Herrera-Estrella et al., 1983) and the plasmid pCGN1055 containing the hygromycin-resistant gene hpt or hph (Zhu et al., 1990). The negative control line used in this study was Xo which was untransformed callus derived from Xanthi. The leaves used in this study were from RKH221 which was regenerated from callus KH221. Southern hybridization indicated that npt II was inserted into the genome of KH221 and segregation tests demonstrated that the segregation of kanamycin resistance in RKH221 backcrossed to Xo was 1:1, consistent with a single gene insertion model (K. Hughes, pers. comm.).

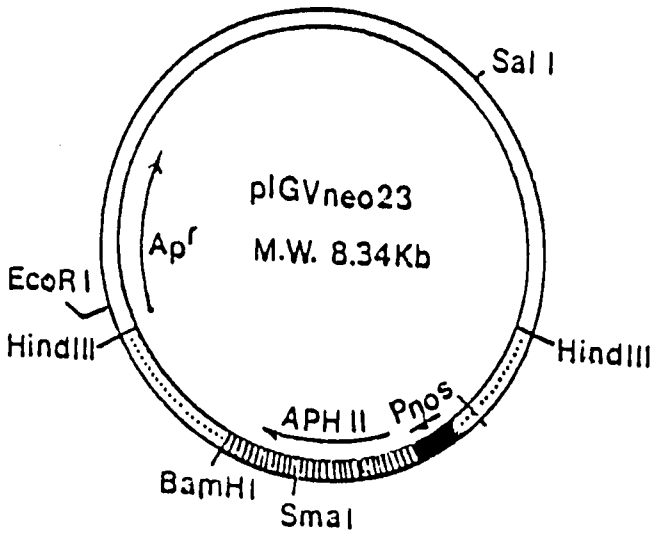

Figure 1. The map of pLGVno23. The following notations are used: ▭ - pBR322 sequences; ▭ - pTiC58 fragment Hind III-23; ▬ - nos promoter; ¦ - border sequences; ▭ - aminoglycoside phosphotransferase coding area.

B. CULTURE CONDITIONS

Calli of cell lines KH221 and Xo were maintained on M3 medium (Meyer et al., 1985; Appendix I) with 1 mg/l naphthaleneacetic acid (NAA) and 0.2 mg/l kinetin, solidified with 0.8% agar and autoclaved. The medium for KH221 was supplemented with 75 mg/l filter-sterilized kanamycin added after autoclaving. The growth chamber in which the calli were maintained provided 9/15 hours of light/dark at 25° C.

C. HORMONE TREATMENT OF LEAF SEGMENTS

Leaves from the middle part of a plant RKH221 (regenerated from callus KH221) were sliced into small pieces (about 1 cm^2) and mixed well. The slices were transferred to 20 ml of solution containing only M3 salts (Meyer et al., 1985; Appendix I). Plant hormones 2,4-D (1.0 to 2.5 μg/ml) or 6-benzyl-aminopurine (BAP, 2.5 to 7.5 μg/ml) were added to the medium. After a 24 hour incubation in a Percival growth chamber (9 hours light/15 hour darks, 25°C), protein was extracted. Some leaf slices were transferred to 20 ml of M3 solution containing 5-azacytidine (10 to 20 μM) instead of plant hormones.

D. CALLUS TREATMENT

Calli were transferred into 250 ml flasks containing 100 ml of autoclaved liquid M3 medium with 1 mg/l NAA and 0.2 mg/ml kinetin and cultured on a rotary shaker at 100 rpm for two days before they were transferred into 40 ml fresh medium and treated with 5-10 μM 5-azacytidine, 0.1-30 mM hydroxyurea, 2.5 mM cytidine, 2.5 μg/ml α-amanitin and/or 5, 10, or 20 μM cycloheximide. Calli were maintained in this medium for 5 days. 5-Azacytidine, hydroxyurea, cytidine, α-amanitin, and cycloheximide were filter-sterilized prior to being added to the medium.

E. NPT II ENZYME ACTIVITY ASSAY

The assay method used in this study was described previously by Zhu et al. (1991) with a few modifications. Briefly, after 5 day treatments, 250 mg of calli treated with hydroxyurea alone or hydroxyurea combined with 5-azacytidine, 250 mg of calli treated with cycloheximide alone or cycloheximide combined with 5-azacytidine or 200 mg of calli treated with α-amanitin, 5-azacytidine and/or cytidine was ground in an Eppendorf tube containing 100 μl extraction buffer (Zhu et al., 1991, Appendix II). Manipulations were performed in a cold room at 9°C. Total protein concentrations of samples were determined by the Bradford method (Bradford,

1976). Extractions equaling 45 μg total protein were applied to a 10% non-denaturing polyacrylamide gel (in 1 M Tris, pH8.8) with a 4% polyacrylamide stacking gel (in 1 M Tris, pH6.8) and electrophoresed in running buffer (Tris 5 mM, glycine 40 mM) at 40 v/ cm in the cold room overnight. The assay for NPT II activity was conducted by overlaying the polyacrylamide gel with a layer (40 ml) of 1% agarose in reaction buffer (Appendix II) containing 50 μg/ml of kanamycin and 5 μCi/ml of (Γ-P^{32})ATP. After 1 hour, the phosphorylated kanamycin was blotted onto P81 phosphocellulose paper (Whatman) by placing P81 paper on the agarose overlay. A stack of paper towels was placed on top. Weights (approximately 1 kg) were added above the stack of paper towels. After 3 hours, the P81 paper was removed and washed in 1% SDS (sodium lauryl sulfate) in a shaking water bath at 50° C for 30 minutes, followed by 10 minutes in 200 ml distilled water at 50° C. Autoradiography was carried out with two intensifying screens at -70° C overnight. The X-ray films were scanned with a LKB2400 Gelscan XL (LKB Produkter, LKB Produkter AB, Box 305, S-161 26 Broma, Sweden). Density curves were analyzed, and the number of absorbance units per millimeter was calculated with the LKB2400 Gelscan XL software package (GSXL).

For each experiment, Xo was used as a negative control. A sample of KH221 cultured in M3 medium without 5-azacytidine, hydroxyurea or cytidine, was also included in each experiment.

Purified NPT II enzyme from 5 Prime - 3 Prime, Inc (5603 Arapahoe Ave., Boulder, CO 80303) was used as a positive control.

Remaining calli from NPT II assays were frozen in liquid nitrogen and stored in a tightly sealed plastic box at -70° for DNA and RNA isolation.

F. RNA AND DNA ISOLATION

RNA was isolated from frozen calli according to a protocol for plant RNA preparation used by Ausubel et al. (1987), but with guanidinum thiocyanate extraction buffer as used by Chirgwin et al. (1979). All glassware was baked at 200° C for at least 3 hours. All plastic and dH_2O were autoclaved. All other solutions and buffers were treated according to the two protocols (Ausubel et al., 1987; Chirgwin et al., 1979) to eliminate RNAse. Approximately 5 g of callus from each sample was ground into a fine powder with a pestle and mortar. Two volumes of guanidinium thiocyanate extraction buffer (Chirgwin et al., 1979; Appendix II) were added to the powder immediately. The frozen mixture was transferred into a sterile screw-top tube and incubated in a water bath at 60° C for 10 min. One volume of phenol/chloroform/isoamyl alcohol (PCI, 25/24/1, the phenol was redistilled and buffered according Sambrook et al., (1989)) was added to the tube. The tube was inverted gently to mix. After centrifuging at 12,000

rpm at 4° C in a Sorvall Superspeed RC2-B for 10 min, the supernatant was removed to a new tube without disturbing the interface and re-extracted with PCI. The supernatant from this step was washed with 1 volume of chloroform/isoamyl alcohol (24/1) to remove traces of phenol. After centrifuging at 12,000 rpm, the supernatant was transferred into a new tube and 0.1 volume of 3 M sodium acetate, and 2 volumes cold 100% ethanol were added to precipitate nucleic acids. The tube was left at -20° C for 30 min. The nucleic acid pellet was collected after centrifuging at 17,000 g for 10 min, and washed with 1 ml 70% cold ethanol and then 1 ml of 100% cold ethanol. The pellet was transferred into 1 ml sterile water and left in room temperature to dissolve. One fourth volume of sterile 10 M LiCl was added to make a final concentration of 2 M LiCl. After storage at -70° C overnight, the solution was microfuged for 30 min at 4° C to precipitate the RNA pellet. The supernatant was saved for DNA precipitation with ethanol and sodium acetate (Sambrook et al., 1989). The RNA pellet was washed with 1 ml of 2 M LiCl followed by 1 ml of 70% ethanol. RNA was stored in 100% ethanol at -70° C.

DNA was also isolated according the CTAB (cetyltrimethylammonium bromide) method reported by Rogers and Bendich (1988). Briefly, 5 g fresh callus was frozen with liquid nitrogen and ground to a powder in a mortar and pestle. Dry ice was added to make sure the powder would not thaw. The powder was transferred into a 50-ml screw-top tube and 1

volume of 2 X CTAB extraction buffer (Appendix II) was added. The mixture was incubated in a water bath at 65°C for 10 minutes. One volume of chloroform/isoamyl alcohol (24: 1) was added to the mixture. Contents were mixed well by inverting the tube very gently. The mixture was centrifuged at 17,000 g for 10 minutes and the top phase was transferred into a new tube without disturbing the interface containing denatured protein. Chloroform/isoamyl alcohol was added to the tube and the tube was inverted very gently. The mixture was centrifuged again at 17,000 g for 10 minutes to obtain the top phase, and then 1/10 volumes of the 10% CTAB (10% CTAB, 0.7 M NaCl) solution was added and mixed. Another chloroform/isoamyl alcohol extraction was performed and the solution was centrifuged again to obtain the top phase. Instead of precipitating nucleic acids with an equal volume of CTAB precipitation buffer used by Rogers and Bendich (1988), 1/10th volume of 3 M sodium acetate was added to the solution and mixed well before adding 2 volumes of 100% cold ethanol (the yield is higher with ethanol precipitation). The mixture was incubated at -20°C for 20 minutes and nucleic acids were isolated by centrifuging at 15,000 g for 10 minutes. The pellet was washed with 1 ml 70% cold ethanol and followed by 1 ml 100% cold ethanol. DNA was stored in 100% ethanol at -20° C.

G. Bam HI/Hind III, AND Eco RI DIGESTION OF KH221 DNA

The procedure described by Sambrook et al. (1989) was followed. Ten μg KH221 total DNA was dissolved in 36 μl distilled water. For Eco RI digestion, 4 μl of Biolabs' NE buffer for Eco RI (New England Biolabs, Inc., Beverly, MA) was added to the DNA solution. Then, 90 units of Biolabs' Eco RI was added and the mixture was incubated at 37° C for 3 hours. For Hind III/Bam HI digestion, 4 μl Biolabs' NE buffer 2 was added to 36 μl DNA solution containing 10 μg DNA. Then, 90 units of Hind III was added and the mixture was incubated at 37° C for 3 hours. After incubation, 6 μl of NE buffer 2 and 10 μl of 22 mg/ml NaCl were added to the mixture, followed by 90 units of Biolabs' Bam HI. The mixture was incubated at 37° C for 3 hours. The digestion mixtures were precipitated by ethanol (Sambrook et al., 1989). Digested DNA was dissolved in 25 μl TE buffer.

H. pLGVneo23 PLASMID ISOLATION, Bam HI AND Hind III DIGESTION

E. coli strain HB101, which contains pLGVneo23, was stored in 50% LB medium (Sambrook et al., 1989; Appendix III) and 50% glycerol at -70° C. Culture and harvesting of the bacteria was according to Sambrook et al. (1989). Bacteria were streaked on a plate containing 25 ml LB medium. A single

colony was transferred to 25 ml of LB medium with 60 mg/l ampicillin and incubated overnight at 37° C with shaking at 120 rpm. Twelve ml of the culture was added to 1 L of LB containing 60 mg/l ampicillin. After a 4 hour incubation, 170 mg/l chloramphenicol was added to amplify pLGVneo23 and the culture was incubated at 37° C overnight with shaking. The cultures were centrifuged at 5,000 g for 5 minutes at 5° C. The pellets were washed and resuspended in 1/2 volume of cold STE (Sambrook et al., 1989; Appendix III) and centrifuged again. Pellets were resuspended in 72 ml STE and poured into 500 ml flasks. Ten mg lysozyme was added into 10 ml STE, mixed with the bacterial-STE mixture and incubated at room temperature for at least 15 minutes. The digestion was monitored with a microscope to make sure that the bacteria became round in shape, an indication of complete digestion of cell walls. At this point, 80 ml of 20% SDS was added and mixed well. After 5 minutes, 80 ml of 0.4 N NaOH was added and mixed well. After 10 minutes, 160 ml of cold potassium acetate (KAc) solution (3 M KAc, 5 M glacial acetic acid) was added and the mixture was shaken very hard several times before it was incubated at 4° C for 20 minutes. The lysate was centrifuged at 15,000 g for 10 minutes and the supernatant was filtered through four layers of cheesecloth into a 250-ml centrifuge bottle. Isopropanol (0.6 volume) was added, mixed well, and incubated at room temperature for 10 minutes. Nucleic acids were recovered by centrifuging at 15,000 g for

10 minutes. The supernatant was removed and bottles were inverted to drain on paper towels.

Sixteen ml of TE (Appendix III) was added to nucleic acid pellets and the solution was transferred into 15 ml tubes. CsCl was added to the solution to make a final concentration of 1 g CsCl/ml and 0.8 ml of 10 mg/ml ethidium bromide solution was added to every 10 ml of the solution. The solution was pipetted into Beckman quick-seal centrifuge tubes, the tubes were balanced, filled with CsCl-TE-ethidium bromide solution and sealed. The tubes were wrapped in aluminum foil to protect DNA-ethidium bromide from light-induced degradation. The solution was centrifuged at 20° C at 45,000 rpm for 40 hours in a Beckman L8-70M Ultracentrifuge.

The lowest DNA band, which represents closed circular plasmid DNA, was transferred with a disposable syringe fitted with 18 gauge needles into new Beckman quick-seal centrifuge tubes and CsCl added as described above. The DNA-CsCl solution was centrifuged at 45,000 rpm at 20° C for 20 hours. The lowest DNA band was transferred into a 15 ml screw-top tubes. An equal volume of water-saturated butyl alcohol was added to the solution to extract ethidium bromide. The top organic phase was discarded and the procedure was repeated several times until no pink color could be seen in the top phase. The DNA solution was transferred into dialysis tubing which had been boiled in TE buffer for 20 minutes. Dialysis against TE buffer for 2 hours and against new TE buffer for

another 3 hours was performed. Then the DNA solution was dialyzed against 50 ml glycerol and 50 ml TE buffer at 4° C overnight. The solution was transferred into Eppendorf tubes and stored at -20°.

To obtain 10 μg of the Bam HI-Hind III fragment of pLGVneo23, which contains npt II, 30 μg of purified plasmid was needed. Plasmid DNA was digested with Bam HI and Hind III according to Ausubel et al. (1987). The digest was loaded on a 0.7 % agarose preparative gel containing 0.5 μg/ml ethidium bromide and electrophoresed in TBE buffer (Appendix III) until the dye front reached the bottom of the gel. The gel portion containing the 2.8 kb band, the middle of three bands, was cut and trimmed with a sharp knife, and the gel slice was put into dialysis tubing with enough TE buffer added to immerse the gel slice. The tubing was subjected to electrophoresis in TE buffer at 100 V for 2 hours. The tubing was taken out and examined with a UV light to make sure that all of the fluorescence was from tubing walls rather than from the gel slice. The gel slice was taken out and the tubing was electrophoresed at 100 V for 1.5 minutes with the current reversed to drive DNA from the tubing wall into solution in the tubing. The solution was transferred into a 15 ml tube and 0.7 volumes of water saturated butyl alcohol was added into the tube and mixed well to remove ethidium bromide. The pink top phase was discarded. The plasmid fragment was precipitated with 0.1 volume of cold 3 M NaAc and 2.5 volumes

of cold 100% ethanol and the pellet was resuspended in 30 μl TE buffer and stored at -20° C.

I. SOUTHERN BLOT AND HYBRIDIZATION

Ten μg of total DNA isolated from KH221 or Xo, and 100 pg of the Bam HI-Hind III fragment of pLGVneo23 which was digested with Msp I (Sambrook et al., 1989) were loaded on a 0.7% agarose gel and subjected to electrophoresis at 5 V/cm in TBE buffer for 5 hours (Ausubel et al., 1987).

Southern Blot and Hybridization were conducted according to Amersham (1985). After electrophoresis, the gel was exposed to UV for 10 minutes to fragment the DNA. The gel was then denatured in denaturing solution (Amersham, 1985) for 40 min and then neutralized in neutralizing solution (Amersham, 1985) for 40 min. The gel was blotted with Hybond-N membrane overlaid by a stack of paper towels and weights for 12 hours at room temperature.

After blotting, the membrane was washed in 2 X SSC (Amersham, 1985; Appendix III), wrapped in Saran Wrap and exposed to UV light from Transilluminator Model TV 36 (Ultra-Violet Products, Inc., San Cabriel, CA) with the DNA side down for 5 min. The membrane was prehybridized in 25 ml prehybridization solution (Amersham, 1985: Appendix III) with denatured salmon sperm DNA (0.5 mg) for 8 hours at 65° C with gentle shaking. The prehybridization solution was replaced by

25 ml hybridization solution (Amersham, 1985; Appendix IV) with 50 ng of the Bam HI-Hind III fragment of pLGVneo23 labelled with ^{32}P. Labeling was by random primer using a random primer kit (Bethesda Research Laboratories Life Technologies, Inc., Maryland) according to the recommendations of the manufacturer. Hybridization was carried out overnight at 65° C. The membrane was washed in a series of decreasing concentrations of SSC, the final concentration being 0.1 X SSC according to Amersham (1985). Autoradiography was done with two intensifying screens at -70° C for 48 hours.

J. RNA SLOT HYBRIDIZATION

A Bio-Rad 48 hole slot apparatus was used to assay total RNA levels of the samples. Five μg of total RNA was denatured and applied to a Hybond-N membrane according to Sambrook et al. (1989). Northern hybridization was conducted according to Amersham (1985). Total RNA was hybridized with 50 ng of the Bam HI-Hind III fragment of pLGVneo23, which was labelled with ^{32}P as described above. After washing with high stringency buffer (Amersham, 1985), autoradiography was done with XRP-1 Kodak film without an intensifying screen for 4 hours at room temperature.

K. ELISA ANALYSIS

NPT II ELISA kits were purchased from 5 Prime - 3 Prime, Inc. (Boulder, CO). Preliminary tests indicated that the extraction buffer (Zhu et al., 1990; Appendix I) interfered with the ELISA assay. Therefore, 0.25 M Tris buffer (pH7.8) was used to extract protein from tissue for ELISA tests.

The procedure was performed according to manufacturer's instructions. Briefly, 200 mg tissue was ground in 100 µl 0.25 M Tris buffer (pH7.8). Protein concentrations were measured by the Bradford procedure (Bradford, 1976), and were diluted to 400 µg/ml, 80 µg/ml, and 40 µg protein/ml. Microwells were coated with NPT II coating-antibodies in coating buffer for 2 hours at 37° C. After washing with 1X washing buffer (supplied by the manufacturer), 400 µl of 1X dilution buffer (supplied by the manufacturer) was added to each well and incubated for 30 min at room temperature to block all additional protein binding sites in the wells. Two hundred µl of standard curve solution (purified NPT II supplied by the manufacturer) and 200 µl of sample were added to the appropriate wells. For each test, 200 µl of 1X blocking/dilution buffer was added to 2 wells as a reagent blank and 200 µl of diluted extract from Xo callus was used as a negative control. Two to three replicates were performed for each sample. After 2 hours at room temperature, the wells

were emptied and washed with 1X washing buffer, and 200 μl diluted biotinylated antibody to NPT II (dilution was according to instructions supplied with each lot of the kit by the manufacturer) were added to the wells and incubated at room temperature for 1 hour. The wells were emptied and washed with 1X wash buffer, and Streptavidin Conjugated Alkaline Phosphatase (diluted according to the instructions) was added. After 30 minutes incubation at room temperature, the wells were emptied and washed again with 1X wash buffer. The substrate for alkaline phosphatase, para-nitrophenyl phosphate (2 mg/ml in diethanolamine buffer) was added to the wells and incubated for 30 minutes in the dark. The reaction was stopped by adding 50 μl of stop solution (containing 2 N sodium hydroxide, supplied by the manufacturer) to each well. The wells were read at 405 nm against the reagent blank with a microwell reader.

Readings for the negative control, Xo, were subtracted from the final reading for transgenic samples. The concentrations of NPT II in the samples were calculated according to the manufacturer's protocol.

L. MALATE DEHYDROGENASE ACTIVITY ASSAY

The activity of malate dehydrogenase was measured according to Kirst (1988). The stock buffers are: 1) Assay buffer: 1 M Tris-HCl, pH7.5, and 33 mM $MgSO_4$; 2) 2.4 mM

oxaloacetate (prepared fresh daily); 3) 5 mM NADH. For large numbers of samples, the following cocktails were made from the above stocks: 1) Assay mixture: 1.2 ml assay buffer, 0.3 ml NADH and 9.18 ml distilled water; 2) Blank mixture: 1.2 ml assay buffer, 1.2 ml oxaloacetate, and 9.54 ml distilled water. For each sample, 5 μl extraction buffer was added to 995 μl of blank mixture. This mixture was used to zero the spectrophotometer at 340 nm. The activity of malate dehydrogenase was measured by adding 5 μl of sample and then 100 μl oxaloacetate (from 2.4 mM stock) to 895 μl of the assay mixture. The absorbance was measured immediately at 340 nm (t = 0), and then measured again 1.5 min later (t = 1.5). A fixed reaction time was used for each sample. The enzyme activity (E) was expressed as $E/min = (A_0 - A_t)/t$. A_0 is the absorbance at t = 0, A_t is the absorbance at t = 1.5 min and t is the reaction time in minutes. Each assay was repeated three times and the average of 3 measurements was determined. Each experiment was repeated twice.

M. EXOGENOUS NPT II ACTIVITY ANALYSIS

Transformed KH221 calli were incubated with 0, 10 or 30 μM 5-azacytidine, or 0 or 30 mM hydroxyurea for 5 or 8 days. Crude extracts were obtained by grinding callus in extraction buffer. Endogenous NPT II activity was destroyed by boiling extracts for 5 minutes, which was confirmed by the

disappearance of the NPT II band in the gel assay. Seventy-five pg NPT II (5 Prime - 3 Prime, Inc.) was added to a volume of extract containing 45 μg cellular protein and the volumes of all the samples were made equal by adding extraction buffer. The mixtures were incubated on ice for 1.5 hours before assaying for NPT II activity. Protein was measured according to Bradford (1976). Experiments above were repeated with untransformed Xo calli, however, the cell extract was not boiled prior to adding exogenous NPT II.

N. STATISTICAL ANALYSIS

A Student's T-test was conducted to analyze the data whenever it was feasible. A paired design t-test was used to compare paired data (across experiments). A standard t-test was used to compare data within a single experiment (Campbell, 1989). In both cases, a two-tailed design was used.

CHAPTER III

RESULTS

A. HYBRIDIZATION OF THE BAM HI-HIND III FRAGMENT OF PLGVneo23 TO TOTAL KH221 AND Xo DNA

In previous studies (Zhu et al., 1990), KH221 was obtained by transforming protoplasts of Nicotiana tabacum cv. Xanthi with the plasmid pLGVneo23 containing npt II and the plasmid pCGN1055 containing hpt (or hph). To determine if npt II was inserted into the plant genome, total DNA was isolated from KH221 and Xo, electrophoresed on an 0.7% agarose gel and blotted to Hybond-N membrane as described in Methods. The membrane containing total DNA isolated from KH221 and Xo was hybridized with the Bam HI-Hind III fragment containing the npt II gene of pLGVneo23. Our results showed that the ^{32}P labelled probe hybridized to the undigested KH221 DNA band with high molecular weight at the top of the gel (Plate 1, Fig. 1, Lane 3), whereas it did not hybridize to DNA from the negative control line, Xo (Plate 1, Fig. 1, Lane 2).

The undigested DNA in Lane 3 is slightly degraded and appears as a diffuse band. The experiment has been repeated several times to verify hybridization to the high molecular weight undigested KH221 DNA. Genomic DNA from KH221 was

Plate 1. Hybridization of the Bam HI-Hind III fragment of pLGVneo23 to total KH221 and Xo DNA.

Figure 1. Hybridization of the Bam HI-Hind III fragment of pLGVneo23 to total KH221 and Xo DNA. Lane 1, Lambda DNA digested with Hind III hybridized with ^{32}P labelled lambda DNA digested with Hind III. Lane 2, 10 μg Xo DNA. Lanes 3, 4 and Lane 5, 10 μg KH221 DNA. Lane 3, undigested KH221 DNA. Lane 4, 10 μg KH221 DNA digested with Bam HI/Hind III. Lane 5, 10 μg KH221 DNA digested with Eco RI.

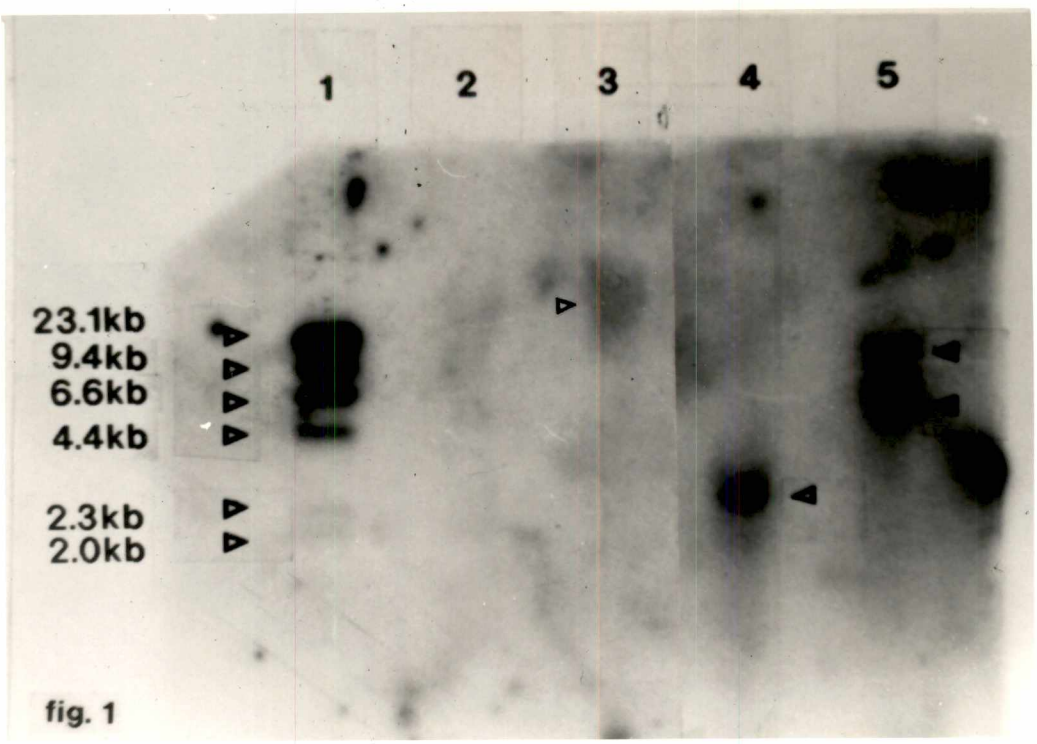

fig. 1

digested with Bam HI/Hind III, and with Eco RI respectively. Bam HI and Hind III digestion yielded a fragment approximately 2.8 kb in size, which hybridized to the npt II probe (Plate 1, Fig. 1, Lane 4). Eco RI digestion yielded two fragments hybridized with the probe. The sizes of these two fragments were between 4.4 kb to 9.4 kb (Plate 1, Fig. 1, Lane 5).

B. VERIFICATION OF THE NPT II ASSAY

In this study, the activity of NPT II was primarily assayed by the reaction in which NPT II transfers ^{32}P onto kanamycin. After autoradiography, three or four bands can usually be seen (Plate 2, Fig. 1 and 2). Only the lowest band is ^{32}P-labelled kanamycin, representing the location of NPT II. This was proven by including the positive control, bacterial NPT II, on each gel assay. NPT II produced only one band at the same position as the lowest band produced by the crude extracts from samples (Plate 2, Fig. 2, and Fig. 4), and by including the negative control, Xo, which produced all of the bands except the lowest one (Plate 2, Fig. 1).

An experiment was conducted to investigate if other bands represent a kanamycin phosphotransferase or a nonspecific phosphotransferase. If only(Γ-P^{32})ATP, instead of both (Γ-P^{32})ATP and kanamycin, was included in the

Plate 2. The effects of 5-azacytidine, hydroxyurea and hormones on KH221 and Xo calli.

Figure 1. Verification of the NPT II assay. Panel A. Both (Γ-^{32}P)ATP and kanamycin were added as reaction substrates. Lane 1, Xo. Lane 2, Xo plus 100 pg bacterial NPT II. Lane 3, KH221 treated with 10 μM 5-azacytidine. Lane 4, KH221 treated with 30 μM 5-azacytidine. Lane 5, KH221 treated with 30 mM hydroxyurea. Panel B. Only (Γ-P^{32})ATP was added as a reaction substrate. Lane 1, Xo. Lane 2, Xo plus 100 pg bacterial NPT II. Lane 3, KH221 without any treatment. Lane 4, KH221 treated with 10 μM 5-azacytidine. Lane 5, KH221 treated with 30 μM 5-azacytidine. Lane 6, KH221 treated with 30 mM hydroxyurea.

Figure 2. Effect of hormones on NPT II activity in RKH221 leaf slices. Lane 1, sample without any treatment. Lanes 2-4: samples treated with different concentrations of 2,4-D. Lane 2, 1 μg/ml. Lane 3, 1.5 μg/ml. Lane 4, 2.5 μg/ml. Lanes 5-6: samples treated with different concentrations of 5-azacytidine. Lane 5, 10 μM. Lane 6, 20 μM. Lanes 7- 9: samples treated with different concentrations of BAP. Lane 7, 2.5 μg/ml. Lane 8, 5 μg/ml. Lane 9, 7.5 μg/ml. Lanes 10 -13: different amounts of standard NPT II. Lane 10, 25 pg. Lane 11, 50 pg. Lane 12, 100 pg. Lane 13, 200 pg.

Figure 3. Effect of hydroxyurea on KH221 DNA and RNA. Lane 1, RNA from sample treated with 10 μM 5-azacytidine. Lane 2, RNA from sample treated with 30 mM hydroxyurea. Lane 3, RNA from sample treated with 2.5 mM cytidine. Lane 4, RNA from sample without any treatment. Lane 5, DNA from sample treated with 10 μM 5-azacytidine. Lane 6, DNA from sample treated with 30 mM hydroxyurea. Lane 7, DNA from sample treated with 2.5 mM cytidine. Lane 8, DNA from sample without any treatment.

Figure 4. Effect of an unknown inhibitory factor on the activity of bacterial NPT II added into crude extracts from Xo. Lane 1 to Lane 6: 75 pg bacteria NPT II was added to crude extracts equaling 45 μg protein. Lane 1 and Lane 2, untreated Xo. Lane 3 and Lane 4, 10 μM 5-azacytidine-treated Xo. Lane 5 and Lane 6, 30 mM hydroxyurea-treated Xo. Lane 7 to Lane 9: positive control. Lane 7, 50 pg NPT II. Lane 8, 75 pg NPT II. Lane 9, 100 pg NPT II.

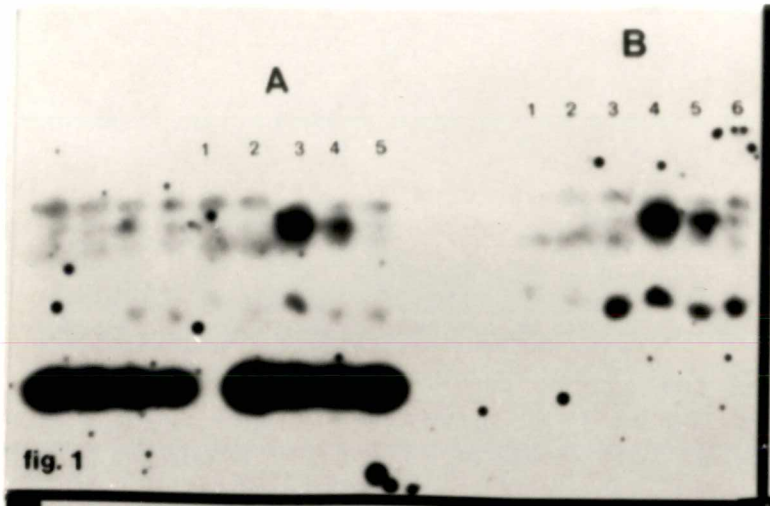

fig. 1

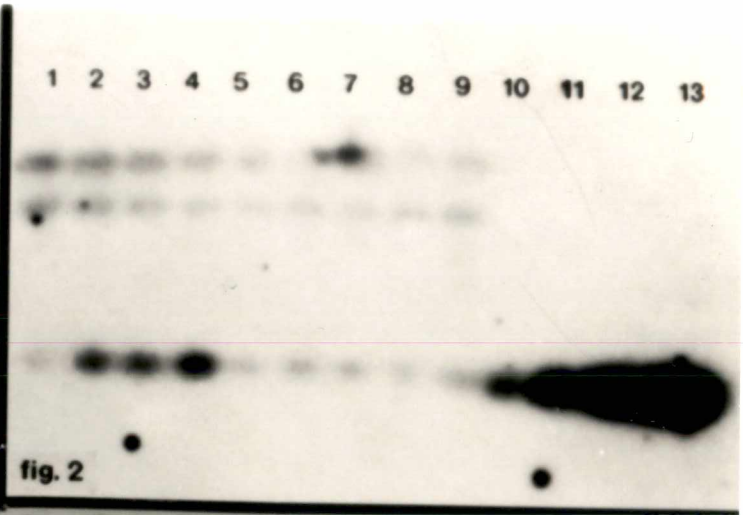

fig. 2

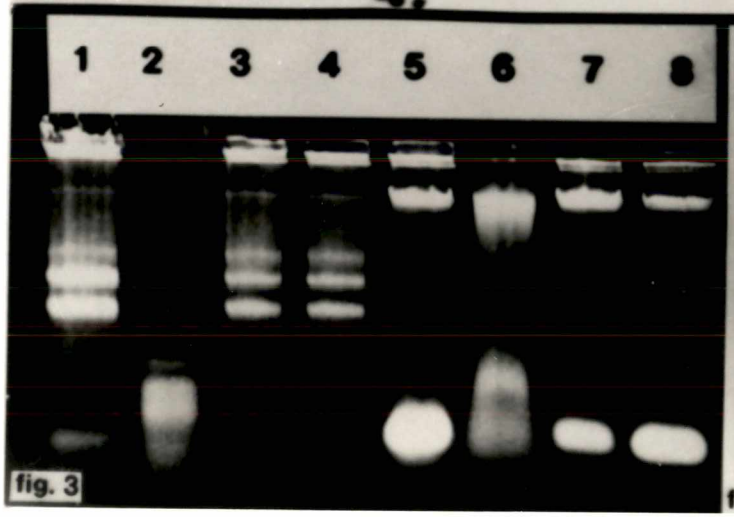

fig. 3

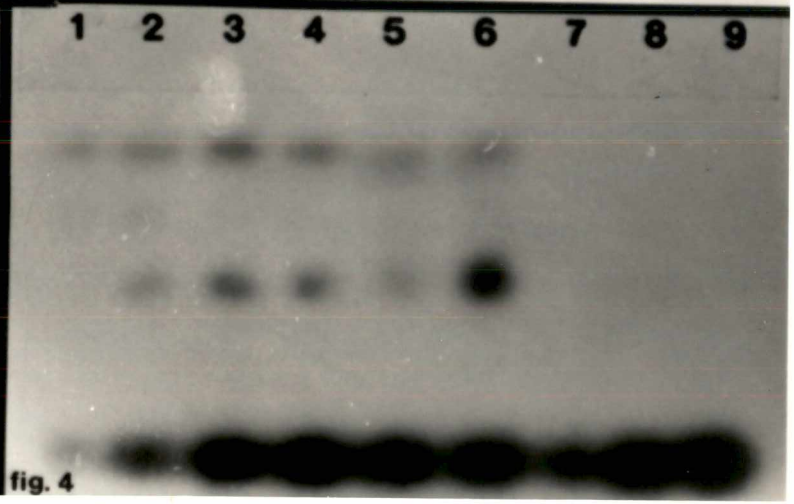

fig. 4

reaction as a substrate, only the lowest band disappeared whereas all other bands remained (Plate 2, Fig. 1). This result indicates that all other bands were produced by reactions which did not require kanamycin as a substrate, and were not produced by NPT II.

C. ANALYSES OF ENDOGENOUS NPT II ACTIVITY

1. EFFECT OF 5-AZACYTIDINE ON NPT II ACTIVITY IN KH221 CALLUS

Zhu et al. (1991) reported that a 10 day treatment with 3 μM 5-azacytidine increased NPT II activity in callus lines XL102, XL103, and XL201. KH221 used in this study has the same origin as the lines used in the work by Zhu et al. (1990). However, KH221 was cotransformed with both *npt II* and the gene for hygromycin resistance *hph*.

Calli were treated with different concentrations of 5-azacytidine for 5 days, and NPT II activity was assayed. We found that NPT II activity in KH221 callus was low, 4.4 absorbance units/mm (Table 1), Nevertheless, treatment with 5-azacytidine resulted in an increased level of NPT II in KH221 calli. After 5-azacytidine treatment for 5 days, NPT II activity was two or three times greater (12.3 absorbance units/mm for a 30 μM 5-azacytidine treatment). The effective concentrations of 5-azacytidine ranged from 10 μM

Table 1. Effect of 5-azacytidine on NPT II activity in KH221 callus[1].

	Untreated	5-Azacytidine (μM)			
		5	10	20	30
Mean	4.4	9.1	8.7**	11.6**	12.3**
STD[2]	1.76	3.70	4.87	3.58	3.72
N[2]	13	3	13	3	3

1. Unit is absorbance units/mm from densitometer scans. Forty five μg protein was loaded for each sample.
2. STD: standard deviation; N: sample size.

** Significantly different from untreated at $p<0.01$.

to 30 μM (Table 1). Paired design Student's T-tests demonstrated that there were significant differences in NPT II activity between untreated calli and calli treated with 5-azacytidine ranging from 10 μM to 30 μM ($p<0.01$). A 5-day 10 μM 5-azacytidine treatment was sufficient to significantly increase NPT II activity and therefore, 10 μM 5-azacytidine treatment was adopted for subsequent studies.

2. EFFECT OF HORMONE TREATMENT ON NPT II ACTIVITY IN RKH221 LEAF SLICES

An et al. (1988, 1990) reported that wounding and auxin treatment, especially by 2,4-D, induced pnos-cat gene expression in leaf slices from transgenic tobacco plants. KH221 contains the same promoter with a different reporter gene (npt II). To determine if auxin induced the same response in RKH221 plants, leaf slices were treated with 2,4-D or 6-benzyl-aminopurine (BAP) for 24 hours, and NPT II activity was assayed by the gel assay as described in Chapter II, Materials and Methods. Results showed that 2,4-D, in concentrations ranging from 1 μg/ml to 2.5 μg/ml, increased pnos-npt II expression within 24 hours, whereas BAP (2.5 μg/ml to 7.5 μg/ml) was not able to induce NPT II activity. Our results also showed that a 24 hour-treatment with 10 or 20 μM 5-azacytidine did not increase NPT II activity in RKH221 leaf slices (Fig 3.). The results were in agreement with An et al.'s report (1990) that auxin, including both 2,4-D and NAA, increased the activity of the pnos promoter whereas BAP did not.

We also conducted an ELISA analysis for NPT II protein in leaf slices treated with 2,4-D which showed an increase of NPT II activity and for leaves treated with BAP which did not show an increase of NPT II activity as described above. The ELISA assay (Table 2) showed that there was 0.85 μg NPT II/mg

Table 2. ELISA test of NPT II in hormone-treated RKH221 leaf slices[1].

Rep[2]	Untreated	2,4-D (μg/ml)			NAA (μg/ml)		
		1.0	1.5	2.5	2.0	4.0	8.0
1)	0.5	0.8	1.0	1.8	0.8	0.9	1.9
2)	1.2	1.5	1.5	1.5			
Mean	0.85	1.15	1.25	1.65			

Rep[2].	Untreated	BAP (μg/ml)			5-azacytidine (μM)	
		2.5	5.0	7.5	10	20
1)	0.5	0.5	0.4	0.5		
2)	1.2	0.93	0.93	0.95	0.97	0.98
Mean	0.85	0.72	0.67	0.73		

1. Unit is μg NPT II/mg protein.

2. Rep: replications.

total cellular protein in untreated leaf slices, whereas there was two times more NPT II protein in leaf slices treated with 2.5 μg/ml 2,4-D, or treated with 8.0 μg/ml NAA. In contrast, the amount of NPT II in leaf slices treated with BAP up to 7.5 μg/ml was not higher than that in the untreated control.

Studies also showed that there was 0.98 μg NPT II/mg protein in leaf slice treated with 10 or 20 μM 5-azacytidine for 24 hours (Table 2), which was not different from levels observed in untreated leaf slices. Therefore, levels of NPT II activity (assayed by gel assay) in leaf slices treated with hormones were in agreement with the amounts of NPT II protein (assayed by ELISA).

3. EFFECT OF HYDROXYUREA ON NPT II ACTIVITY IN CALLI TREATED WITH 5-AZACYTIDINE

5-Azacytidine is a well known demethylating agent. Incorporation of 5-azacytidine into newly replicated DNA, replacing cytosine, is considered a necessary prerequisite for demethylation (Jones, 1984). Once incorporated, it irreversibly binds to and inhibits methylases (Jones, 1984). Hydroxyurea inhibits DNA replication and should therefore prevent the incorporation of 5-azacytidine into DNA (Hepburn et al., 1983). If demethylation plays a role in *npt II* expression induced by 5-azacytidine, hydroxyurea should block the effect of 5-azacytidine.

To test the hypothesis that hydroxyurea blocks the effect of 5-azacytidine, calli were treated with 10 μM 5-azacytidine combined with 0.1 mM to 30 mM hydroxyurea for 5 days and NPT II activity was assayed. Results showed that 10 μM 5-azacytidine significantly increased NPT II activity (from

3.88 absorbance units/mm in untreated calli to 5.90 absorbance units/mm in treated calli; Table 3). NPT II activity in calli treated with 10 μM 5-azacytidine in combination with 30 mM hydroxyurea was actually higher than in the absence of hydroxyurea (8.15 absorbance units/mm). In contrast to Hepburn et al. (1983), our data indicated that 30 mM hydroxyurea in combination with 5-azacytidine did not reduce the effect of 5-azacytidine on NPT activity, and there was no significant difference in NPT II activity between calli treated with 10 μM 5-azacytidine alone and calli treated with 10 μM 5-azacytidine in combination with 30 mM hydroxyurea. There is, however, a significant difference in NPT II activity between untreated calli and calli treated with 10 μM 5-azacytidine or calli treated with 10 μM 5-azacytidine in combination with 30 mM hydroxyurea ($p<0.05$) according to the Student's T-Test (Table 3). Lower concentrations of hydroxyurea also appeared to increase NPT II activity, although the number of replicates was too small for statistical analysis.

4. EFFECT OF CYTIDINE ON NPT II ACTIVITY IN CALLI TREATED WITH 5-AZACYTIDINE

5-Azacytidine is an analogue of cytidine. Therefore, in the DNA replication process, 5-azacytidine and cytidine

Table 3. Effect of hydroxyurea on 5-azacytidine-increased NPT II activity in KH221 Callus[1].

	Untreated	Azacytidine (10 μM)	Azacytidine (10 μM) + Hydroxyurea (mM)			
			0.1	1.0	10	30
Mean	3.88	5.90*	11.7[n]	15.6[n]	14.7[n]	8.15*
STD[2]	2.76	1.72	6.65			3.00
N[2]	8	9	2	1	1	8

1. Unit is absorbance units/mm from densitometer scans. Forty five μg protein was loaded for each sample.
2. STD: standard deviation; N: sample size.

n not tested statistically.

* significantly different from untreated at $P<0.05$.

compete for the same sites in DNA. If 5-azacytidine increases NPT II activity by demethylating DNA, cytidine might block the effect of 5-azacytidine by blocking incorporation of 5-azacytidine (Hepburn et al., 1983).

Calli were treated with 10 μM 5-azacytidine and plus cytidine ranging from 0.05 mM to 2.5 mM for 5 days. Table 4 shows that NPT II activity was 4.4 absorbance units/mm in untreated calli, whereas it was 8.2 absorbance units/mm

Table 4. Effect of cytidine on 5-azacytidine-increased NPT II activity in KH221 callus[1].

	Untreated	Azacytidine (10 μM)	Azacytidine (10 μM) +Cytidine (mM)				
			0.05	0.15	0.30	1.0	2.5
Mean	4.4	8.2**	4.1[n]	5.6[+]	10.6[n]	5.1[n]	7.5[+]
STD[2]	4.6	5.9		8.2			4.6
N[2]	5	5	1	3	1	1	5

1. Unit is absorbance units/mm from densitometer scans. Forty five μg protein was loaded for each sample.
2. STD: standard deviation; N: sample size.

** Significantly different from control (untreated callus) at $P<0.01$.

\+ Not significantly different from 5-azacytidine-treated callus.

n Not tested.

in 10 μM 5-azacytidine-treated calli, a difference which was highly significant ($p<0.01$). NPT II activity in calli treated with 10 μM 5-azacytidine in combination with 2.5 mM cytidine was 7.5 absorbance units, which was not significantly different from that in calli treated with 10 μM 5-azacytidine

only. Therefore, in contrast to Hepburn et al. (1983), it was found that 2.5 mM cytidine had no effect on the increase of NPT II activity caused by 10 μM 5-azacytidine (Table 4). As for the lower concentrations of cytidine indicated in Table 4, the sample sizes are too small to conduct a statistical analysis. Therefore, no conclusion can be drawn for lower concentrations of cytidine in this study.

5. EFFECT OF HYDROXYUREA ON NPT II ACTIVITY

Since hydroxyurea was not able to block 5-azacytidine-increased NPT II activity and preliminary experiments indicated that hydroxyurea alone increased NPT II activity, calli were treated with hydroxyurea in concentrations ranging from 0.1 mM to 30 mM for 5 days. Surprisingly, hydroxyurea alone at all concentrations tested increased NPT II activity. NPT II activity in untreated calli was 4.4 absorbance units/mm, while it was 9.5 absorbance units/mm in calli treated with 5-azacytidine alone. Calli treated with hydroxyurea in concentrations ranging from 0.1 to 30 mM had approximately twice the level of NPT II activity (Table 5). The increase was significantly different from that of the control ($p<0.01$). The data show that hydroxyurea was as effective as 10 μM 5-azacytidine in increasing NPT II activity in KH221 calli.

Table 5. Effect of hydroxyurea on NPT II activity in KH221 callus[1].

	Untreated	5-Azacytidine (10 μM)	Hydroxyurea (mM)			
			0.1	1.0	10	30
Mean	4.4	9.5**	10.0**	10.7**	12.3**	10.9**
STD[2]	3.1	5.4	6.1	6.4	6.7	8.1
N[2]	19	8	3	4	4	9

1. Unit is absorbance units/mm from densitometer scans. Forty five μg protein was loaded for each sample.
2. STD: standard deviation; N: sample size.

** Significantly different from control (untreated callus) at $P<0.01$.

6. EFFECT OF HYDROXYUREA ON TOTAL CELLULAR PROTEIN

Hydroxyurea is a very effective inhibitor of DNA replication. In the 5 day period of hydroxyurea treatment, no growth of the calli could be detected by eye, while calli without any treatment showed rapid growth as determined by an increase in callus size.

To understand better the effect of hydroxyurea on

callus and NPT II activity, calli were treated with 10 μM 5-azacytidine or 30 mM hydroxyurea for 5 days and protein concentrations were determined. Table 6 shows the effect of hydroxyurea on total protein. The concentration of total cellular protein in untreated calli was 1.711 mg/g fresh tissue, whereas it was 1.584 mg/g fresh tissue in calli treated with 10 μM 5-azacytidine, which was not significantly different from that in the untreated control. In contrast, the protein concentration in calli treated with 30 mM hydroxyurea (0.602 mg/g fresh tissue) was reduced to approximately 1/3 of that in the control. A Student's T-Test analysis indicated that the protein concentration in calli treated with 30 mM hydroxyurea was significantly different from that of the control ($p<0.05$).

7. EFFECT OF HYDROXYUREA ON DNA AND RNA FRAGMENTATION

Hydroxyurea has been reported to cause DNA fragmentation, possibly because DNA synthesis is initiated but elongation is inhibited (Nyce et al., 1986). To determine if hydroxyurea had the same effect on DNA in tobacco, calli were treated with 10 μM 5-azacytidine, 2.5 mM cytidine, or 30 mM hydroxyurea separately for 5 days. DNA and RNA were isolated from calli, and electrophoresed on an agarose gel. Results (Plate 1, Fig. 4) indicated that DNA fragmentation was caused by hydroxyurea. Such DNA fragmentation was not found in DNA

Table 6. Effect of 5-azacytidine and hydroxyurea on total cellular protein[1].

	Untreated	5-Azacytidine (10 μM)	Hydroxyurea (30 mM)
Mean	1.711	1.584	0.602*
STD[2]	1.573	1.315	0.564
N[2]	18	18	10

1. Unit is mg protein/g fresh tissue.
2. STD: standard deviation; N: sample size.

* significantly different from untreated control at $p<0.05$.

from control, 5-azacytidine- or cytidine-treated calli (Plate 1, Fig. 4).

Additionally, RNA species with high molecular weight disappeared in the RNA samples from 30 mM hydroxyurea-treated calli. This is especially obvious for the strong RNA bands assumed to be ribosomal RNA (Plate 1, Fig. 4). Reduction of RNA length was not found in the samples treated with 5-azacytidine or cytidine (Plate 1, Fig. 4).

8. EFFECTS OF 5-AZACYTIDINE OR HYDROXYUREA ON STEADY-STATE LEVELS OF RNA

Analysis of steady-state levels of mRNA does not necessarily reflect transcription *in vivo* because some transcripts may be degraded rapidly after transcription; However, there is good reason to assume that steady-state levels of nuclear mRNA reflect the majority of transcription in plants (Okamuro and Goldberg, 1989). If 5-azacytidine increases the activity of NPT II by demethylating DNA and therefore increasing or inducing transcription, then, an increase of transcription might be detected by RNA-DNA hybridization of *npt II*-RNA. An RNA slot blot was conducted to examine the effect of 5-azacytidine and hydroxyurea on steady-state levels of *npt II*-mRNA. Total RNA (5 μg) was hybridized with the *Bam* HI-*Hind* III fragment of pLGVneo23 as described in the Chapter II Materials and Methods. Results indicated that there was no difference in *npt II*-mRNA levels between untreated calli and calli treated with 5-azacytidine or hydroxyurea (Table 7). The steady-state level of mRNA in the control was 0.18 absorbance units, whereas it was 0.14 absorbance units/mm in calli treated with 10 μM 5-azacytidine. This difference was not statistically significant. In addition, the steady-state level of mRNA in calli treated with 30 mM hydroxyurea was 0.21 absorbance units/mm, also not significantly different from that of the control.

Table 7. Effect of 5-azacytidine and hydroxyurea on steady-state levels of mRNA in KH221 callus[1].

	Untreated	5-Azacytidine (10 μM)	Hydroxyurea (30 mM)
Mean	0.18	0.14 [3]	0.21[4]
STD[2]	0.038	0.045	0.135
N[2]	3	3	3

1. Unit is absorbance units/mm from densitometer scans. Five μg of total RNA was loaded for each sample.
2. STD: standard deviation; N: sample size.
3. Not significantly different from untreated sample at p=0.054.
4. Not significantly different from untreated sample at p=0.62.

9. EFFECT of α-AMANITIN ON NPT II ACTIVITY IN CALLI TREATED WITH 5-AZACYTIDINE

α-amanitin is an inhibitor of eukaryotic RNA polymerase II. It inhibits the polymerase at low concentrations (Lewin, 1987). Dixit et al (1989) reported that a level as low as 1 μg/ml α-amanitin inhibited *in vitro* transcription of the metallothionein gene. Mercoyrol et al. (1989) showed that

0.04 μg/ml α-amanitin inhibited the formation of phosphodiester bonds with a UTP (uridine triphosphate) substrate in RNA synthesis catalyzed by wheat-germ RNA polymerase. If 5-azacytidine increases the level of npt II transcription, α-amanitin might block the effect of 5-azacytidine. To test the hypothesis, calli were treated with 2.5 μg/ml α-amanitin combined with 10 μM 5-azacytidine, or 30 mM hydroxyurea for 5 days. Treatment with α-amanitin had no detectable effect. Table 8 shows that NPT II activity was 9.5 absorbance units/mm in calli treated with 10 μM 5-azacytidine and it was 9.1 absorbance units/mm in calli treated with 10 μM 5-azacytidine in combination with 2.5 μg/ml α-amanitin, and a Student's T-Test indicated that the difference was not significant. Because RNA concentration was not measured, there is no evidence to show that α-amanitin indeed entered cells and we cannot determine if the lack of effect was due to lack of entry or to failure of α-amanitin to block the 5-azacytidine effect.

Table 8. Effect of α-amanitin on 5-azacytidine-increased NPT II activity in KH221 callus[1].

	Untreated	α-amanitin (2.5 μg/ml)	5-azacytidine (10 μM)	α-amanitin[3] +5-azacytidine
Mean	4.1	5.1	9.5[4]	9.1[4]
STD[2]	3.97	1.83	4.90	3.96
N[2]	6	6	5	5

1. Unit is absorbance units/mm from densitometer scans. Forty five μg protein was loaded for each sample.
2. STD: standard deviation; N: sample size.
3. 2.5 μg/ml α-amanitin and 10 μM 5-azacytidine.
4. Untreated and α-amanitin treated are not different, p=0.41.
   5-azacytidine-treated and 5-azacytidine plus azacytidine-treated are not different, p=0.17.

10. EFFECT OF CYCLOHEXIMIDE ON NPT II ACTIVITY IN CALLI TREATED WITH 5-AZACYTIDINE

Cycloheximide is an inhibitor of eukaryotic protein synthesis (Alberts et al., 1983). It has been widely used to determine if protein synthesis is required for gene expression

(Huang et al., 1989; Lam, et al., 1989; Morohashi, 1989). Oppenoorth (1979) reported that 0.28 μg/ml cycloheximide was able to inhibit adventitious root formation. We treated calli with 10 μM cycloheximide combined with 10 μM 5-azacytidine. After 5 days, total protein in calli treated with cycloheximide was reduced. Results indicated that cycloheximide was not able to reduce the increased level of NPT II induced by of 5-azacytidine (Table 9). NPT II activity was 7.9 absorbance units/mm in calli treated with 10 μM 5-azacytidine alone. This was not significantly different from 7.8 absorbance units/mm for NPT II activity in calli treated with 10 μM 5-azacytidine in combination with 10 μM cycloheximide.

11. EFFECT OF CYCLOHEXIMIDE ON TOTAL CELLULAR PROTEIN AND NPT II ACTIVITY

Because 10 μM cycloheximide was not able to inhibit the stimulating effect of 5-azacytidine on NPT II activity, and preliminary experiments seemed to indicate that cycloheximide was able to cause an increase of NPT II activity. Calli were treated with 5 μM, 10 μM, and 20 μM cycloheximide in M3 medium for 5 days. NPT II activity and protein levels were assayed in crude extracts. It was found that cycloheximide reduced total cellular protein. The reduction was dose-dependent. Total cellular protein was 1.981 mg/g fresh callus in untreated calli, 0.847 mg/g fresh

Table 9. Effect of cycloheximide on 5-azacytidine-increased NPT II activity in KH221 calli[1].

	Untreated	5-azacytidine (10 μM)	5-azacytidine (10 μM) +cycloheximide (10 μM)
Mean	2.5	7.9	7.8
STD[2]	1.0	3.6	1.7
N[2]	6	6	6

1. Unit is absorbance units/mm from densitometer scans. Forty five μg protein was loaded for each sample.
2. STD: standard deviation; N: sample size.
3. 5-azacytidine-treated and 5-azacytidine plus cycloheximide-treated are not different, p=0.17.

callus in calli treated with 5 μM cycloheximide, 0.595 mg/g fresh callus in calli treated with 10 μM cycloheximide, and 0.447 mg/g fresh callus in calli treated with 20 μM cycloheximide. The protein level in calli treated with 20 μM cycloheximide is approximately 1/5 of that in untreated tissue (Table 10). Protein levels in untreated calli and in cylcoheximide-treated calli were significantly different ($p<0.01$).

Table 10. Effect of cycloheximide on total cellular protein[1].

	Untreated	cycloheximide		
		5 μM	10 μM	20 μM
Mean	1.981	0.847**	0.595**	0.447**
STD[2]	0.237	0.141	0.086	0.230
N[2]	4	4	4	4

1. Unit is mg protein/g fresh tissue.
2. STD: standard deviation; N: sample size.

** Untreated and 5 μM cycloheximide-treated are significantly different at $P<0.01$; Untreated and 10 μM of cycloheximide-treated are significantly different at $P<0.01$; Untreated and 20 μM of cycloheximide-treated are significantly different at $P<0.001$.

Interestingly, cycloheximide alone caused an increase in NPT II activity, an effect that was also dose-dependent. NPT II activity was 2.15 absorbance units/mm in calli treated with 5 μM cycloheximide, 3.02 absorbance units/mm in calli treated with 10 μM cycloheximide and 3.61 absorbance units/mm in calli treated with 20 μM cycloheximide (Table 11). Student's T-Tests indicated that difference in NPT II activity between untreated calli and the calli treated with

Table 11. Effect of cycloheximide on NPT II activity in KH221 callus[1].

	Untreated	Cycloheximide		
		5 μM	10 μM	20 μM
Mean	0.85	2.15**	3.02**	3.61**
STD[2]	0.789	0.437	0.295	0.758
N[2]	4	4	4	4

1. Unit is absorbance units/mm from densitometer scans. Forty five μg protein was loaded for each sample.
2. STD: standard deviation; N: sample size.

** There is significant difference between untreated and 5 μM cycloheximide-treated, $p<0.01$; There is significant difference between untreated and 10 μM cycloheximide-treated, $p<0.001$; There is significant difference between untreated and 20 μM cycloheximide-treated, $p<0.001$.

cycloheximide was highly significant ($p<0.01$).

12. EFFECT OF 5-AZACYTIDINE ON THE AMOUNT OF NPT II PROTEIN

The data above indicated that the 5-azacytidine-increased NPT II activity was not due to an increase in levels of *npt II* RNA. Further, the increase could not be blocked by the protein synthesis inhibitor cycloheximide, indicating that current protein synthesis was not involved in the observed increase of NPT II activity. To determine further the influence of 5-azacytidine on NPT II activity, ELISA tests were conducted. Crude extracts were made from control samples and calli treated with 10 μM of 5-azacytidine for 5 days, and the amount of NPT II enzyme was detected with ELISA. The level of NPT II protein in untreated calli was 0.82 μg/mg protein, and 0.96 μg/mg protein in calli treated with 10 μM 5-azacytidine for 5 days (Table 12). The difference was not statistically significant.

13. EFFECTS OF 5-AZACYTIDINE OR HYDROXYUREA ON MALATE DEHYDROGENASE ACTIVITY

Malate dehydrogenase is a "housekeeping enzyme" which converts oxaloacetic acid to malic acid with NADPH as a cofactor in plant chloroplasts and with NADH as a cofactor in

Table 12. ELISA determination of NPT II protein in KH221 callus treated with 5-azacytidine[1].

	untreated	5-azacytidine (10μM)
Mean	0.82	0.96
STD[2]	0.759	0.693
N[2]	5	5

1. Unit is μg NPT II/mg protein.
2. STD: standard deviation; N: sample size.
3. Untreated and treated are not significantly different at p=0.519.

plant mitochondria (Goodwin & Mercer, 1983). To determine if 5-azacytidine and hydroxyurea affect callus metabolism, we selected malate dehydrogenase to monitor the effect of 5-azacytidine and hydroxyurea. Calli treated with 10 μM 5-azacytidine or 30 mM hydroxyurea for 5 days were ground and extracts were assayed for malate dehydrogenase activity. A Student's T-Test indicated that there was no significant difference in the activity of malate dehydrogenase between untreated calli and calli treated with 10 μM 5-azacytidine, or 30 mM hydroxyurea. The activity of malate dehydrogenase in untreated calli was 0.053 units/mg protein, whereas it was

0.058 units/mg protein in calli treated with 10 μM 5-azacytidine, and 0.061 units/mg protein treated with 30 mM hydroxyurea (Table 13).

D. ANALYSIS OF EXOGENOUS NPT II ACTIVITY

To determine if treatment with 5-azacytidine affects NPT II levels posttranslationally, transgenic KH221 calli were treated with 0 or 10 μM 5-azacytidine, or with 30 mM hydroxyurea in M3 medium for 5 or 8 days. Crude extracts from these calli were boiled to destroy endogenous NTP II activity. Seventy-five pg bacterial NPT II was added to the boiled solution and the mixture was incubated on ice for 90 minutes before assaying for NPT II activity. Results indicated that a factor present in boiled extracts affected exogenous NPT II activity (Table 14). In extracts from 5-day-treated calli, NPT II activity was 3.28 absorbance units/mm in calli treated with 10 μM 5-azacytidine, and 2.95 absorbance units/mm in calli treated with 30 mM hydroxyurea, compared to 2.83 absorbance units/mm in untreated calli. Statistically, there was no difference in NPT II activity between untreated calli and calli treated with 10 μM 5-azacytidine or with 30 mM hydroxyurea. However, after an 8 day treatment, NPT II activity (6.77 absorbance units/mm) in calli treated with 10 μM 5-azacytidine was significantly different from the NPT II activity (4.97 absorbance units/mm) in untreated calli

Table 13. Effect 5-Azacytidine or hydroxyurea on malate dehydrogenase activity in KH221 callus[1].

	Untreated	Azacytidine (10 μM)	Hydroxyurea (30 mM)
Mean	0.053	0.058	0.061
STD[2]	0.021	0.024	0.036
N[2]	21	20	12

1. Unit is units/mg protein.
2. STD: standard deviation; N: sample size.
3. Untreated and calli are not significantly different at p=0.452.

Table 14. Effect of 5-Day and 8-Day 5-azacytidine and hydroxyurea treatments of KH221 callus on exogenous NPT II activity[1].

	Untreated	Azacytidine (10 μM)	Untreated	Hydroxyurea (30 mM)
5-Day Treatment				
Mean	2.83	3.28	2.83	2.95
STD[2]	0.94	0.66	0.94	0.66
N[2]	5	5	5	5
8-Day Treatment				
Mean	4.97	6.77*	2.28	2.79**
STD[2]	2.71	3.28	0.69	0.71
N[2]	7	7	5	5

1. Unit is absorbance units/mm from densitometer scans.

2. STD: standard deviation; N: sample size.

* Significantly different from untreated at $P<0.05$.

** Significantly different from untreated at $p<0.01$.

($p<0.05$). There was also a significant difference between 2.79 absorbance units, the NPT II activity in calli treated with 30 mM hydroxyurea, and 2.27 absorbance units, the NPT II activity in untreated calli ($p<0.01$).

The experiment was repeated with untransformed calli to determine if the azacytidine and hydroxyurea effect was a function of transgenic callus. After Xo calli were treated with 5-azacytidine or hydroxyurea for 5 or 8 days, crude extracts were made, and 75 pg bacterial NPT II was added to the solution. The mixture was incubated on ice for 90 minutes before assaying for NPT II activity. Results showed that after a 5 day treatment, there was no significant difference in NPT II activity between untreated calli, and calli treated with 30 μM 5-azacytidine, or calli treated with 30 mM hydroxyurea (Table 15). However, after an 8 day treatment, a significant difference was observed. NPT II activity in untreated calli was 2.20 absorbance units/mm, which was significantly different from 2.95 absorbance units/mm, the NPT II activity in calli treated with 10 μM 5-azacytidine ($p<0.05$), from 5.39 absorbance units/mm, the NPT II activity in calli treated with 30 μM 5-azacytidine ($P<0.01$), and from 3.95 absorbance units/mm, the NPT II activity in calli treated with 30 mM hydroxyurea ($p<0.05$).

In the course of our investigation, we found that several authors reported that an unknown inhibitory factor exists in crude extracts from eukaryotic cells including plant

Table 15. Effect of 5-Day and 8-Day 5-azacytidine and hydroxyurea treatments of Xo callus on exogenous NPT II activity[1].

	Untreated	Azacytidine (10 μM)	Azacytidine (30 μM)	Hydroxyurea (30 mM)
5-Day Treatment				
Mean	3.89		4.70	3.96
STD[2]	0.38		0.70	0.10
N[2]	3	0	3	3
8-Day Treatment				
Mean	2.20	2.95*	5.39**	3.95**
STD[2]	0.87	0.42	0.35	1.11
N[2]	6	6	4	5

1. Unit is absorbance units/mm from densitometer scans.
2. STD: standard deviation; N: sample size.

* Significantly different from control (untreated callus) at $P<0.05$.

** Significantly different from control (untreated callus) at $P<0.01$.

cells (Colbère-Garapin et al., 1981; Platt and Yang, 1987; Staebell et al., 1990). Our results also showed that, in agreement with Staebell's report (1990) indicating an inhibitory factor existed in crude extracts from Nicotiana tabacum cv. Xanthi, an inhibitory factor also existed in our system (Plate 1, Fig. 5). Our results showed that the activity of 75 pg of bacterial NPT II added into crude extracts from Xo treated with 10, 20 and 30 μM 5-azacytidine or 30 mM hydroxyurea was similar to that of 75 pg NPT II in positive controls (bacteria NPT II added to the same amount of NPT II extraction buffer instead of crude extracts), whereas the activity of 75 pg bacteria NPT II added into crude extract of untreated Xo was only similar to that of 50 pg NPT II in positive controls (Plate 1, Fig. 5). These results indicated that there was a stronger inhibitory factor (or factors) in the crude extracts from untreated Xo than in the crude extracts from 5-azacytidine- or hydroxyurea-treated Xo.

CHAPTER IV

DISCUSSION

KH221 is a transgenic line derived from protoplasts transformed with pLGVneo23 (Zhu et al., 1990). Southern hybridization indicated that npt II was inserted into the genome of KH221. After Bam HI and Hind III digestion, only one band (2.8 k) hybridized with the npt II probe. However, Eco RI digestion of KH221 DNA produced two bands which hybridized to the npt II probe. Since there is no internal Eco RI site in the npt II sequeneces (Beck et al., 1982; Depicker et al., 1982; Herrera-Estrella et al., 1983), this result suggests that two copies of npt II were inserted into the KH221 genome. The segregation of kanamycin resistance in RKH221 backcrossed to Xo (1:1), on the other hand, is consistent with a single gene insertion model (K. Hughes, pers. comm.). This might indicate that there are two copies of npt II inserted into the KH221 genome. but , only one insertion is active. The second insertion might be inactive due to the influence of flanking sequences or some structural deficiencies.

These studies have demonstrated that 5-azacytidine treatment increases NPT II activity in a transgenic tobacco line, KH221. This is consistent with studies by Zhu et al. (1991) showing that 5-azacytidine increased NPT II activity in

the transgenic tobacco lines XL102, XL103 and XL201.

5-azacytidine is a well-known DNA demethylating agent and also an effective chemical for inducing the expression of many suppressed genes (Jones, 1984). Numerous studies have suggested a connection between DNA demethylation and gene activation caused by 5-azacytidine. There is no evidence, however, proving a direct cause-effect relationship between DNA demethylation and gene activation induced by 5-azacytidine. Our studies were undertaken to determine if DNA demethylation was the cause of 5-azacytidine-increased NPT II activity in transgenic KH221 calli. Several lines of evidence indicate that there might be other regulatory effects of 5-azacytidine in our system. These are:

1) Hydroxyurea and cytidine did not block 5-azacytidine-increased NPT II activity: In our studies, 5-azacytidine increased NPT II activity in KH221 calli after a 5 day treatment. If 5-azacytidine functions as a demethylating agent through incorporation into newly replicated DNA strands (Jones, 1984), the inhibition of DNA replication should prevent the incorporation of 5-azacytidine into DNA, and therefore, 5-azacytidine would not be effective in activating genes as reported. Hepburn et al. (1983) reported that 10 mM hydroxyurea, an inhibitor of cell division and DNA replication, could block the stimulating effect of 30 μM 5-azacytidine on nopaline synthase in a line of flax. In contrast to Hepburn's studies, while 30 mM of hydroxyurea

clearly inhibited cell division of KH221 calli and reduced the total cellular protein of the calli, it did not block the effect of 10 μM of 5-azacytidine on inducing NPT II activity in the calli. In fact, hydroxyurea treatment actually increased NPT II activity, even at very low treatment levels.

If it is necessary for 5-azacytidine to be incorporated into DNA to activate a silent gene, cytidine, an analogue of 5-azacytidine, should reduce incorporation of 5-azacytidine into nascent DNA. Therefore, cytidine might reduce the induction of NPT II by 5-azacytidine. Hepburn et al. (1983) reported that cytidine could significantly reduce the stimulation of 5-azacytidine on nopaline synthase when the concentration of cytidine was four-fold higher than that of 5-azacytidine. In contrast, our results showed that even at 2.5 mM (250 fold), cytidine was not able to reduce the effect of 10 μM 5-azacytidine on NPT II activity.

The experiments involving hydroxyurea and cytidine suggest that the increase of NPT II activity caused by 5-azacytidine is independent of DNA replication, which is generally considered a prerequisite for 5-azacytidine-mediated DNA demethylation. Wilks et al. (1984), however, reported that hydroxyurea did not inhibit DNA demethylation of the chicken vitellogenin II gene, and concluded that either demethylation of this gene was independent of DNA replication or an alternative demethylation mechanism was involved in which an enzymatic process removed the methyl groups from

methylated cytosine or removed the methylated cytosine.

2) Npt II mRNA levels: Whatever the demethylation mechanism, if DNA demethylation is the cause of the increase of enzyme activity, it should increase the transcription of genes. Hepburn et al. (1983) suggested that 5-azacytidine increased nopaline synthase transcription based on the increase of mRNA level induced by 5-azacytidine; Klass et al. (1989) demonstrated that 5-azacytidine increased steady-state mRNA levels of a T-DNA gene. Our studies, however, indicated that 5-azacytidine did not increase the level of steady-state npt II RNA in 5-azacytidine-treated samples, whereas at the same time, NPT II activity in the treated samples increased. This suggests that 5-azacytidine might not impose its effect on NPT II at the transcriptional level. It may be argued that the level of steady-state RNA does not reflect the transcription rate of genes because some transcripts are quickly degraded after transcription. However, the levels of steady-state RNA reflect the transcription rate of a majority of transcripts in plant cells (Okamuro and Goldberg, 1989).

3) α-amanitin had no effect on 5-azacytidine-increased NPT II activity: α-amanitin, an inhibitor of RNA synthesis in eukaryotic cells, provides a convenient method to determine if a gene is controlled at transcription level. Williamson and Quatrano (1988) reported 5 μg/ml of α-amanitin did not block the effect of ABA on the early methionine-labeled polypeptide (Em)-protein clone, p1015, whereas it blocked the effect of

ABA on the 7S globulin clone p511 of wheat, and concluded that p511 was controlled at the transcriptional level whereas p1015 was not. In our studies, 2.5 μg/ml of α-amanitin was used to probe the regulatory level of npt II by 5-azacytidine. The results showed that 2.5 μg/ml of amanitin did not block the effect of 5-azacytidine on NPT II, which might indicate that the effect of 5-azacytidine is not at the transcriptional level. However, it should be pointed out that the steady-state level of RNA in the α-amanitin-treated samples was not measured and overall cellular protein levels were unaffected. Therefore, we do not have evidence to show that α-amanitin indeed entered the cells.

4) Cycloheximide had no effect on 5-azacytdine-increased NPT II activity: Cycloheximide, an inhibitor of cytoplasmic protein synthesis, has been widely used to determine if current protein synthesis is involved in a process, and if the regulation of an enzyme is at the translational level. Williamson and Quatrano (1988) reported that cycloheximide had no effect on the stabilization of p1015 mediated by ABA, and suggested that protein synthesis was not involved and Em expression was not controlled at translational level. Morohashi (1989) showed that cycloheximide did not inhibit the increase of oxidation rate of malate, which was regulated by phytochrome, and concluded that the increase of oxidation of malate was independent of cytoplasmic protein synthesis. Similar methodology was used by Lu et al. (1990)

who reported that cycloheximide was not able to inhibit nitrate reductase activity induced by BA. Furthermore, Foster et al. (1991) showed that cycloheximide did not inhibit the effect of 5-azacytidine on increasing total cellular copper content, and concluded that DNA demethylation was not involved.

In our studies, KH221 calli were treated with 10 μM cycloheximide in combination with 10 μM of 5-azacytidine. The inducing effect of 5-azacytidine was not blocked by cycloheximide, suggesting that 5-azacytidine-increased NPT II activity was not regulated translationally. Furthermore, it was found that although cycloheximide ranging from 5 to 20 μM drastically reduced the total cellular protein, it significantly increased NPT II activity by itself. This could occur if cycloheximide reduced total protein vs. NPT II protein disproportionally. If there were more NPT II molecules in the 45 μg total protein loaded on gel, compared to that of the untreated control, an increase in NPT II activity would be observed. Nevertheless, this result indicates that current protein synthesis is not required for the 5-azacytidine-increased increase of NPT II activity, and suggests that the effect of 5-azacytidine on NPT II activity might be posttranslational.

5) Levels of NPT II protein were not affected by 5-azacytidine whereas NPT II activity was affected: Further support for a posttranslational mechanism is provided by ELISA

analysis which showed that although 5-azacytidine increased NPT II activity as measured by the gel assay (Table 1), it did not increase the level of NPT II protein as measured by ELISA (Table 12). To verify the reliability of ELISA, leaf slices from RKH221 were treated with the auxin, 2,4-D and the cytokinin BAP. An et al. (1990) showed that auxin, especially 2,4-D, regulated the nopaline synthase promoter (fused with the reporter gene cat), so that more cat was transcribed and translated. BAP had no effect on the promoter and therefore, no BAP-induced increase of CAT activity was found. The construct used in our studies has same promoter, pnos, but fused to the npt II coding sequence. If 2,4-D increases the activity of pnos, npt II transcription will be increased, and in turn NPT II translation will be increased which can be detected by ELISA. Our results showed that, in agreement with the report of An et al. (1990), 2,4-D increased NPT II activity, as assayed by the gel assay method (Plate 1, Fig. 3), in leaf slices within 24 hours whereas BAP did not. The ELISA test also showed that 2,4-D increased the amount of NPT II protein whereas BAP did not (Table 2). Therefore, 2,4-D increased NPT II activity (as measured by gel assay) and increased the amount of NPT II protein in leaf slices (as measured by ELISA). This is consistent with the hypothesis that 2,4-D increased transcription of npt II resulting in the observed concurrent increase in NPT II protein and activity. Further support for transcriptional regulation by 2,4-D is

given by An et al. (1990). They reported that wounding and 2,4-D treatment acted on a 10-base pair Z element in pnos promoter.

In calli treated with 10 μM 5-azacytidine for 5 days, however, the gel assay showed that 5-azacytidine significantly increased NPT II activity in calli (Table 1), but ELISA did not detect any significant increase of NPT II protein in calli (Table 12). Comparing the ELISA data for NPT II protein in calli treated with 5-azacytidine (Table 12) and in leaf slices treated with hormones (Table 2), it is quite clear that 5-azacytidine increases NPT II activity though a mechanism different from that of auxin which increases the transcription and translation of NPT II. The non-parallel relationship between NPT II activity and the amount of NPT II protein indicates that increase in NPT II activity caused by 5-azacytidine was not due to synthesis of NPT II protein. Therefore, the effect of 5-azacytidine on NPT II in calli might be posttranslational.

6) Crude extracts affected bacterial NPT II activity: In vitro tests in which standard NPT II enzyme was added to extracts from 5-azacytidine-treated calli indicated that the 5-azacytidine-increased NPT II activity is posttranslational and due to the presence and the level of a second factor. This unknown factor inhibited the activity of bacterial NPT II added into crude extracts from untreated Xo, indicating 5-azacytidine and hydroxyurea affected this unknown factor. One

of the possible reasons that cycloheximide increased NPT II activity could be that, it inhibits the synthesis of the unknown factor. In our experiments, the crude extracts from KH221 were boiled to destroy endogenous NPT II activity whereas the crude extracts from Xo were not boiled. Therefore, the effect of crude extracts from these two lines may not be comparable.

Unknown inhibitors of NPT II have been reported by several authors. Colbère-Garapin et al. (1981) purified cell extracts from mammalian cell lines through a Sephadex G200 column to remove strong inhibition of NPT II. Staebell et al. (1990) reported a low-molecular-weight inhibitor of NPT II in extracts of a transgenic cell line of Nicotiana tabacum cv. Xanthi. Platt and Yang (1987) reported certain inhibitors of NPT II present in the extracts of a transgenic cell line of Nicotiana tabacum cv. Havana 425, which caused a 10-fold reduction of the activity of NPT II in assays. At this point, we do not know if the inhibitor which was affected by 5-azacytidine and hydroxyurea in our system is identical to the inhibitors reported by the above authors.

Differences might exist between in vitro and in vivo studies. Crude extracts of calli treated with 5-azacytidine or hydroxyurea for 5 days did not exhibit lowered activity of bacterial NPT II when it was added into the extracts. The activity of bacterial NPT II was inhibited by crude extracts treated with 5-azacytidine or hydroxyurea for 8 days.

However, a 5-day treatment of 5-azacytidine or hydroxyurea clearly increased the endogenous NPT II activity in the treated calli. This might suggest that the in vitro study does not reflect the in vivo mechanism exactly. However, one possibility might be that endogenous NPT II was subjected to the effect of an unknown inhibitory factor for 5 days when calli were in culture, whereas the exogenous NPT II was only subjected to the effect of the unknown factor for 90 minutes on ice. Our results also indicated there was variation of the effect of the unknown inhibitory factor in the crude extracts on bacterial NPT II. This variation might be caused by physiological variation of different callus cultures. In addition, somaclonal variation might occur during a long term subculture of a callus (Dixon, 1985), which might contribute to the variation observed in our experiments.

Our data suggested that DNA demethylation caused by 5-azacytidine is not the only mechanism for 5-azacytidine-increased NPT II activity. Rather, 5-azacytidine might affect NPT II activity by a posttranslational mechanism. Recently, Foster et al (1991) reported that 5-azacytidine increased the activity of metallothionein genes by increasing the cellular content of copper. Hydroxyurea and cycloheximide treatments enabled them to conclude that the increase of copper levels by 5-azacytidine is through a mechanism other than DNA demethylation.

An unexpected finding in these studies was that

hydroxyurea itself caused as much of an increase in NPT II activity as 5-azacytidine. Although we have not found any report indicating that hydroxyurea induces gene expression in plants, there are many studies showing that hydroxyurea induces gene expression in animals. Mariani and Schimke (1984) reported that hydroxyurea could cause gene amplification after the it was removed and hence caused Chinese Hamster ovary cells to show a 100-fold increase in methotrexate resistance. Platt et al. (1984) reported that hydroxyurea enhanced fetal hemoglobin synthesis in sickle cell anemia. A similar finding was made by Letvin et al. (1984) in anemic monkeys. Interestingly, DeSimone et al. (1982) showed that 5-azacytidine also induced fetal hemoglobin synthesis. Datta et al. (1991) found that both 5-azacytidine and hydroxyurea were able to induce changes in hemoglobin proportions, and their effect could be blocked by aspirin. Initially, it was suggested that DNA hypomethylation caused by 5-azacytidine played a role in inducing the hemoglobin gene expression (DeSimone et al., 1982). Later, it was suggested that hypomethylation may not be necessary because hypomethylation did not always increase gene expression (Dover and Charache, 1989). Actually, hydroxyurea has been reported to cause hypermethylation (Nyce et al., 1986). Hypermethylation was also found in amplified DNA of Neurospora crassa (Bull and Wootton, 1984) and in amplified ribosomal RNA genes in a rat cell line (Sugino and Nakayama, 1980). It was

implied that hypermethylation caused by hydroxyurea and gene amplification might be linked (Nyce et al., 1986).

If hydroxyurea increases NPT II activity by increasing the transcription of npt II, there might be a difference in the level of steady-state RNA between untreated and hydroxyurea-treated samples. However, we were not able to detect any difference between them.

Because hydroxyurea caused a decrease in total cellular protein in treated calli, one possible explanation for the observed increase in NPT II activity is that there were more NPT II molecules in the 45 μg total protein from hydroxyurea-treated samples than that in the 45 μg total protein from the untreated samples. In addition to that, in vitro tests involving standard NPT II enzyme and the extracts from hydroxyurea-treated calli indicated that there is an unknown inhibitory factor in the extracts. Hydroxyurea was able to reduce the effect of this factor on NPT II activity.

Our studies also showed that hydroxyurea caused DNA fragmentation and reduced the length of RNA. DNA fragmentation caused by hydroxyurea is probably due to its suppressive effect on DNA polymerization of newly synthesized DNA, which leads to the accumulation of fragments with low molecular weights (Nyce et al., 1986). DNA fragmentation in turn might cause incomplete transcription of some RNA species, resulting in the disappearance of RNA species with higher molecular weights. It seems that hydroxyurea affected rRNA.

However, we do not know if the disappearance of assumed rRNA bands is because of the overall effect of hydroxyurea on DNA fragmentation or because of some specific effect of hydroxyurea on rRNA genes or rRNA specifically.

To determine if the effects of 5-azacytidine or hydroxyurea on NPT II activity have an overall metabolic effect on the cells, we measured the activity of a "housekeeping" enzyme, malate dehydrogenase. Our results are similar to that of Hepburn et al (1983) in that neither 5-azacytidine nor hydroxyurea significantly affect the activity of malate dehydrogenase specifically.

In conclusion, our study showed that treatment with 10 μM 5-azacytidine was sufficient to increase NPT II activity in a transgenic tobacco line KH221 in 5-day cultures which indicated that npt II might be subjected to the regulation of DNA methylation. However, this study has shown that 5-azacytidine did not increase the steady-state level of mRNA of npt II, and it did not increase the synthesis of NPT II protein which was determined by ELISA analysis while it increased the activity of NPT II. In addition, a DNA replication inhibitor, hydroxyurea, a RNA transcription inhibitor, α-amanitin, and a protein synthesis inhibitor, cycloheximide, had no effect on the stimulating effect of 5-azacytidine. In fact, hydroxyurea and cylcoheximide themselves increased NPT II activity. Experiments involving exogenous bacterial NPT II and crude extracts from calli

treated with or without 5-azacytidine indicated that 5-azacytidine affected an unknown inhibitory factor which inhibited the activity of NPT II. Our study therefore, indicates that the effect of 5-azacytidine on NPT II activity might be at least partially due to a posttranslational mechanism.

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APPENDICES

Appendix I

M3 Culture Medium

STOCK SOLUTION OR CHEMICALS	NEEDED FOR 1 LITTER MEDIUM
B5-2 Stock	10 ml
H_3BO_3 (0.3 g/L) KI (0.075 g/L) $NaMoO_4.2H_2O$ (0.025 g/L) $CoCl_2.2H_2O$ (0.0025 g/L)	
B5-4 Stock	10 ml
$MgSO_4.7H_2O$ (50 g/L) $MnSO_4.H_2O$ (1.0 g/L) $ZnSO_4.7H_2O$ (0.2 g/L) $CuSO_4.5H_2O$ (0.0025 g/L) $(NH_4)_2SO_4$ (13.4 g/L)	
B5-6 Stock	10 ml
$NaH_2PO_4.H_2O$ (15 g/L)	
Iron Solution	10 ml
$Na_2EDTA.2H_2O$ (3.625 g/L) $FeSO_4.7H_2O$ (2.785 g/L)	
K8P Vitamin Stock (100 ml stock)	10 ml
Niacinamide (10.00 mg/100 ml) Pyridoxine HCl (10.00 mg/100 ml) Thiamine HCl (10.00 mg/100 ml) DL-Calcium Pantothenate (20.00 mg/100 ml) Choline Chloride (10.00 mg/100 ml) Ascorbic Acid (20.00 mg/100 ml) Riboflavin (2.0 mg/100 ml) Biotin (in 0.01 mg/ml stock) (10 ml/100 ml) P. aminobenzoic acid (in 1 mg/ml stock) (0.2 ml/100 ml) Vitamin A (in 1 mg/ml stock) (0.1 ml/100 ml) Vitamin D_3 (in 1 mg/ml stock) (0.1 ml/100 ml) Vitamin B_{12} (in 1 mg/ml stock) (20.0 ml/100 ml)	
K8P Sugar Stock (100 ml stock)	10 ml
Fructose (2.5 g/100 ml) Ribose (2.5 g/100 ml) Xylose (2.5 g/100 ml)	

Mannose (2.5 g/100 ml)
Rhamnose (2.5 g/100 ml)
Cellobiose (2.5 g/100 ml)
Sorbitol (2.5 g/100 ml)
Mannitol (2.5 g/100 ml)

K8P Organic Acids Stock (100 ml stock)	10 ml

Sodium pyruvate (0.20 g/100 ml)
Citric acid (0.40 g/100 ml)
Malic acid (0.40 g/100 ml)
Fumaric acid (0.40 g/100 ml)

KNO_3	2.5 g
$CaCl_2.6H_2O$	1.0 g
NH_4NO_3	0.25 g
N-Z amine	0.25 g
myo-inosital	0.050 g
folic acid	0.4 mg
sucrose	20 g

1) pH is adjusted with NaOH or HCl to pH5.7
2) K8P vitamin, organic acids and sugar stocks were stored at -20° C.
3) To make K8P organic acids stock, add chemicals to 50 ml distilled water, and adjust to pH5.5 with NH_4OH. Then bring volume to 100 ml.
4) To make K8P vitamin stock, vitamin A, vitamin D_3 and vitamin B_{12} were dissolved in 95 % ethanol.

Appendix II

NPT II Extraction Buffer

Chemicals	Stocks	needed for 10 ml	Final Concentration
EDTA (pH7.5)	0.5 M	0.8 ml	40 mM
NaCl	1 M	1.5 ml	150 mM
NH_4Cl	1 M	1.0 ml	100 mM
DL-Dithiothreitol	1 M	0.15 ml	15 mM
Tris-HCl (pH7.5)	1 M	0.12 ml	12 mM
Leupeptin		1.3 mg	
Soybean trypsin inhibitor	5 mg/ml	0.6 ml	0.3 mg/ml
Sucrose		342 mg	100 mM

Reaction Buffer

Chemicals	Final Concentration
Tris-maleate (pH7.1)	67 mM
MgCl	42 mM
NH_4Cl	400 mM

Guanidinium Thiocyanate Extraction Buffer for DNA and RNA

Chemicals	Final Concentration
Guanidinium thiocyanate	4 M
Sodium N-Lauroylsarcosine	0.5%
Sodium citrate (pH7.0)	25 mM
Sigma 30% antifoam	0.1%

Adjust to pH7.0 with 1 N NaOH. Filter it and add 0.7 ml of 2-mercaptoethanol (0.1 M). Make up 100 ml with distilled water. Store it tightly closed at room temperature up to one month.

2 X CTAB Extraction Buffer for DNA

Chemicals	Final Concentration
Cetyltrimethylammonium bromide (CTAB)	2%
Tris (pH8.0)	100 mM
EDTA (pH8.0)	20 mM
NaCl	1.4 M
Polyvinylpyrrolidone (PVP) Mw 40,000	1%

Appendix III

LB Medium for Bacterial Culture

Agents	Final Concentration
Bacto-tryptone	10 g/L
Bacto-yeast extract	5 g/L
NaCl	10 g/L

Dissolve the agents with shaking. Adjust solution to PH7.0 with 5 N NaOH. Sterilize by autoclaving.

STE Buffer

Chemicals	Final Concentration
NaCl	0.1 M
Tris-HCl (pH8.0)	10 mM
EDTA (pH8.0)	1 mM

TE Buffer (pH8.0)

Chemicals	Final Concentration
Tris-HCl (pH8.0)	10 mM
EDTA (pH8.0)	1 mM

TBE (Tris-borate) Buffer (5 X)

Chemicals	Final Concentration
Tris base	54 g/L
Boric acid	27.5 g/L
EDTA (0.5 M, pH8.0)	20 ml/L

The working solution is 0.5 X.

SSC Buffer (20 X)

Chemicals	Final Concentration
NaCl	3.0 M
Na_3Citrate	0.3 M

Prehybridization and Hybridization buffer for DNA Hybridization

Solutions	Volume	Final Concentration
20 X SSC[1]	7.5 ml	6 X SSC
100 X Denhardt's solution[2]	1.25 ml	5 X Denhardt's solution
10% SDS	1.25 ml	0.5%

1. 20 X SSC: 3.0 M NaCl
0.3 M Na_3citrate

2. 100 X Denhardt's solution: 2% (w/v) BSA
2% (w/v) Ficoll
2% (w/v) PVP (polyvinyl pyrollidone)

After adding 20 X SSC and 100 X Denhardt's solution, warm the mixture to 40° C before adding 10% SDS. Make up to 25 ml with distilled water.

VITA

Wei Shao was born on 9 October, 1955 in Longnan, Jiangxi Province, P. R. China. He received his elementary school education in Longnan, and Nidu County and middle school education in Nidu, Guanchan County. His high school education was received in Ganzhou City, Jianxi Province. He attended Lanzhou University in China from 1978 to 1982. After receiving his B.S. in Biology, he worked in Jiling Agricultural University as a teaching assistant from 1982 to 1986. He came to the Botany Department of the University of Tennessee in the fall of 1986, and received his M.S. in Botany under the supervision of Dr. James D. Caponetti in the winter of 1988. He enrolled in the Ph.D program in the same department under the supervision of Dr. Karen W. Hughes in the spring of 1989.