THE UNIVERSITY OF CALGARY

Functional Architecture of Alkaloid Biosynthetic Gene Promoters from Opium Poppy

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

NOVEMBER, 1997

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Abstract

A multi-enzyme biosynthetic pathway produces the isoquinoline alkaloid sanguinarine in opium poppy. Tyrosine/dopa decarboxylase (TYDC) represents the entrypoint, and berberine bridge enzyme (BBE) operates at the branchpoint which commits a common precursor to sanguinarine biosynthesis. These genes are induced in response to wounding and/or treatment with a fungal elicitor. Promoters from genes encoding the two TYDC isoforms (*TYDC6* and *TYDC7*) and BBE (*BBE1*) were fused to the GUS reporter gene and progressively deleted from the 5′ end. The resulting constructs were introduced into cultured opium poppy cells via particle bombardment and the transient expression of GUS was assayed. Positive regulatory regions were functionally identified in all of the promoters, and regions which seemed to repress transcription were localized in the *TYDC7* promoter. Deleting the positive regulatory regions in the *TYDC6* and *BBE1* promoters resulted in a significant decrease in GUS expression, while the removal of the regulatory regions in the *TYDC7* promoter produced unexpected results.

Acknowledgements

There are so many people who contributed to this work in one way or another. I hope that I manage to thank them all in this brief section. First, I would like to acknowledge Dr. Peter Facchini for teaching me more than just the technical skills I required for my degree. The lessons I learned from him are invaluble. As well, I would like to thank both Peter and Mrs. Catherine Yost for their contibution to this work. Specifically, Peter assembled some of the figures included here (Figures 14, 19, 23, and 29) and helped to generate the internal deletion constructs while Catherine did the library screening to originally isolate the genomic clones for *TYDC6*, *TYDC7*, and *BBE1*. Throughout my first year in Calgary, Peter and Catherine were happy to help with ligations, sequencing, or almost any other task and I know that I could not have finished this degree as quickly as I did without their assistance.

I thank all of my committee members for their advice and for agreeing to participate in the examination of this thesis, and would like to specifically express my appreciation to Dr. Thorpe and Dr. Moloney for all the support I received from them in the past few months. Similarly, I would also like to thank Dr. Yeung and Dr. Reid for your concern and assistance. As well, I want to sincerely thank Dr. Gijs van Rooijen for reading and reviewing my thesis for me, and for offering to do so. Once again, your support really meant a lot to me.

My family has always been a huge part of everything I have ever done and this degree is no exception. I want to tell all of you, Grandma, Bill, Sandra and Blair, Jim and Loretta, and of course, Mom and Dad, how much it means to me to know that no matter what happens out here in the big bad world, I can always count on you guys.

Now for all of my wonderful friends. I would not have made it through this last year without the understanding and support of my very dear friend Nicole Ramesar-Fortner, so thank you doesn't seem sufficient, but that is all that I can really say. I hope you know how much I appreciate all the talks (and the Kleenex!). I would also like to thank Cindy, Steve, Catherine, and Lisa for their assistance with my work, for their understanding, and for a lot of fun times. And Larry, Scott, Ben, Simon, Kattir, Joe, Harm, Dave, Dana, Sarita, Kat, Soheil, Indra, Jill, Janice, Lleva and Claudio for being a part of my time here in Calgary and for generally just being nice people that I will always remember with a smile.

Last but not least, I would like to thank Rick. For everything.

For my parents, Bud and Betty Johnson.

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Abbreviations and Symbols

^{32}P: phosphorous 32

^{35}S: sulphur 35

A_{260}: absorbance at 260 nm wavelength

A_{280}: absorbance at 280 nm wavelength

A_{595}: absorbance at 595 nm wavelength

Amp^r: ampicillin resistant

ATP: adenosine triphosphate

BBE: berberine bridge enzyme

bp: base pairs

BSA: bovine serum albumin (fraction V)

cDNA: complementary deoxyribonucleic acid

cpm: counts per minute

Ci: Curie

Δ: indicates gene deletion

dCTP: deoxycytidine triphosphate

DNA: deoxyribonucleic acid

dNTP: deoxynucleoside triphosphates

EDTA: ethylenediaminetetraacetic acid

EtOH: ethanol

EtBr: ethidium bromide

gDNA: genomic deoxyribonucleic acid

GUS: β-glucuronidase

h: hour

IPTG: isopropylthiogalactoside

kb: kilo base pairs = 10^3 base pairs

LB-Amp_{100}: LB media with 100 μg/mL final concentration of ampicillin

LB-Kan_{100}: LB media with 100 μg/mL final concentration of kanamycin

LMP: low melting point

Luc: luciferase

MBq: megabecquerels = 10^6 Becquerels

MeJA: methyl jasmonate

min: minute

mRNA: messenger RNA

MU: methylumbelliferone

MUG: 4'methyl umbelliferyl glucuronide

ORF: open reading frame

%(v/v): percent volume by volume = mL/100 mL total volume

%(w/v): percent weight by volume = g/100 mL total volume

pmol: pico mole

RLU: relative luciferase unit

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

RT: room temperature

SD: standard deviation

SDS: sodium dodecylsulfate

SSC: salt with sodium citrate

ssDNA: single stranded DNA

t: time

Tris: tris(hydroxymethyl) methylamine

TYDC: tyrosine/dopa decarboxylase

U: units of enzyme activity

UTR: untranslated region

V: volts

x: times

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

X-gluc: 5-bromo-4-chloro-3-indolyl glucuronide

1. Introduction

1.1 Secondary metabolism

Plants produce a diverse array of organic compounds, some of which are ubiquitous, and some which are restricted to certain plant families or even to particular species. The widely distributed compounds are generally intermediates of primary metabolism, and as such are required by the plant to function efficiently on a day to day basis. Examples of primary metabolites include constituent compounds such as protein α-amino acids, sugars, nucleic acids, and fatty acids. Those compounds which do not seem to contribute to the economy of the plant are called secondary metabolites, due to their apparent secondary role (Haslam, 1986).

There are tens of thousands of secondary metabolites known, and based on structural diversity, they can easily be categorized into three main families. Terpenoids represent the largest and the most widely distributed group of natural products in plants. All terpenes are derived from the mevalonic acid pathway, are generally insoluble in water, and are the result of successive head to tail condensations of isoprene building blocks. Phenolic compounds are chemically diverse aromatic substances which are primarily formed in plants from the precursors phenylalanine and tyrosine via the shikimate pathway. The third category of plant secondary metabolites are all derived from amino acids, and are therefore known as nitrogen-containing compounds. Alkaloids and glycosides are the most well known members of this group.

For decades these secondary natural products were believed to serve no purpose in the plants producing them. In fact, Krebs referred to them as "ballast", and dismissed them as products of mutations which resulted in characteristics which were neither beneficial or harmful so were not subject to selection either for or against their expression (Haslam, 1986). Relatively

recent discoveries, however, have revealed new information about these compounds, and a picture is beginning to emerge which suggests that secondary metabolites do have important ecochemical functions in the defense of the plant.

1.2 Plant defense responses

In natural habitats, plants are surrounded by a great number of potential predators and pathogens. Roots are exposed to bacteria, nematodes, and fungi living in the soil, and aerial organs are subject to being fed upon by an infinite number of insects, and herbivorous animals. To defend themselves against being infected or eaten, plants have developed a wide range of responses which are activated when the plant is either wounded or challenged by a pathogen. Some of these responses rely on the deployment of a diverse arsenal of chemical weapons which can be used either passively, or aggressively. These chemicals are naturally occurring products which do not participate in the primary metabolic pathways of the plant producing them, so therefore belong to that group of compounds known as secondary metabolites.

In vegetative plant tissues, if cells are challenged with a pathogen, a number of defense mechanisms are triggered. Some of these responses result in the fortification of cell walls, whereby an actual physical barrier is formed to impede the infection. This can be achieved by depositing newly synthesized carbohydrates in the cell wall (Terras *et al.*, 1995), or by inducing enzymes which cross-link proteins already present there (Bradley *et al.*, 1992). Another approach to combat an invading pathogen involves the induction of specific intracellular responses. Pathogenesis-related (PR) proteins, and/or phytoalexins, (low molecular weight secondary compounds with antimicrobial properties), can be synthesized at the site of infection, and in some cases, even in distant, unaffected tissues (Ward *et al.*, 1991). Finally, there can be an incompatible reaction between a specific pathogen and plant,

known as a hypersensitive reaction, which results in the formation of small necrotic lesions at the site of invasion (Alonso *et al.*, 1995). Using any one of these mechanisms alone, or in combination with one another, plants are able to protect themselves from many of the threats in their environment. Many of these inducible defense responses can also be initiated by wounding, which is really not surprising since a breach in the integrity of the cell must exist prior to, or concurrently with, pathogen infection (Truernit *et al*, 1996).

1.3 Isoquinoline alkaloids

Alkaloids are nitrogenous compounds that belong to the broad category of plant secondary metabolites. They have traditionally only been of interest because many alkaloids have profound physiological effects on people and animals. In recent years however, it has become increasingly apparent that alkaloids may play an important role in the defense of the plant against invading pathogenic organisms and/or grazing herbivores.

There are seven classes of alkaloids, and all are characterized according to the amino acid from which they are derived. The three most well-known classes are the indole alkaloids, which are synthesized from tryptophan, the nicotine and tropane alkaloids, derived from ornithine, and the isoquinoline alkaloids, which have tyrosine as their amino acid precursor. In addition to being the largest class of alkaloids in terms of structural diversity, the isoquinolines are perhaps the most well understood class. In fact, it was the isolation of the benzylisoquinoline morphine, which started the entire field of alkaloid research (Bisset, 1985).

Isoquinoline alkaloids are produced in certain plants of the Papaveraceae, Berberidaceae, Ranunculaceae, Fumariaceae, and Menispermaceae families (). Many of these alkaloids are used as pharmaceuticals because of their pronounced biological activities, and many of them are still isolated from the plants which produce them because their complex chemical structure prevents commercial synthesis. The medicinal

isoquinolines include morphine (an analgesic), codeine (a cough suppressant and also an analgesic), berberine (an antimicrobial used to treat eye and intestinal infections), and tubocurarine (a muscle relaxant). Many of these compounds are found in the notorious flowering plant, the opium poppy (*Papaver somniferum* L.).

1.3.1 Opium poppy

Economically, the most important member of the Papaveraceae is the opium poppy. This plant produces over 40 isoquinoline alkaloids, many of which have pharmacological properties, and extracts of opium poppy have been used throughout history for medicinal purposes (Lindner, 1985). In 1500 BC, the Ebers Papyrus described it as a remedy to prevent the excessive crying of children (Brownstein, 1993). Ever since its isolation in 1806, morphine has been administered to alleviate pain. Other Papaver alkaloids, namely codeine, papaverine, noscapine, and thebaine, are also widely used as pharmaceuticals (Bisset, 1985). By the thirteenth century, opium (the dried latex of the plant in which the alkaloids accumulate) was available throughout Asia, India and all parts of Europe (Brownstein, 1993), although its use was not restricted to medicinal purposes. In the third millennium BC, the ancient Sumerians are believed to have cultivated the opium poppy for use as a narcotic (since they called it the "plant of joy"), and manuscripts prepared in the sixteenth century document abuse of opium (Brownstein, 1993). By the late 1800s, addiction to opium and/or morphine was recognized as a serious problem and an alternative was sought. The Bayer company synthesized the first novel opiate in 1898 and marketed the product, O,O-diacetylmorphine (heroin), as a cough suppressant, claiming that it was more potent than morphine and free from abuse liability (Brownstein, 1993). Thus, *Papaver somniferum* was not only one of the first medicinal plants, it was also responsible for starting the science of alkaloid biochemistry.

1.3.2 The biosynthetic pathway of isoquinolines in opium poppy

One of the most well understood pathways for a plant-derived secondary metabolite is that which results in the production of sanguinarine (Figure 1). All of the enzymes involved have been characterized (Kutchan and Zenk, 1993) and in two instances the genes encoding them have been cloned (Facchini and DeLuca, 1994; Dittrich and Kutchan, 1991; Facchini *et al.*, 1996b). The elucidation of this pathway was carried out in the laboratory of Meinhart Zenk with an inducible cell suspension culture of the Papaveraceae family member *Eschscholtzia californica*. Under normal conditions plant cell cultures do not produce significant amounts of secondary metabolic products, but if they are challenged by a pathogenic organism, or often even components of one, the levels of secondary products can be greatly elevated. Substances capable of inducing secondary metabolite production are known as elicitors. Addition of elicitors to cell cultures induces expression of the genes encoding secondary product biosynthetic enzymes. The ability to elevate gene expression levels and the relative abundance of the proteins they encode has lead to the identification, characterization, and purification of many alkaloid biosynthetic enzymes, and the availability of purified enzymes has allowed for the cloning of some of the genes.

The first two steps in the biosynthesis of all isoquinoline alkaloids occur concurrently, and involve the decarboxylation of L-tyrosine to L-dopamine by tyrosine/dopa decarboxylase (TYDC), as well as the conversion of L-tyrosine to 4-hydroxyphenylacetaldehyde (Rueffer and Zenk, 1987). Dopamine and 4-hydroxyphenylacetaldehyde are then condensed to form the central isoquinoline alkaloid precursor (S)-norcoclaurine by norcoclaurine synthase (NS). (S)-norcoclaurine is then converted to (S)-reticuline by an O-methyltransferase, an N-methyltransferase, a phenolase, and finally another O-methyltransferase. All of these enzymes have been at least partially

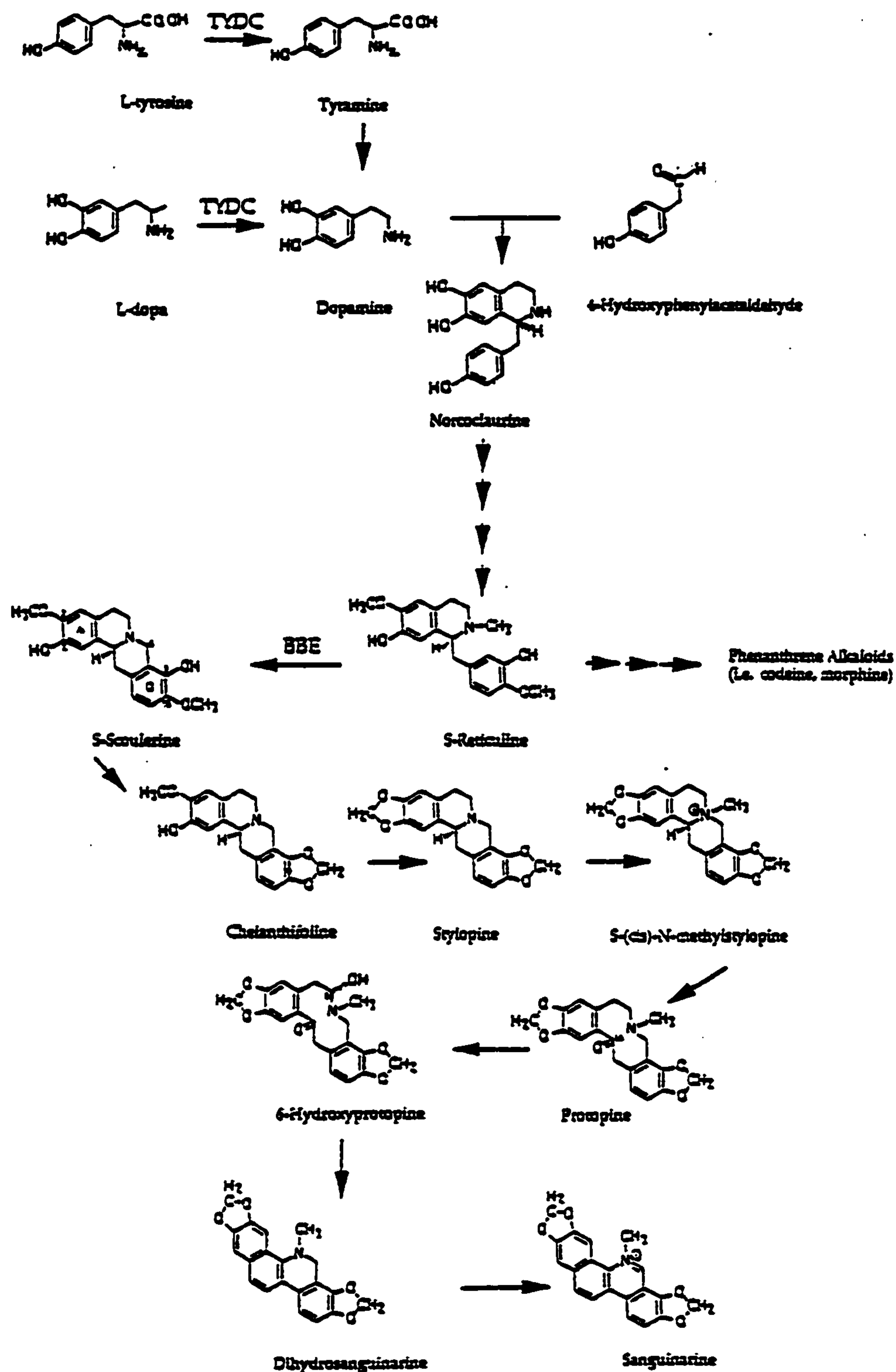


Figure 1: The isoquinoline alkaloid biosynthetic pathway in opium poppy.

purified and characterized (Kutchan and Zenk, 1993). (S)-reticuline is the branchpoint intermediate for almost all of the benzylisoquinolines in higher plants. In opium poppy, this metabolite serves as the precursor for both the major alkaloid endproducts, morphine and sanguinarine. (S)-reticuline is converted to (S)-scoulerine when an N-methyl bridge is formed by the berberine bridge enzyme. This is a unique conversion in nature and cannot be achieved using current organic chemistry techniques. (S)-cheilanthifoline is formed from (S)-scoulerine and then converted to (S)-stylopine by two consecutive cytochrome P_{450}-dependent oxidase reactions. Subsequently, (S)-stylopine is subjected to an N-methyltransferase to form *cis*-N-methylstylopine, which is in turn converted to protopine via another cytochrome P_{450}-dependent monooxygenase. Protopine must undergo three more conversions to form sanguinarine, but only two of them are enzyme mediated. Protopine is first hydroxylated to form 6-hydroxyprotopine, which spontaneously rearranges to form dihydrosanguinarine. Dihydrosanguinarine then serves as the substrate of the last oxidation reaction to form the antimicrobial phytoalexin sanguinarine.

Despite the fact that the enzymology and chemistry of this pathway has been studied extensively and is now well characterized, little is known about the basic biology of benzoisoquinoline alkaloid biosynthesis. The relationships between plant development, alkaloid biosynthetic gene expression, and accumulation of specific alkaloids are only now being investigated.

Until recently little research has been directed toward understanding the mechanisms regulating the enzymes involved. Enzymatic steps which may function in metabolic regulation include those that operate at entry points and branch points in alkaloid biosynthesis. Tyrosine/dopa decarboxylase (TYDC), is likely to play an important regulatory role in sanguinarine biosynthesis because it operates at the interface of primary and

secondary metabolism in a manner analogous to the well-established regulatory functions of phenylalanine ammonia lyase (PAL) in phenylpropanoid metabolism. Another putative regulatory step is catalyzed by the berberine bridge enzyme (BBE) in which the branchpoint intermediate (S)-reticuline is committed to sanguinarine production and diverted from morphine biosynthesis. Genes encoding these enzymes have recently been cloned and their expression patterns have been characterized (Dittrich and Kutchan, 1991; Facchini and DeLuca, 1994; Facchini *et al.*, 1996b).

1.3.3 TYDC gene family

Key regulatory functions are often associated with enzymes that operate at the entry point to, or at branch points within, a pathway. The first step in isoquinoline alkaloid biogenesis is the conversion of the amino acids tyrosine and dopa into tyramine and dopamine, respectively, via tyrosine/dopa decarboxylase (TYDC). Recently, TYDC from opium poppy was cloned and found to be present in the genome as a family of genes that encode two different isoforms of the enzyme, which can be represented by *TYDC1* and *TYDC2* (Facchini and DeLuca, 1994). Each isoform is encoded by 6 - 8 genes. Four clones were originally isolated and their sequences were compared. *cTYDC1* and *gTYDC4*, (another gene encoding a *TYDC1*-like protein) shared greater than 90% nucleotide sequence identity, as did *cTYDC2* and *cTYDC3*. When the sequences of *cTYDC1* and *cTYDC2* were compared, however, they showed less than 73% identity. Interestingly, both isoforms accept either tyrosine or dopa as substrates with similar efficiency (Facchini and DeLuca, 1995b). Northern blot analysis determined that the *TYDC* genes are regulated in a differential-, temporal- and tissue specific manner. When poppy cell cultures are treated with a fungal elicitor or with methyl jasmonate (MeJA), *TYDC1*- and *TYDC2*-like transcripts accumulate at different rates, to different levels, and remain elevated for different lengths of time (Facchini *et al.*, 1996a). As well, *TYDC1*-like transcripts accumulate in

the roots of the plant while *TYDC2*-like genes are expressed in both the root and the stem (Facchini and DeLuca, 1995a). Since sanguinarine accumulates only in the root, and morphine accumulates only in the aerial tissues, this differential expression of the TYDC isoforms suggests that *TYDC1*-like genes could be coordinately regulated with the enzymes involved in sanguinarine biosynthesis, while *TYDC2*-like genes are coupled to the branch pathways responsible for the production of phenanthrene (i.e. morphine) or benzylisoquinoline (i.e. noscapine) alkaloids (Facchini and DeLuca, 1995a). To test this hypothesis, genes encoding enzymes from these specific branch pathways would have to be cloned and their expression patterns compared to those of *TYDC* genes.

1.3.4 BBE gene family

In 1991, Dittrich and Kutchan purified the berberine bridge enzyme (BBE) from an elicited *Eschscholtzia californica* L. cell culture, and after determining portions of the protein sequence, were able to isolate a corresponding cDNA clone. BBE catalyzes the conversion of (S)-reticuline to (S)-scoulerine, the first committed step in the biosynthesis of the benzophenanthridine alkaloid sanguinarine. Recently, we used the full-length coding region of the *E. californica BBE1* cDNA as a probe to screen a poppy genomic library. Fragments which cross-hybridized were subcloned and further characterized. This led to the isolation of a clone encoding a functional BBE protein in opium poppy (Facchini *et al.*, 1996b). When the expression patterns of this enzyme were determined by Northern analysis and compared with the patterns of the two TYDC isoforms, *BBE1* did not resemble *TYDC1* as expected. In fact, *BBE1* appears to be expressed in a manner most similar to *TYDC2*. Treatment of poppy cell suspension cultures with MeJA or with a fungal elicitor induced *BBE1* expression, and in whole plant tissues *BBE1* transcripts were found at the highest levels in the roots of the plant, but were also present in the stems. Since BBE is involved in

sanguinarine production, and its expression pattern resembles that of TYDC2, a second hypothesis could be that the regulation of *TYDC2*-like genes is coupled to isoquinoline alkaloid biogenesis in general, whereas *TYDC1*-like genes are involved in other defense-related responses or tyramine-requiring plant processes.

1.4 Objectives of this project

It has recently been determined that the genes encoding two TYDC isoforms and BBE are transcriptionally regulated and that their expression is inducible with the addition of a fungal elicitor or MeJA (Facchini *et al.*, 1996a,b). Analyzing the promoters of these three genes will allow for the determination of the sequence and location of putative *cis*-acting elements responsible for inducible expression, and will provide some insight as to whether these steps in the pathway are coordinately regulated or involve uncoupled signaling mechanisms.

To accomplish this project, the following experiments were performed:

1. The promoters of TYDC1-like, TYDC2-like and BBE1 were isolated and fused to GUS.
2. A nested deletion series of each promoter was created.
3. Each promoter was sequenced and the transcription start sites were mapped.
4. Using particle bombardment techniques, each promoter construct was transiently expressed in cultured opium poppy cells to locate regions involved in regulating the inducible transcription of the genes.
5. Internal deletion constructs were created to demonstrate the importance of these regulatory regions.

2. Materials and Methods

2.1 Materials

2.1.1 Biochemical reagents

All chemicals used to carry out this research were of analytical grade and were purchased from one of the following suppliers:

Fisher Scientific Company (Ottawa, ON, Canada)

Sigma Chemical Co. (St. Louis, MO, USA)

ICN Biomedicals, Inc. (Aurora, OH, USA)

Boehringer Mannheim Biochemicals (Mannheim, Germany)

Rose Scientific Ltd. (Edmonton, AB, Canada)

BDH Chemicals, Inc. (Poole, UK)

Bathesda Research Laboratories (BRL; Gaithenburg, MD, USA)

BioRad (La Jolla, CA, USA)

Jersey Lab Supply (Livingston, NJ, USA)

All restriction and modifying enzymes were purchased from Promega (Ottawa, ON, Canada), Pharmacia (Uppsala, Sweden), or New England Biolabs (Missasuaga, ON, Canada), except Sequenase™ and Mung Bean Nuclease which were obtained from USB (Cleveland, OH, USA) and Stratagene (La Jolla, CA, USA) respectively.

Radioactive isotopes were purchased from Amersham Life Sciences (Arlington Heights, IL, USA).

2.1.2 Plant materials

Cell suspension cultures of opium poppy (*Papaver somniferum* L., cv Marianne, cell line 2009 SPF) were maintained in 90-95 $\mu E/m^2/s$ light at 23 °C in Gamborg 1B5C media (described in 2.1.6).

2.1.3 Bacterial strains

E. coli strain DH10B (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*RΔ(*ara, leu*)7697 *ara*D139, *gal*U *gal*K

nupG rspL), available in the laboratory, was used for all plasmid transformations.

2.1.4 Oligonucleotides

All oligos used to carry out this research are listed in Table 1. All oligos were synthesized on a Millipore/Waters Cyclone Plus oligonucleotide synthesizer using the manufacturer's protocol and Millipore chemicals.

2.1.5 Cloning vectors

The vectors used in the cloning of *TYDC6*, *TYDC7*, and *BBE1* promoters are outlined in Table 2.

2.1.6 Growth media

Bacterial cultures were grown at 37°C in LB media (5g/L bacto-yeast extract, 10 g/L bacto-tryptone, 10 g/L NaCl) while shaking at 250 rpm, or on solid LB agar plates (5 g/L bacto-yeast extract, 10 g/L bacto-tryptone, 10 g/L NaCl, 15 g/L bacto-agar) at 37°C. To select for transformants, antibiotics (either ampicillin or kanamycin) were added to the LB media following autoclaving to a concentration of 100 µg/mL (Sambrook *et al.*, 1989).

Cell suspension cultures were grown in Gamborg's 1B5C media (B5 salts and vitamins, 100 mg/L myo-inositol, 1 g/L hydrolyzed casein, 20 g/L sucrose, and 1 mg/L 2,4-D), and maintained on a shaker set to 80 rpm in diffuse light at 23°C.

2.1.7 Fungal elicitor preparation

Elicitor preparations were prepared as outlined in Facchini *et al.* (1996b). *Botrytis* mycelium cultures were grown in 1B5C plant cell culture media on a gyratory shaker at 22°C in the dark for 6 days, homogenized autoclaved, and centrifuged. The sterile supernatant was then used as an elicitor.

2.2 Methods

Table 1: Oligonucleotides used in this research.

Primer Name	Sequence (5' - 3')
TYDC6pro1	GAT GTG ATT CCG TTC ATA
TYDC6pro2	TTT CTG CAA TCT GAT TAA
TYDC6pro3	CCC ACA GAG TGT GAT TCA
TYDC6pro4	TTC CAT CAT TAC TAG CAG
TYDC6pro5	ATG CTG GGA ATG GCT CAA A
TYDC6pro6	AAT ACA CCA TTA GGC ACG TC
TYDC6pro7	ACT AGT TTC TTC TTG TCA
TYDC6pro8	TGG CTT CAA GGT AGT TAG
MPTYDC6	NNN NNN AAG CTT GAC TAA CTA CCT TGA AGC CA
Δ1TYDC6-1	NNN NNN CTG CAG GAC TAA CTA CCT TGA AGC CA
Δ1TYDC6-2	NNN NNN CTG CAG GTT TAC AAA CGT GGG TTC GC
TYDC7pro1	TTA AAT TCA GTA GTG CCA
TYDC7pro2	AGT TGT GAA GTG AGA TAG
TYDC7pro3	TTG GAG CTA TGA TTA GCC
TYDC7pro4	GTA GCA ATA TTA ATA GCA
TYDC7pro5	TAT CTA CAA GGA CAG TTG
TYDC7pro6	TTC AAG GCT ACT GCA GCA
TYDC7pro7	TGG GCT AAT CAT AGC TCC
TYDC7pro8	GGT ACC GAA GGT GTA AGG
TYDC7pro9	GGA GTT TGA TGA CCG GAG
-165TYDC7	NNN NNN AAG CTT TTA AAT TCA GTA GTG CCA GA
-5TYDC7	NNN NNN AAG CTT ACT TCA CAA CTT GTA AAG AA
Δ1TYDC7	NNN NNN CTG CAG TGC TAC TTA TTA GTT GTT GC
Δ2TYDC7	NNN NNN GGA TCC CCT TAC ACC TTC GGT ACC AA
Δ3TYDC7	NNN NNN GGA TCC GCA AAC TCT CTC CGG TCA TC
BBEpro1	AGG CTT CTC TAA TGT CCG
BBEpro2	TGA TAC ACG TAG CGT CAT
BBEpro3	GCC AAT GAT TCA TCA TCC
BBEpro4	GCT ACA TAG TAT TGG CTT
BBEpro5	AAT GTT GTC AGT ACT GTT
BBEpro6	AAG CCA ATA CTA TGT AGC
BBEpro7	CCA CAA GAT ACC CAA TCA
BBEpro8	GCA CGT GGG AGT AAA CGC

Table 1: Oligonucleotides used in this research.

Δ1BBE1-1	NNN NNN CTG CAG GAA TCA CCC TTG GTT GAG G
Δ1BBE1-2	NNN NNN CTG CAG AAG CCA ATA CTA TGT AGC AA
Δ2BBE1-1	NNN NNN CTG CAG ACG CGT TTA CTC CCA CGT GC
Δ2BBE1-2	NNN NNN CTG CAG CCT TGA TTG GGT ATC TTG TG
TYDC6-PE	ACA CAG CGA CAT GCT TTC AAA GTT A
TYDC7-PE	CTG AAT TCT TCT GGG TCT AAT GGA T
BBE1-PE	GTT TAA ACA TGA CGA GAG GAG ATT A
BSSKUPT7	GGG ATG TGC TGC AAG GCG A
FORWARD	AGT CAC GAC GTT GTA AAA CG
REVERSE	CTT TCC CAC CAA CGC TGA TCA
GUS-REVERSE	GTC CGT ATG TTG TGT GGA AT
T7	GTA ATA CGA CTC ACT ATA GGG C
8-2D-7	CCC CCC GGA TCC GTT GGA GAA GTA CGT CAA
8-2D-8	CCC CCC AAG CTT GAA TTC AGA ATG GGT TAG TC
T3	AAT TAA CCC TCA CTA AAG GG
TYDC6-11	CCC CCC GGA TCC TTG CTG ATT AGT GAG GGA GA
TYDC6-12	ACG TTC AAG CTT ATA GAA GTT GTT GGG AGA TA
TYDC6-13	GAC GTT CTC GAG GTT ACT ATC AGT TTT GCT GAT
TYDC7-12	ACG TGC AAG CTT TTA TCC ACA CCC AAC TCA TC
TYDC7-13	ACT GTC CTC GAG CAG GTG AAA GAA GGT TAT TG

Table 2: Cloning vectors used in the construction of promoter::GUS fusions.

pBluescript SK+	Stratagene cloning vector
pBI-101	promoterless;multiple cloning site: *Hind*III,*Sph*I,*Pst*I,*Sal*I,*Xba*I,*Bam*HI, *Sma*I
pBI-102	modified version of Jefferson's pBI-101
pBI-121	based on pBI-101 (has 35S cloned between *Hind*III and *Bam*HI, so MCS is *Hind*III,*Sph*I,*Pst*I,(35S),*Xba*I,*Bam*HI,*Sma*I)
pBI-122 (-102/35S)	pBI-102 with CaMV 35S promoter (similar to pBI-121)
pBI-221	3.0 kb *Eco*RI - *Hind*III from pBI-121 (has 35S) in pUC-19
pBI-222	≈4.0 kb *Eco*RI - *Hind*III from pBI-122 (-102/35S) in pUC-19
pUC-19	standard cloning vector
pUC-202	pUC-19 with *Eco*RI - *Hind*III from pBI-102 (promoterless)
pUC-222 (-202/35S)	pUC-19 with *Eco*RI - *Hind*III from pBI-122 (-102/35S)

2.2.1 Standard protocols

2.2.1.1 DNA isolations

Plasmid DNA was isolated from *E. coli* using the mini prep method described by Zhou *et al.* (1990). If more DNA was required then 200 mL bacterial cultures were grown and the DNA was purified by PEG precipitation, as outlined in Facchini *et al.* (1996b).

2.2.1.2 DNA restriction enzyme manipulations

All restriction endonuclease digestions, ligation reactions using T4 DNA ligase, end-repair reactions using T4 DNA polymerase, radiolabel incorporation of [α-^{32}P]-dCTP using Klenow polymerase, and addition of labeled phosphate using PNK were carried out according to Sambrook *et al.* (1989).

2.2.1.3 DNA electrophoresis

Electrophoresis was carried out using molecular analytical grade agarose (Bio-Rad) at concentrations of 1% - 1.5% (w/v) in TAE (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). Gels were run at 20 - 150 V in approximately 500 - 800 mL 1x TAE until individual bands could be resolved in the presence of ethidium bromide (Sambrook *et al.*, 1989).

2.2.1.4 DNA sequencing

Double-stranded DNA was sequenced using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) according to the procedure outlined by the manufacturer of Sequenase™, a recombinant T7 DNA polymerase (United States Biochemical). Sequencing reactions were run on a 6% polyacrylamide gel, at constant amperage in 1x TBE (10.8 g Tris base, 5.5 g boric acid, 2 mL 0.5M NaEDTA, dH_2O to 1 L) for approximately 3 h.

2.2.1.5 DNA probe preparations

DNA fragments were amplified by PCR, isolated on agarose gels, and used to synthesize radiolabeled probes by random hexamer

priming and incorporation of deoxynucleotide triphosphates (dNTPs) and [α-^{32}P]-dCTP using Klenow polymerase (Sambrook *et al.*, 1989). Labeling reactions were passed through P-60 biogel matrix (BioRad) to remove unincorporated radioactivity and the specific activity of the purified probe was determined in a scintillation counter (2200CA, Canberra Packard).

2.2.1.6 Bacterial transformation

Competent *E. coli* strain DH10B cells (stored at -80°C) were added to ligation reaction, mixed, and left on ice for 45 min. Cells were then heat shocked at 42°C for 45s and placed back on ice for 2-5 min. After the addition of 500 μL of LB media, the mixture was incubated at 37°C for 30 min-1h. Cells were then plated on LB-Amp_{100} or LB-Kan_{100} plates with 40 μL of 25 mg/mL X-Gal and 4 μL of 10 mg/mL IPTG if appropriate and allowed to grow at 37°C overnight (Sambrook *et al.*, 1989).

2.2.1.7 Deletion series

An Exo III/Mung Bean Nuclease deletion kit was purchased from Stratagene and used according to the instructions of the manufacturer to create a series of unidirectional nested deletions in each promoter construct (pUC-TYDC6::GUS, pTYDC7::GUS, and pUC-BBE1::GUS). If the deletion series was not continuous, additional deletions were made by cutting the full length construct with unique restriction endonucleases, excising a fragment, blunting the overhanging ends with T4 DNA polymerase, and then religating.

2.2.1.8 PCR

All PCR reactions were performed with *Taq* polymerase in a Minicycler™ PCR machine (MJ Research) using the standard program of 1 min @ 95°C; 1 min @ 50°C; 1 min @ 72°C for 35 cycles followed by 5 min @

72°C. Final concentrations of dNTPs and primers were always 1 mM and 1 μM, respectively.

2.2.1.9 DNA fragment purification

2.2.1.9.1 LMP-agarose

Digested DNA was electrophoresed on a pre-stained 1% LMP-agarose gel at 50V, the desired fragment was cut out, and the gel containing it was melted at 65°C. After 1.5 volumes of dH_2O were added, the sample was returned to the 65°C heat block for 5 min. The samples were extracted 2x with Tris-phenol, and then extracted with dry butanol until the volume of the aqueous phase was < 100 μL. The DNA was then precipitated with 3 volumes of 95% EtOH, and dried under vacuum (Keon, 1989; unpublished).

2.2.1.9.2 Polyacrylamide

Digested DNA was electrophoresed on 4% non-denaturing polyacrylamide gel (2.7 mL 30% acrylamide, 13.2 mL dH_2O, 4 mL 5x TBE, 140 μL 10% ammonium persulfate, 7 μL TEMED) in a 1x TBE running buffer at 50V for ≈ 2h. The gel was then placed on a TLC plate and illuminated under UV light. Since the DNA bands absorb UV light, areas which cast shadows on the TLC plate were cut from the gel. The DNA was eluted by grinding the gel fragment, allowing it to incubate overnight at 70°C in elution buffer (0.386 g CH_3COONH_4, 0.012 g $Mg(C_2H_3O_2)_2$, 20 μL 0.5M Na-EDTA, 50 μL 20% SDS, dH_2O to 10 mL), spinning it down to remove the gel, and ethanol precipitating the DNA from the supernatant (Sambrook *et al.*, 1989).

2.2.1.10 Biolistics

60 mg gold particles (1.6 µm in diameter; Bio-Rad) were sterilized by vortexing in 100% EtOH for 5 min, washed 2x with sterile dH_2O, and resuspended in 1 mL sterile dH_2O. A 50 µL aliquot of this was removed and 10 µg of a promoter::GUS fusion construct was added to it along with 10 µg of pCaLucNOS (as described in Facchini *et al.*, 1996b), 50 µL of 2.5M $CaCl_2$, and 20 µL 0.1 M spermidine. The gold particles were vortexed and left on ice for 5 min after each addition. The mixture was then vortexed at RT for 4 min, pelleted, resuspended in 100% EtOH, vortexed for another 4 min, pelleted, and finally resuspended in 110 µL of 100% EtOH. For each bombardment, 15 µL of this particle suspension was pipetted and dried onto sterilized macrocarriers (Bio-Rad) and appropriately positioned in the biolistic particle acceleration device (PDS 1000/He, Bio-Rad).

1 mL of 2-4 day opium poppy cell suspension culture was collected over vacuum onto Whatman GF/A microfibre filters. The filter was then placed into a sterile Petri dish and positioned below a macrocarrier stopping screen (Bio-Rad) in the PDS 1000/He. Bombardments were performed under a chamber pressure of 26 mm Hg, and at a He pressure of 1100 psi. Following bombardment, 'shot' cells (still in the Petri dishes) were incubated for 48 h in the dark at RT in 600 µL of sterile 1B5C media.

2.2.1.11 RNA isolation

Total RNA was isolated according to the procedure of Logemann *et al.* (1987), in which powdered plant tissues are resuspended in an 8M guanidine-HCl buffer (30.56 g Guanidine-HCl, 0.78 g MES, 1.6 mL 0.5 M NaEDTA, dH_2O to 40 mL, (pH 7.0), and 3.5 µL/mL β-mercaptoethanol), extracted with phenol/chloroform, and ethanol precipitated.

2.2.1.12 Northern blotting

15 μg of total RNA was electrophoresed on a 1.0% formaldehyde agarose gel in a 1x MOPS (20 mM MOPS, 5 mM NaOAc, and 1 mM EDTA) buffer, and then transferred to a nylon membrane via 10x SSC (150 mM sodium citrate, pH 7.2, and 1.5 M NaCl), (Sambrook *et al.*, 1989). The nylon membrane was then baked at 80°C in a vacuum oven for approximately 1h, placed in a hybridization oven bottle and allowed to pre-hybridize for 20 min - 1h at 65°C in 10 mL of 0.25 M $NaPO_4$, pH 8.0, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA. Purified radiolabeled probe was added to the bottle and left overnight. Blots were then washed for 30 min at 65°C; twice with 100 mL of 2x SSC, 0.1% (w/v) SDS, and twice with 100 mL of 0.2x SSC, 0.1% (w/v) SDS, and autoradiographed with an intensifying screen at -80°C.

2.2.1.13 Protein assay

Protein concentration was determined by the method of Bradford (1976) using BSA as the standard. 10 μL of cell extract was added to 1 mL of protein assay buffer (BioRad) diluted 1:5 with dH_2O, and the absorbance measured at a wavelength of 595 nm.

2.2.1.14 Transcription start site mapping

Antisense oligonucleotides, designated TYDC6-PE, TYDC7-PE, and BBE1-PE, were designed to anneal to sequences approximately 100-150 bp upstream of the putative transcription start site in each promoter. These 20 bp oligos were endlabeled with [γ - ^{32}P] ATP using polynucleotide kinase (Sambrook *et al.*, 1989). ~5 pmole of labeled oligo was then mixed with 10 μg of total RNA, isolated from an elicited cell suspension culture, in 0.4 M KCl for 60 min at RT to hybridize the oligo to the mRNA (Wu *et al.*, 1988). Using the mRNA template, the DNA strand was extended from the primer in

a reaction mix containing 100 mM Tris-HCl (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 15U RNAsin (Promega), and 20 U reverse transriptase (AMV). This reaction mix was incubated at 37°C for 1h. The DNA-RNA hybrid was then purified by extracting with phenol and chloroform, followed by an EtOH precipitation. The resulting pellet was recovered in a solution of 0.1 M NaOH and 1 mM EDTA, incubated at 37°C for 30 min, and reprecipitated. Finally, the pellet was dissolved in 4 μL of sequencing gel loading dye.

To analyze the primer extension product, the entire sample was loaded on a sequencing gel in a well adjacent to a sequencing reaction of the corresponding genomic clone initiated with the same primer (TYDC6-PE, TYDC7-PE, or BBE1-PE).

2.2.1.15 Computer programs

DNA of known sequence was analyzed with respect to restriction enzyme digestion sites using the computer program DNA Strider™ (CEA). Alignments were performed using the MacVector™ (Oxford Molecular Group) software package, and the functional analysis of the promoter::GUS constructs was completed using Excel 5.0™ (Microsoft).

2.2.2 GUS assay

2.2.2.1 Fluorometric assay

48h after bombardment, cultured cells were collected over vacuum, ground to a uniform homogenate in 600 μL of extraction buffer (50 mM KPO_4, pH 7.0; 1 mM EDTA; 10 mM β-mercaptoethanol) (Zhang *et al.*, 1996). This homogenate was then centrifuged at ~16 000 x *g* at 4°C for 20 min and the supernatant collected. 80 μL of this extract was mixed with 320 μL of GUS assay buffer (50 mM $NaPO_4$, pH 7.0; 10 mM β-mercaptoethanol; 10 mM Na-EDTA, 0.1% (w/v) SDS, 0.1% (w/v) Triton-X, and 1 mM 4-

methylumbelliferyl-β-D-glucuronide), and incubated at 37°C for 3h. 100 μL of the reaction was stopped with the addition of 900 μL of 0.2 M Na_2CO_3. The stopped reaction was analyzed in a spectrofluorimeter (Hitachi F-2000, Tokyo, Japan), excitation at 365 nm and emission at 455 nm, to quantify the amount of 4-methylumbelliferone cleaved from 4-methylumbelliferyl-β-D-glucuronide (Jefferson, 1987).

2.2.2.2 Histochemical assay

48h after bombardment, fixation was achieved by infiltrating plant tissues under vacuum for 5 min with a solution containing 41 μL 37% formaldehyde, 50 μL 1M MES, and 1.5 mL 1M mannitol. Tissues were further incubated in this solution for 45 min at RT without vacuum. After rinsing the tissue 3x in 50 mM $NaPO_4$, pH 7.0, 5 mL of histochemical stain (4 mL 50 mM $NaPO_4$, pH 7.0, 1 mL methanol, and 30 μL 250 mM X-gluc) was added. Samples were placed under vacuum for 10 min, and then incubated overnight at 37°C (Jefferson, 1987). To view staining, tissues were observed under a light microscope at an appropriate magnification.

2.2.3 Luciferase assay

20 μL of the bombarded cell extract was mixed with 200 μL of luciferase assay buffer (25 mM Tricine, pH 7.8, 15 mM $MgCl_2$, 5 mM ATP, 0.5 mg/mL BSA and 7 mM β-mercaptoethanol), and incubated at RT for 15 min (Zhang *et al.*, 1996). 100 μL of luciferin (0.5 mM diluted with 1 mM Tricine, pH 7.8, from 10 mM stock; Boehringer Mannheim) was injected into the reaction mixture by the luminometer (Monolight 2010, Analytical Luminescence Laboratories, San Diego, CA), and the light emitted in the first 10s was recorded as relative luciferase units, RLU.

2.3 Plasmid construction

2.3.1 *TYDC6* promoter::GUS fusion

The promoter of *TYDC6* (TYDC6pro) was amplified from template pTYDC6g with primers TYDC6-11 (*Bam*HI) and TYDC6-12 (*Hind*III). The product of this PCR reaction was digested with *Bam*HI and *Hind*III, as was the plasmid vector pBluescript-SK+. The digestion reactions were ethanol precipitated, ligated under standard conditions, and transformed into competent *E. coli* strain DH10B cells. Following transformation, recombinant plasmids, (designated pTYDC6pro), were identified on the basis of blue/white selection in the presence of X-Gal and IPTG on LB-Amp_{100} plates (Sambrook *et al.*, 1989). pTYDC6pro was then digested with *Bam*HI and *Hind*III to generate the promoter fragment, and with *Sal*I to prevent religation of the original construct. After being ethanol precipitated, this digestion reaction was ligated with *Bam*HI and *Hind*III digested pUC-202, a promoterless vector containing the GUS open reading frame and the nopaline synthase terminator. The resulting construct was called pUC-TYDC6::GUS and contained 3000 bp of *TYDC6* promoter sequence upstream of the reporter gene.

2.3.2 *TYDC7* promoter::GUS fusion

The *TYDC7* promoter was amplified from template pTYDC7g with primers TYDC7-12 (*Hind*III) and TYDC7-13 (*Xho*I). The product of this PCR reaction was digested with *Xho*I and *Hind*III, as was the plasmid vector pBluescript-SK+. Again, the digestion reactions were ethanol precipitated, ligated under standard conditions, and transformed into competent *E. coli* strain DH10B cells. Following transformation, recombinant plasmids, (designated pTYDC7pro), were identified on the basis of blue/white selection in the presence of X-Gal and IPTG on LB-Amp_{100} plates (Sambrook *et al.*, 1989). pTYDC7pro was then digested with *Xho*I and *Hind*III to generate the promoter fragment, and with *Eco*RI to prevent religation of this original construct. After being ethanol precipitated, this digestion reaction was ligated with *Xho*I/*Hind*III digested pBI-102, a promoterless vector containing the

GUS open reading frame and the nopaline synthase terminator. The resulting construct was called pBI-TYDC7::GUS and contained ~1300 bp of *TYDC7* promoter sequence upstream of the reporter gene. Since pBI vectors are stringently copied by *E. coli* cells, it is preferable to work with a high copy plasmid vector such as pUC-19 or pBluescript. So, the entire promoter/GUS/NOS expression cassette was cut from pBI-TYDC7::GUS with *Hind*III and *Eco*RI, and ligated into pBluescript-SK+ which was similarly digested. The resulting construct was called pTYDC7::GUS and was used as the full length representative of the *TYDC7* promoter in all further experiments.

2.3.3 *BBE1* promoter::GUS fusion

The *BBE1* promoter was amplified by PCR from template 8-2D-5 with primers 8-2D-7 (*Bam*HI) and 8-2D-8 (*Hind*III). The resulting product was digested with *Bam*HI and *Hind*III, as was the plasmid vector pBI-102. The digestion reactions were ethanol precipitated, ligated under standard conditions, and transformed into competent *E. coli* strain DH10B cells. Following transformation, recombinant plasmids, (designated pBI-BBE1::GUS), were identified on the basis of growth on LB-Kan_{100} plates. pBI-BBE1::GUS was then digested with *Eco*RI and *Hind*III to generate a fragment in which the *BBE1* promoter was 5′ to the open reading frame of the reporter gene β-glucuronidase (GUS) and the nopaline synthase (NOS) terminator. After being ethanol precipitated, this digestion reaction was ligated with pUC-19 which had also been digested with *Eco*RI and *Hind*III. This ligation product was termed pUC-BBE1::GUS and contained ~2600 bp of BBE1 promoter sequence in an orientation which would direct the expression of GUS.

2.3.4 Deletion series constructs created by exo III/mung bean nuclease

The full length *TYDC6, TYDC7* and *BBE1* promoter constructs, pUC-TYDC6::GUS, pTYDC7::GUS and pUC-BBE1::GUS, were digested with *Hind*III to create an opening at the junction of the vector and the promoter. Since exonuclease III progressively digests the 3′ end of double stranded DNA, the 5′ overhang which resulted from the *Hind*III digestion had to be protected. This was accomplished by blunting the overhang with Klenow polymerase and α-thio phosphate dNTPs, which are insensitive to exonuclease III treatment. Once the 3′ end of the *Hind*III site was filled in, pUC-TYDC6::GUS and pUC-BBE1::GUS were digested with *Xba*I to create a 5′ overhang inside the promoter sequence, and pTYDC7::GUS was digested with *Sal*I. From this new cut site, exo III could only digest the 3′-end in one direction, resulting in a progressively shorter promoter. Stopping the exo III digestion at time points between 0 min and 3 min resulted in the generation of a series of promoters ranging from full length to tens of base pairs. Mung bean nuclease was then added to the reactions to specifically digest the remaining 5′ strand of ssDNA, thereby creating a blunt end which was then ligated to the previously blunted *Hind*III site. The size of the promoter in the resulting constructs was analyzed by PCR. *TYDC6* promoter deletions were amplified with the primers TYDC6-11 and reverse; *TYDC7* used TYDC7-13 and T7; and *BBE1* promoter lengths were determined using primers 8-2D-7 and reverse.

A series of constructs which contained promoters of progressively shorter length (Table 3) were sequenced and the transcription initiation sites contained within these promoters were mapped.

2.3.5 Deletion series constructs created by restriction enzyme digestion

The exo III/mung bean nuclease treatment produced a relatively continuous deletion series for all three promoters, but there were some obvious gaps. In the *TYDC6* promoter, for example, the next shortest deletion

Table 3: Deletion series' constructs created by exonuclease III and mung bean nuclease treatment.

TYDC6 constructs	TYDC7 constructs	BBE1 constructs
-3000TYDC6::GUS	-1194TYDC7::GUS	-2628BBE::GUS
-2031TYDC6::GUS	-634TYDC7::GUS	-1860BBE::GUS
-1566TYDC6::GUS	-510TYDC7::GUS	-1250BBE::GUS
-1463TYDC6::GUS	-393TYDC7::GUS	-1070BBE::GUS
-1180TYDC6::GUS	-53TYDC7::GUS	-670BBE::GUS
-793TYDC6::GUS	-33TYDC7::GUS	-320BBE::GUS
-447TYDC6::GUS		-160BBE::GUS
-10TYDC6::GUS		-99BBE::GUS

after -447 was only 10 bp upstream of the transcription start site. Two additional deletions were needed; one which would produce a promoter construct ~250 bp in length, and one which could represent a minimal promoter, containing the putative TATA box but little sequence upstream of that. To make the ~250 bp promoter construct, pUC-TYDC6::GUS (with thefull length promoter) was digested with *Hind*III and *Spe*I because sequencing revealed that there was a unique *Spe*I site at -242 of the promoter and *Hind*III was the original cloning site into which the 5′ end of the promoter was inserted. Following digestion, the sites were blunted via T4 DNA polymerase incorporation of added dNTPs, and the plasmids were religated (Figure 2). To generate the minimal promoter construct, a different approach was taken because there were no unique restriction sites in the appropriate area. A sense primer containing a unique *Pst*I site was designed, called MPTYDC6, and used along with the forward primer to amplify a fragment from the pUC-TYDC6::GUS template which contained 90 bp of promoter, the GUS ORF, and the NOS terminator. The product of this PCR reaction was then digested with *Pst*I and *Eco*RI, and ligated with pUC-19 which had been similarly digested (Figure 3).

In the *TYDC7* promoter, four additional deletion constructs needed to be made to create a relatively continuous series. The longest product of the exoIII/mung bean nuclease treatment was only 634 bp, so an additional construct was needed between the full length promoter at -1194, and this first deletion. The only unique restriction site upstream of -634 was a *Pst*I site at -744. pUC-TYDC7::GUS was digested with *Hind*III and *Pst*I, the ends were blunted with T4 DNA polymerase, and then religated. This approach was also used to create a promoter construct at -287, but pUC-TYDC7::GUS was digested with *Bam*HI instead of *Pst*I (Figure 4). Two other constructs, one at -165 and the other at -5, were created in a manner similar to that employed for

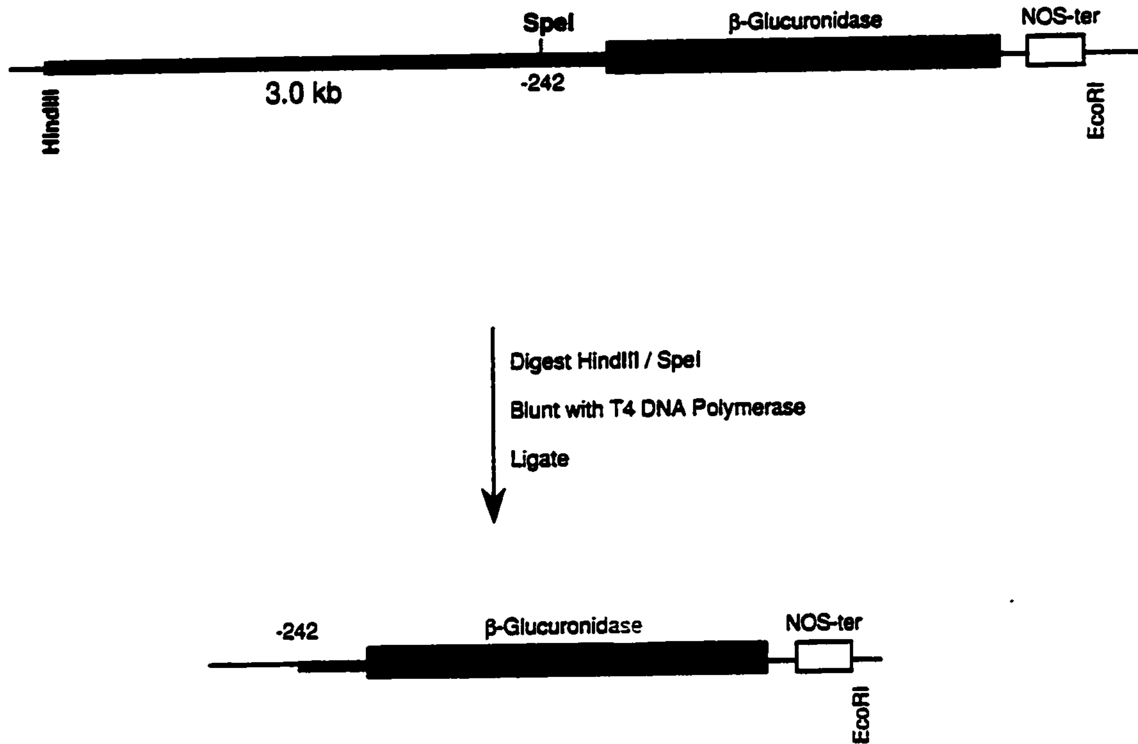


Figure 2: Creating the -242TYDC6::GUS construct

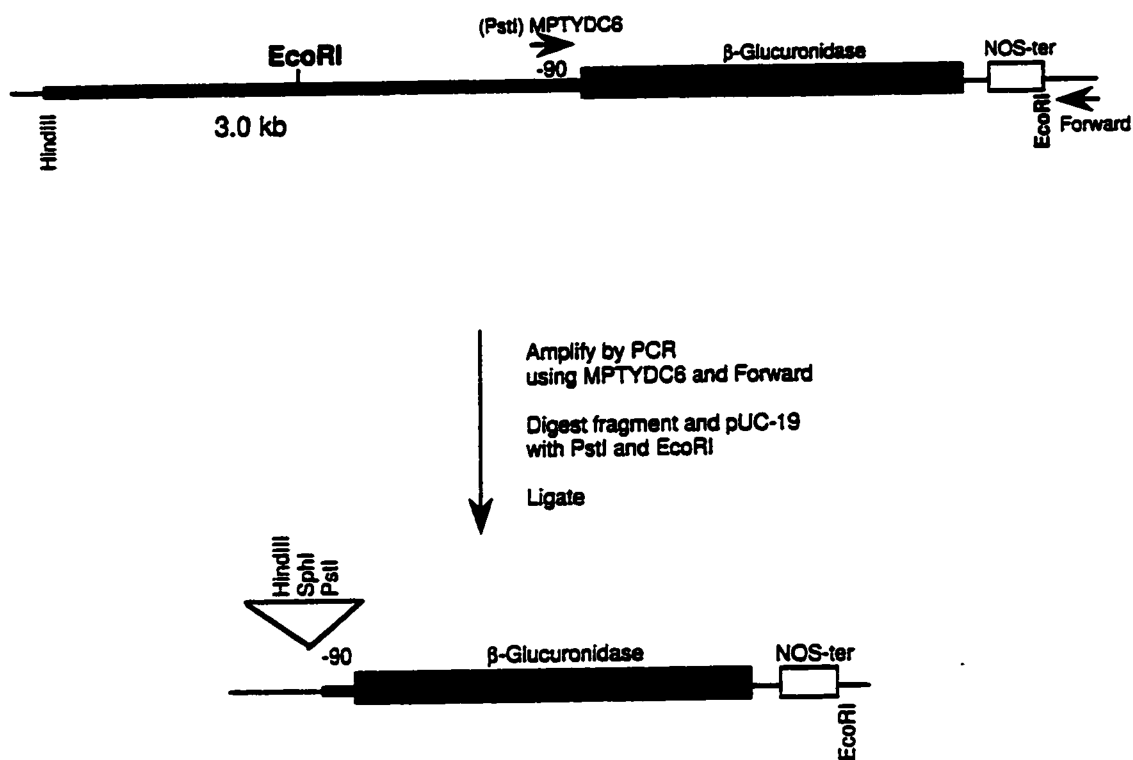
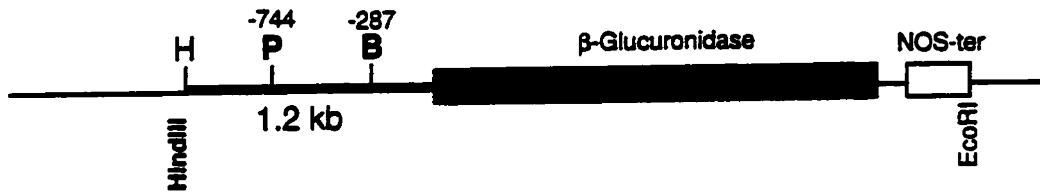


Figure 3: Creation of the -90TYDC6::GUS construct

pTYDC7::GUS



Digest pUC-TYDC7::GUS with HindIII/PstI (-744)
or HindIII/BamHI (-287)

Blunt with T4 DNA Polymerase

Religate

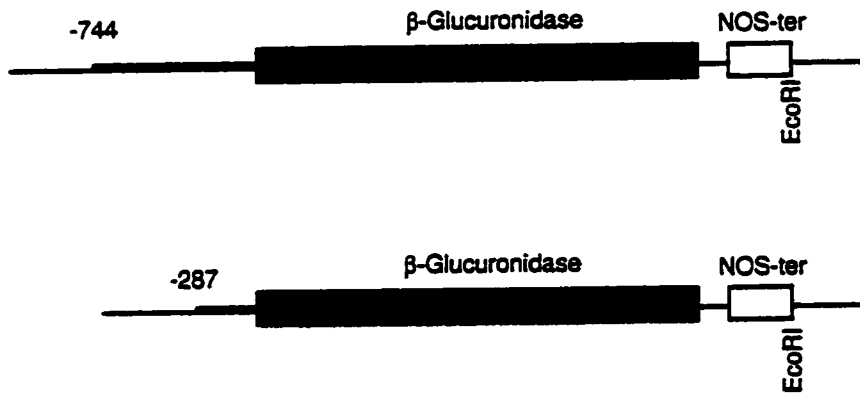


Figure 4: Creating -744TYDC7::GUS and -287TYDC7::GUS constructs

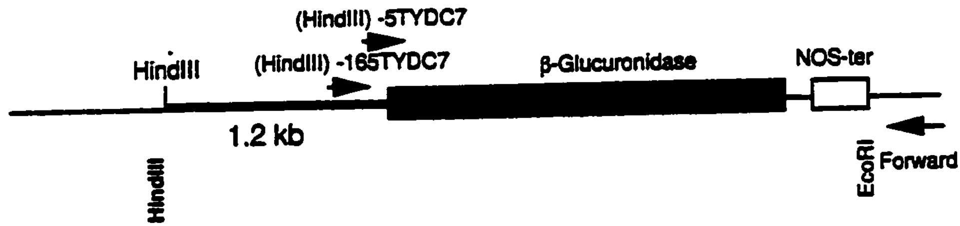
the -90TYDC6 construct. Sense primers (-165TYDC7 and -5TYDC7) were designed with a *Hind*III site and used along with forward primer to amplify a fragment containing either 165 bp or 5 bp of promoter, in addition to the GUS ORF and the NOS terminator. The products of these PCR reactions were digested with *Hind*III and *Eco*RI, and ligated into pUC-19 which was also digested with these enzymes (Figure 5).

The *BBE1* promoter deletion series only required the construction of one new plasmid in order to have a continuous truncation ranging from full length to a minimal promoter. This construct was also relatively easy to make since a unique *Bgl*II site existed in an appropriate location. The full length promoter construct pUC-BBE1::GUS was digested with *Hind*III and *Bgl*II, blunted with T4 DNA polymerase, and then religated to form a construct with 2329 bp of promoter sequence (Figure 6).

2.3.6 Creation of internal deletion constructs

Deletion analysis revealed regions in each promoter which seemed to be responsible for regulating inducible expression. By specifically removing these regions, we hoped to determine whether the regulatory elements contained within were necessary and/or sufficient for the inducible expression of the corresponding genes. The Δ1TYDC6 construct contained the entire 3000 bp of the *TYDC6* promoter except for the sequence between -242 and -90. Δ1TYDC6 was constructed by first amplifying a region of pUC-TYDC6::GUS, which contained the 3′ end of the promoter from -90, the GUS ORF, and the NOS terminator using primers Δ1TYDC6-1 and forward. After purifying this fragment it was digested with *Pst*I and *Eco*RI, and then ligated into pUC-19 which had also been digested with *Pst*I and *Eco*RI. The resulting construct was designated Δ1TYDC6i. The next step was to amplify the 5′ end of the promoter using the primers Δ1TYDC6-2 and reverse, digest this

pTYDC7::GUS



Amplify by PCR
using forward and
-165TYDC7 or -5TYDC7

Digest fragment
and pUC-19
with HindIII and EcoRI

Ligate vector and insert

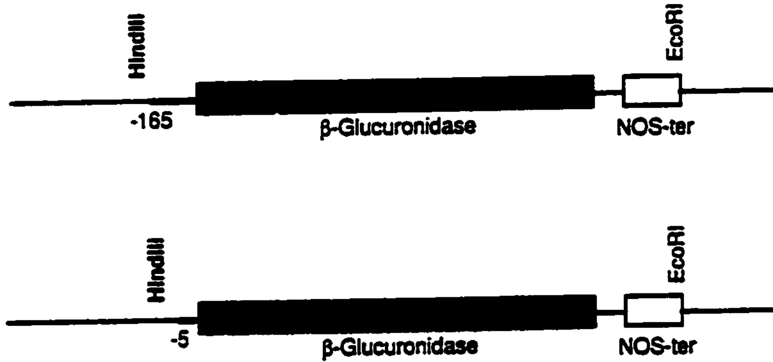


Figure 5: Creating -165TYDC7::GUS and -5TYDC7::GUS constructs

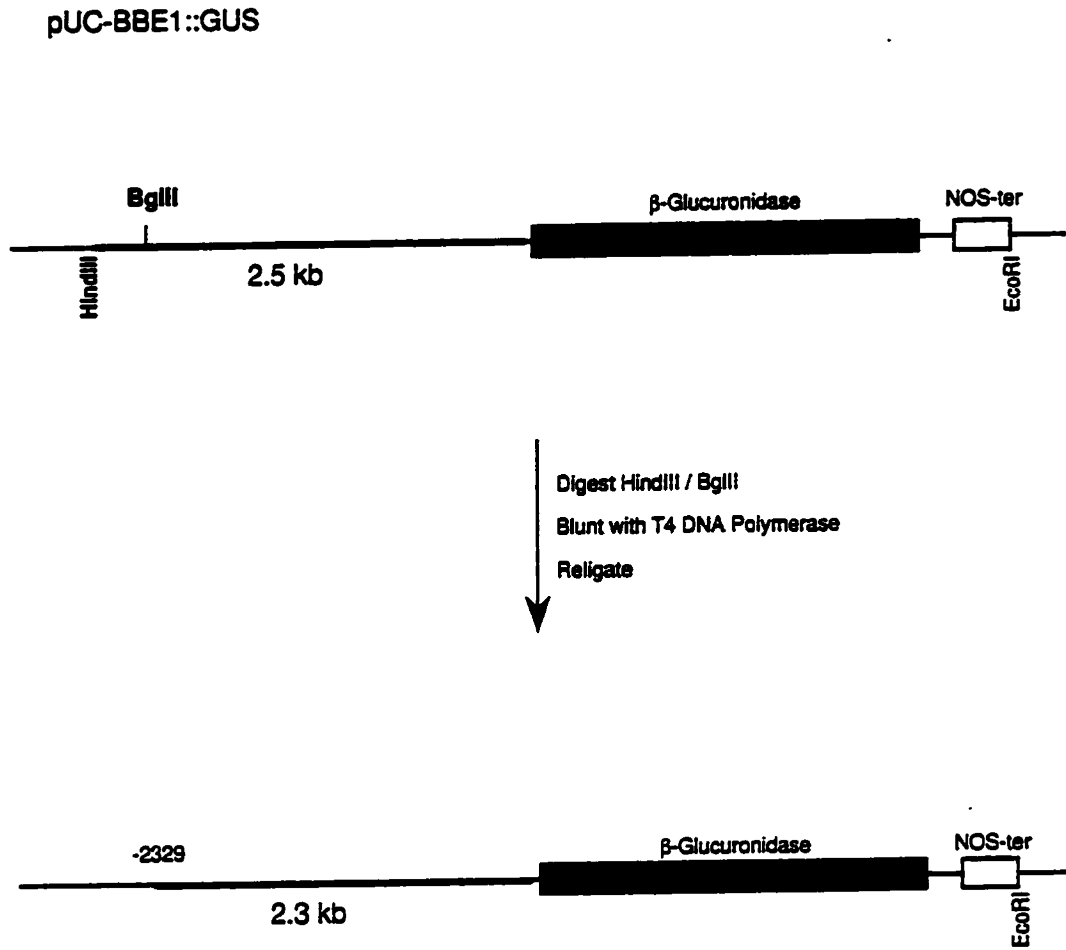


Figure 6: Creating the -2329BBE1::GUS construct

fragment with *Pst*I and *Hind*III, and ligate it into *Pst*I and *Hind*III digested Δ1TYDC6i (Figure 7).

The three *TYDC7* internal deletion constructs, designated Δ1, Δ2, and Δ3, were also complete *TYDC7* promoters from which the sequences between -744 and -634, -393 and -287, or -287 and -53 were deleted respectively. To create Δ1TYDC7, a region of pTYDC7::GUS was amplified by PCR using primers T7 and the sequencing primer TYDC7pro4 (-608). The resulting fragment was then digested with *Hind*III and *Pst*I (there is a *Pst*I site in the promoter at -744) and cloned into pUC-19, which had also been digested with these enzymes. The product of this ligation was named Δ1TYDC7i (for incomplete). To complete the construction of Δ1TYDC7, a second PCR amplified fragment was generated and cloned into Δ1TYDC7i. Primers Δ1TYDC7 and T3 were used to amplify the region between base pair -614 of the promoter and the NOS terminator from the template pTYDC7::GUS. The PCR product and Δ1TYDC7i were then digested with *Pst*I and *Eco*RI, and ligated to form the complete internal deletion construct (Figure 8). To create the Δ2TYDC7 construct the approach had to be slightly modified. To amplify the 5′ promoter region form -1194 to -393, the primers T7 and Δ2TYDC7 were used, and the resulting PCR fragment was digested with *Bam*HI and *Hind*III. pUC-TYDC7::GUS was also digested with these two enzymes to drop out the sequences between -1194 and -287 of the promoter, and the larger fragment (containing the 3′ end of the promoter along with the GUS ORF and the NOS terminator) was isolated on polyacrylamide. Finally, the PCR product was ligated into this digested vector, bringing the base pairs -393 and -287 together (Figure 9). Δ3TYDC7 was generated in the same manner as Δ2TYDC7. The primers Δ3TYDC7 and GUS-REVERSE were used to amplify the sequences

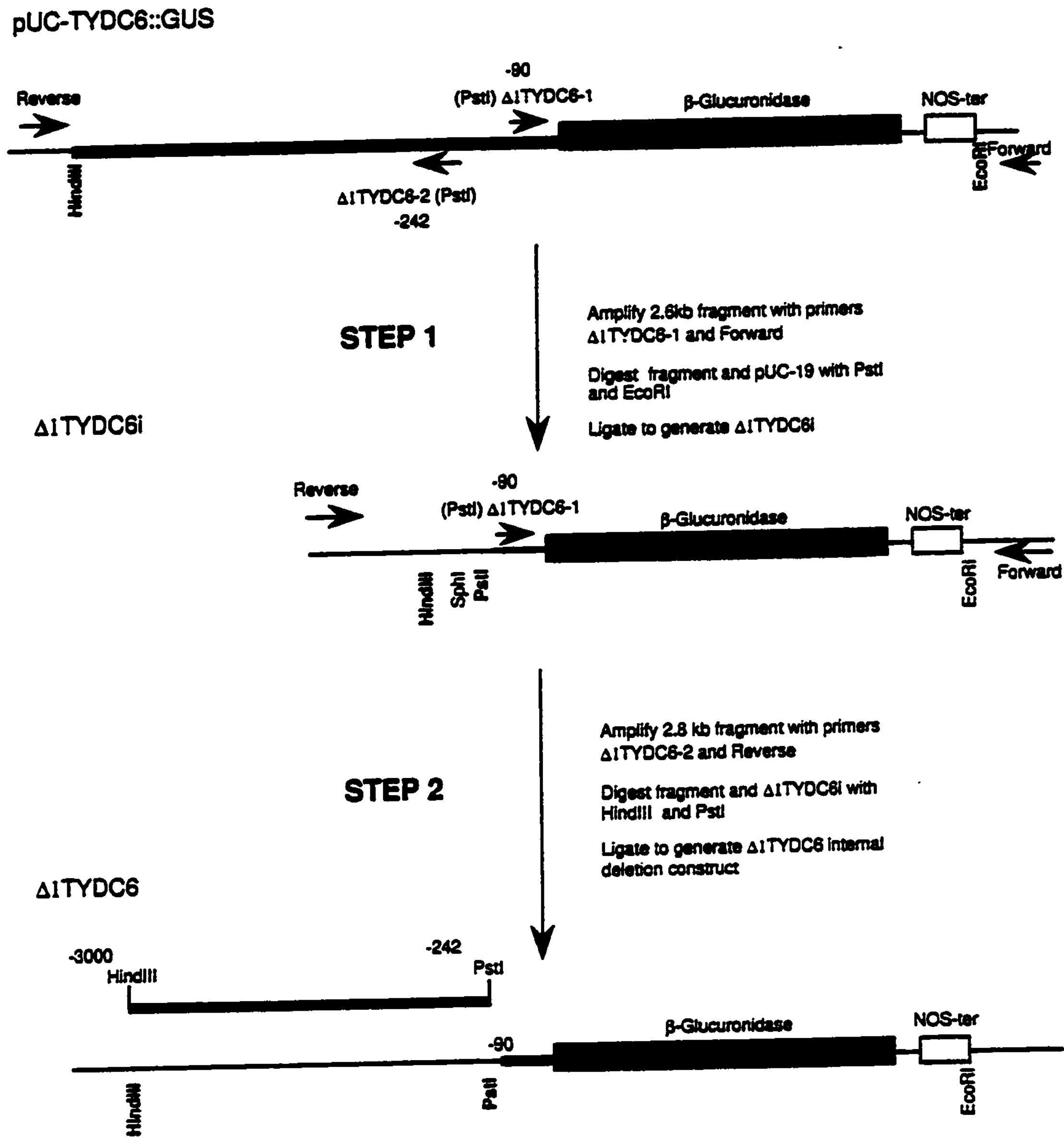


Figure 7: Creating the Δ1TYDC6 internal deletion construct

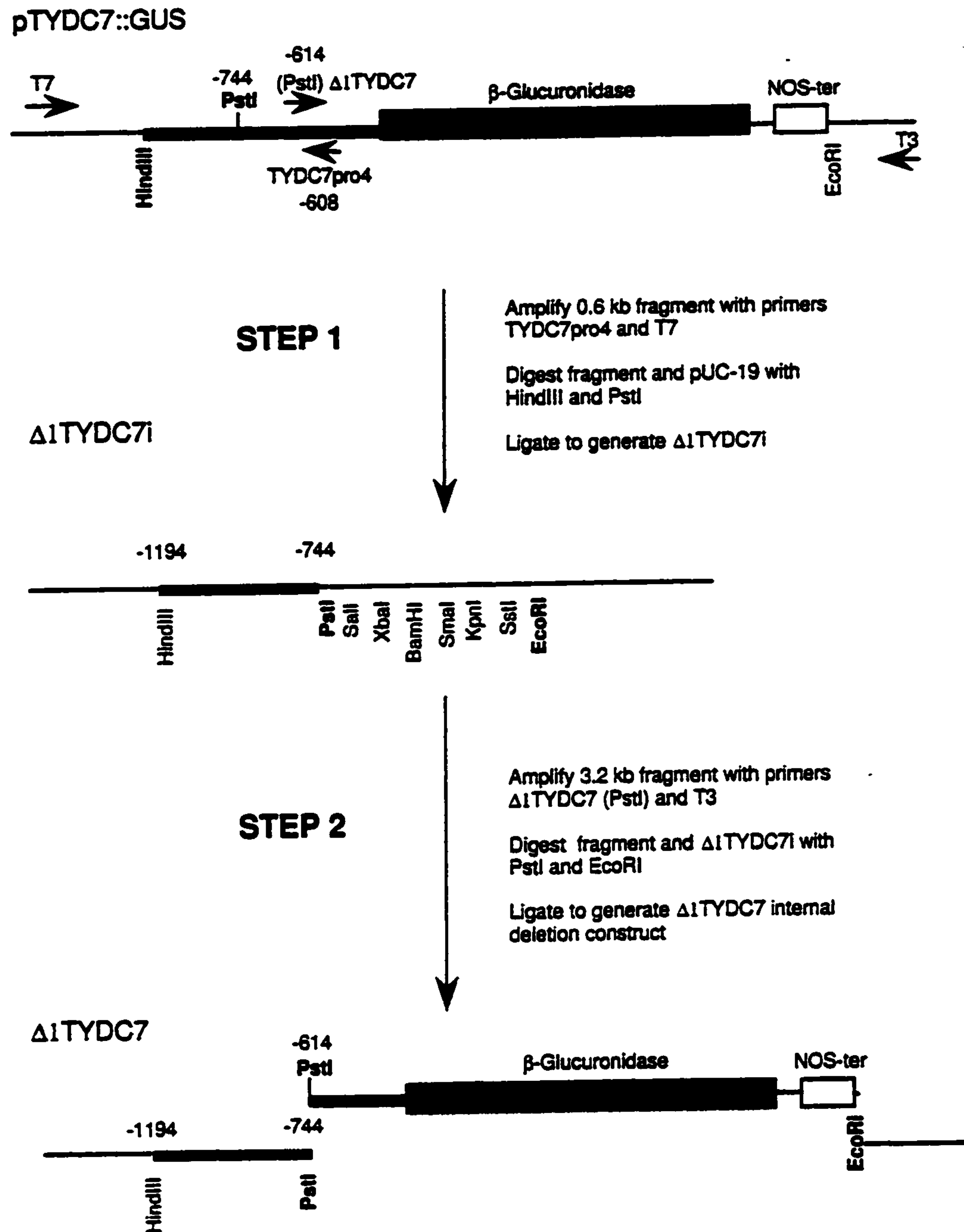


Figure 8: Creating the Δ1TYDC7::GUS internal deletion construct

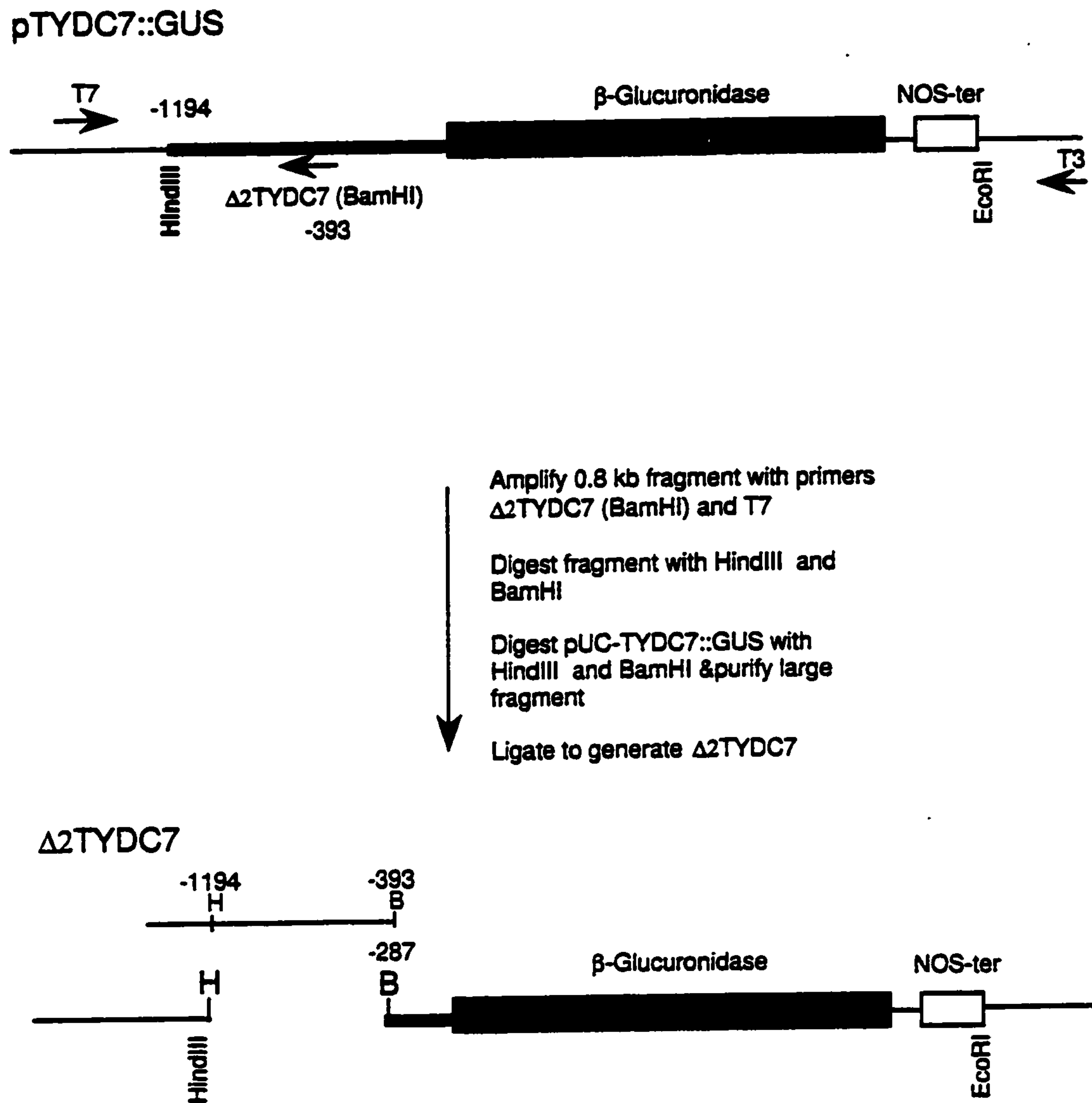


Figure 9: Creating the Δ2TYDC7::GUS internal deletion construct

between -53 of the promoter and a region within the GUS ORF. The product of this PCR reaction was digested with *Bam*HI (Δ3TYDC7 had a *Bam*HI site engineered into it) and *Xho*I which was the original cloning site for the 3′ end of the promoter. pUC-TYDC7::GUS was digested with *Xho*I and *Bam*HI, and the larger fragment was isolated on LMP-agarose. When the two fragments were ligated together, base pairs -287 and -62 became adjacent (Figure 10).

The Δ1BBE1 construct had the sequence between -2068 and -1890 precisely removed, while Δ2BBE1 was missing base pairs -355 to -99. Both of these constructs were created using one approach. The 5′ end of the promoter was amplified by PCR using the primer 8-2D-8 (containing a *Hind*III site) and a specific antisense primer (Δ1BBE-2 for the Δ1BBE1 construct and Δ2BBE1-2 for the Δ2BBE1 construct) which had a *Pst*I site engineered into it. After being digested with *Hind*III and *Pst*I, this insert was subcloned into pUC-19. The resulting plasmids were designated Δ1BBE1i or Δ2BBE1i accordingly. The 3′ end of the promoter, the GUS ORF, and the NOS terminator were amplified using the forward primer and a specifically designed sense primer which also contained a *Pst*I site (Δ1BBE1-1 for the Δ1BBE construct and Δ2BBE1-1 for the Δ2BBE1 construct). Following digestion, this fragment was inserted into the similarly digested Δ1BBE1i or Δ2BBE1i (Figure 11).

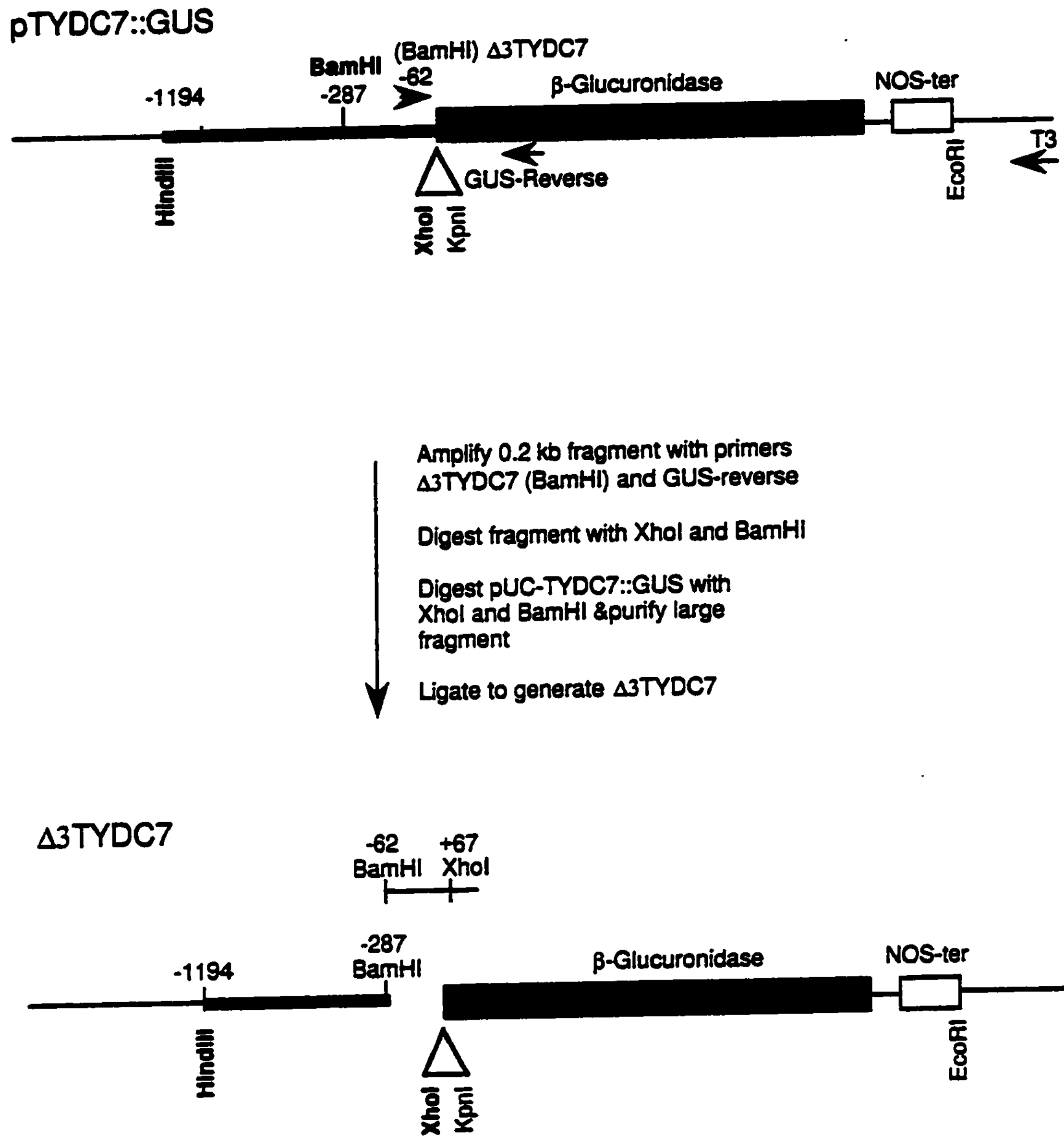


Figure 10: Creating the Δ3TYDC7::GUS internal deletion construct

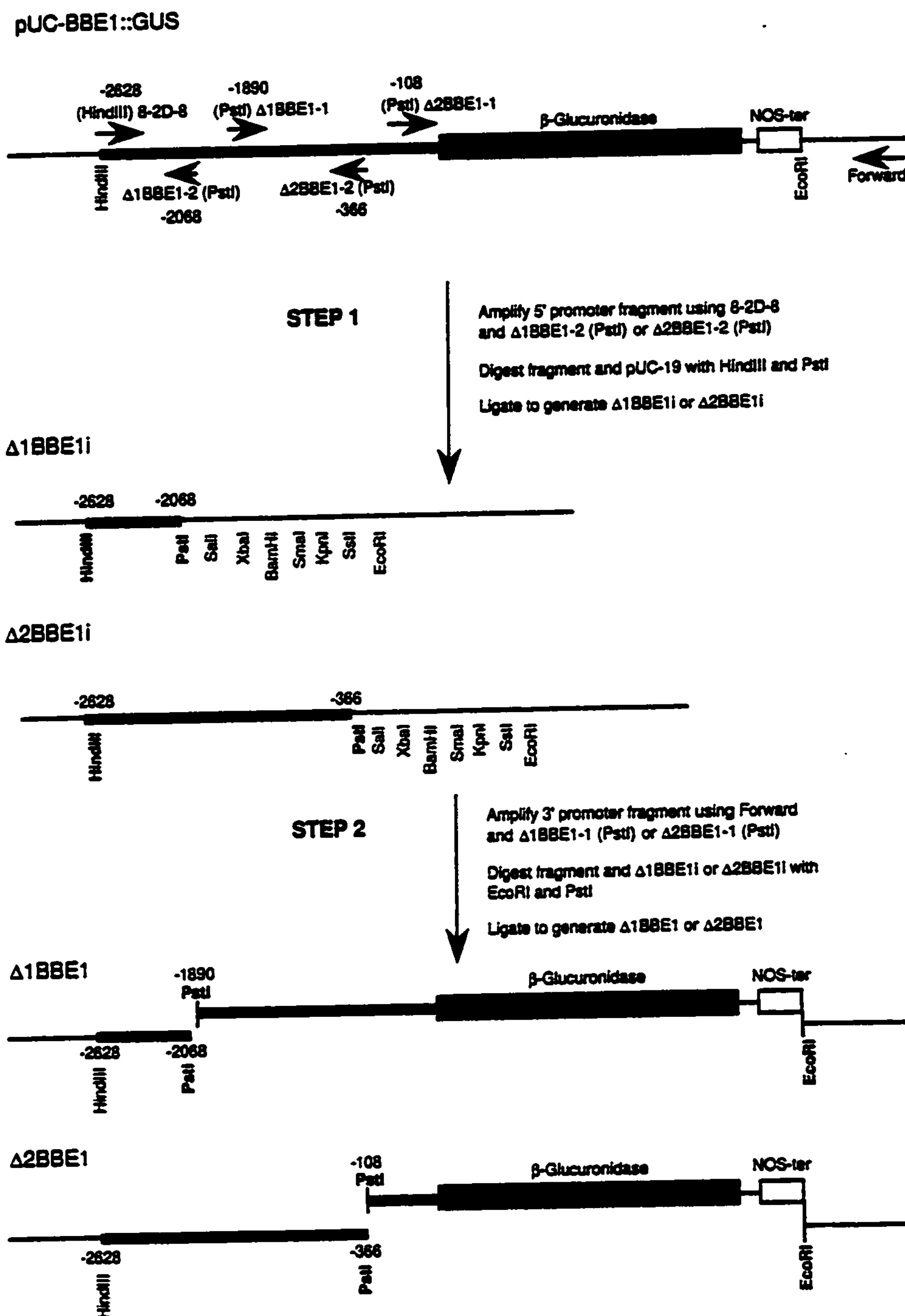


Figure 11: Creating the Δ1BBE1::GUS and the Δ2BBE1::GUS internal deletion constructs

3. Results

3.1 Promoter::GUS fusions

3.1.1 Isolation of TYDC1, TYDC2, and BBE promoters

A poppy leaf genomic library was screened by Catherine Yost with the coding regions of *TYDC1*, *TYDC2*, and *BBE1* (Facchini and DeLuca, 1995; Facchini *et al.*, 1996b). Fragments which hybridized were subcloned, mapped, and partially sequenced to verify their identities with primers designed to sequence the original tyrosine/dopa decarboxylase clones. When the sequences of the clones which hybridized to the coding regions of *TYDC1* and *TYDC2* were compared to those obtained during the original cloning of these genes, discrepancies were noted. The coding regions as well as the 3′ untranslated regions for both of the new *TYDC* clones were sequenced. This sequencing revealed that *TYDC1* and *TYDC2* had not actually been cloned, but other members of the multigene family. The *TYDC1*-like clone was renamed *TYDC6* and the *TYDC2*-like clone was renamed *TYDC7*. At both the nucleotide and amino acid levels, *TYDC1* and *TYDC6* are 97% identical to one another (Figure 12), and *TYDC2* is 96% identical to *TYDC7* (Figure 13). The predicted amino acid sequences of all cloned *TYDC*s were compared (Figure 14). *TYDC1, 4, 5,* and *6* show greater than 90% nucleotide identity when compared to each other, as do *TYDC2, 3,* and *7* (Facchini, unpublished results). When the sequence of any member of one subgroup is compared to any member of the other subgroup, there is less than 73% nucleotide identity (Figure 15).

3.1.2 Creating promoter::GUS expression constructs

As described in Chapter 2 (section 2.3), the promoters of *TYDC6* and *BBE1* were subcloned into pUC-19 and the TYDC7 promoter was cloned into pBluescript so that the activity level of each promoter could be determined.

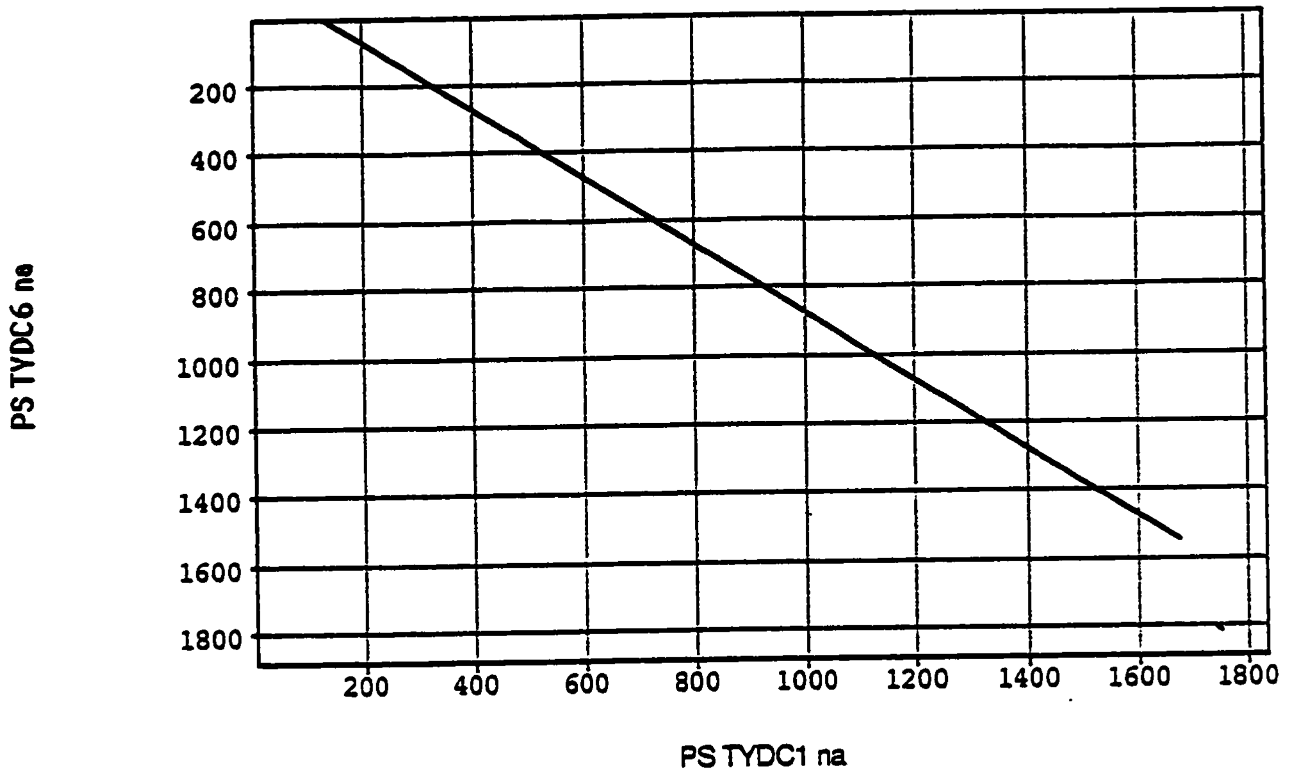


Figure 12: Pustell DNA matrix (MacVector 6.0) showing the nucleotide homology through the coding region for the clones *TYDC1* and *TYDC6* (minimum 65% identity over 30 bp window).

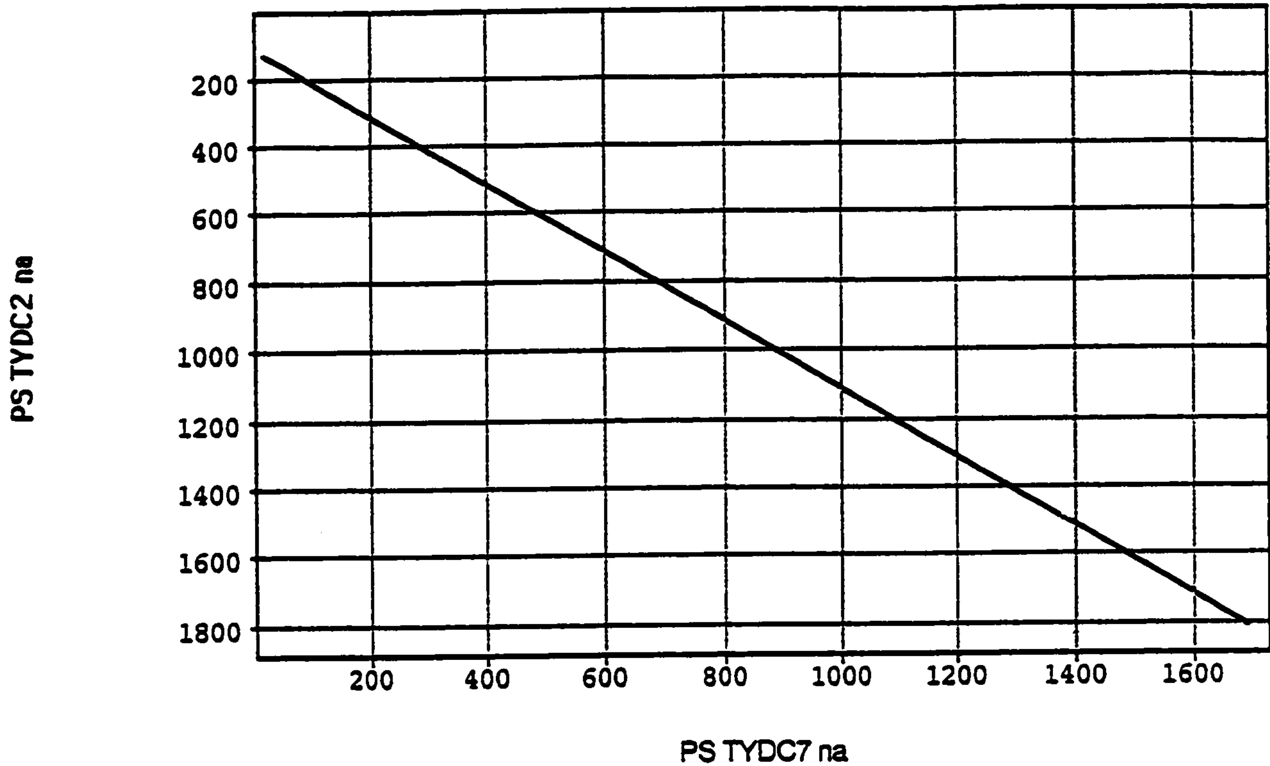


Figure 13: Pustell DNA matrix (MacVector 6.0) showing the nucleotide homology through the coding region for the clones *TYDC2* and *TYDC7* (minimum 65% identity over 30 bp window).

```
TYDC1  MGSLPA-NNFE-SMSLCSQNPLDPDEFRRQGHMIIDFLADYYKNVEKYPV   48
TYDC6  ::::::-::::-:::::::::::::::::::::::::::::::::::::::   48
TYDC5  :::::T-D:L:-:::I:::::::::::::::::::::::::::::-:VSS   47
TYDC4  :::::T-G:L:-:::IS::::::::::::::::::::::::::::S:::   48
TYDC2  ::::NTEDVL:N:SAFGVT:::::E::::::::::::::::::RD:::::   50
TYDC7  ::::NTEDVL:N:SAFGVT:::::E::::::::::::::::::RD:::::   50

TYDC1  RTQVDPGYLKKRLPESAPYNPESIETILEDVTNDIIPGLTHWQSPNYFAY   98
TYDC6  :S::E:::::::::::::::::::::::::::::::::::::::::::::   98
TYDC5  :S:AN::-SQQT:::T::NHS:::::::Q::Q::::::I:::::::::::   96
TYDC4  :S::E::-:R::::::::N:S:::::::Q::::::::::::::N::::::   98
TYDC2  :S::E::::R:::::T:::::::::::::Q:::TE:::::::::::::Y::  100
TYDC7  :S::E::::R:::::T:::::::::::::Q:::TE:::::::::::::Y::  100

TYDC1  FPSSGSIAGFLGEMLSTGFNVVGFNWMSSPAATELESIVMNWLGQMLTLP  148
TYDC6  ::::::::::::::::::::::::::::::::::::::::::::::::::  148
TYDC5  ::::::V:::::::::S:::::::::::::::::::::::::::::N::  146
TYDC4  :::::::T::::::::::::::::::::::::::::::::::::::M::  148
TYDC2  ::::::V:::::::::::::::::::::::::::::::V::D:F:K::N::  150
TYDC7  ::::::V:::::::::::::::::::::::::::::::::::D:F:K::N::  150

TYDC1  KSFLFSSD---GSSGGGGVLQGTTCEAILCTLTAARDKMLNKIGRENINK  195
TYDC6  ::::::::---:::::::::::::::::::::::::::::::::::::::  195
TYDC5  :::::::::DNA::::::-:::::::::::::::::S:::::::::::::  195
TYDC4  ::::::::---::::::::::::::::::::::::::::Y:::T::::::::  195
TYDC2  E:::::------::--::::::::S::::::::::::::::::::::HIGR  193
TYDC7  E:::::------:T--:::::::::S::::::::::::::RK:::::::HIGR  193
TYDC3                                     ::::::RK:::::::HIGR   19

TYDC1  LVVYASDQTLSALQKAAQIAGINPKNFLAIATSKATNFGLSPNSLQSTIL  245
TYDC6  ::::::::::HC:::::::::::::::::R::::::::H:::::::::::::  245
TYDC5  ::::::::::HC:::::::::::::::::R::::::::D:::::QA:L::::  245
TYDC4  ::::::::::HC:::::::::::::::::R::::::::::::::::H::::  245
TYDC2  ::::G::::HC:::::::V::::::::R::K:F:ENS::::AAT:REV::  243
TYDC7  ::::G::::HC:::::::V::::::::YR:VK:F::NS:::AAAT:KEV::  243
TYDC3  ::::G::::HC:::::::V::::::::::R:VK:F::NS:::AAST:REV::   69

TYDC1  ADIESGLVPLFLCATVGTTSSTAVDPIGPLCAVAKLHGIWVHIDAAYAGS  295
TYDC6  ::::::::::::::::::::::::::::::::::::::::::::::::::  295
TYDC5  ::::::::::::::::::::::::::::::::E:::QF:::::V:::::::  295
TYDC4  ::::::::::::::::::::::::::::::::E:::MY:::::V:::::::  295
TYDC2  E:::A::I:::V:P::::::::::::::::::I:E:::EYEM:::V:::::::  293
TYDC7  E:::A::I:::V:P::::::::::::::::::I:E:::EYEM:::V:::::::  293
TYDC3  E:::A::I:::V:P::::::::::::::::::I:E:::EYEM::::::::::::  119

TYDC1  ACICPEFRHFIDGVEDADSFSLNAHKWFFTTLDCCCLWVKDSDSLVKALS  345
TYDC6  ::::::::::::::::::::::::::::::::::::::::::::::::::  345
TYDC5  ::::::::::::::::E::::::::::::::::::::::::::NA::::::  345
TYDC4  ::::::::::::::::E::::::::::::::::::::::::::::::::::  345
TYDC2  ::::::::::::::::E:::::::::::::::::::::::::PSA::::::  343
TYDC7  ::::::::::::::::E::::::::::::::::::::::::::SA::::::  343
TYDC3  ::::::::::::::::E:::::::::::::::::::::::::PS:::::::  169

TYDC1  TSPEYLKNKATDSKQVIDYKDWQIALSRRFRSMKLWLVLRSYGIANLRTF  395
TYDC6  ::::::::::::::::::::::::::::::::::::::::::::::::::  395
TYDC5  ::::::::::::::::::::::::::::::::::::::::V::::S:  395
TYDC4  :N:::::::::E::::::::::::::::::::::::::::::::::::::  395
TYDC2  :N::::R::::E:R::V:::::::::::::::L:::::::::::VT:::N:  393
TYDC7  :N::::R::::E:R::V:::::::::::::::L:::M::::::::T:::N:  393
TYDC3  :N::::R::::E:R::V:::::::::::I::::::::::M:::::::VT:::N:  229

TYDC1  LRSHVKMAKHFQGLIGMDNRFEIVVPRTFAMVCFRLKPAAIFRKKIV-ED  444
TYDC6  ::::::::::::::::::::::::::::::::::::::::::::::::-::  444
TYDC5  :::::::::::D:::A::K:::::::N:::::::::::::::::NG:LG-:N  444
TYDC4  :::::::::::::::::::::::GN:F::::::::::::::T:::KQ::::-:N  444
TYDC2  :::::::::T:E:::C::G::::T::::::::::::::L:P--KTI:VY-DN  440
TYDC7  :::::::::T:E::::::G::::T::::::::::::::L:P--TTI:VY-DN  440
TYDC3  :::::R:::T:E::VGA:R:::::T::::::::::::::L:P--KTI:VY-DN  267

TYDC1  ----DHIEAQT-----NEVNA---KL----LESVNASGKIYMTHAVVGGV  478
TYDC6  ----:::::::-----:::::---::----::::::::::::::::::::  478
TYDC5  GVDYNC::EK:-----::I:S---::----:::::::S::::::::::::  482
TYDC4  ----EY::::::-----::T::---::----::::::::R::::::::::::  478
TYDC2  GVHQNGNGVVPL-RDE::NLVLAN::NQVY::T:::T:SV::::::::::  489
TYDC7  GVHQNGNGVVAV-HNE::TLLLAN::NQVY::T:::T:SV::::::::::  489
TYDC3  GVHQNGNGVIAVLRNE::ELVLAN::NQVY:RQ:K:T:SV::::::::::  317

TYDC1  YMIRFAVGATLTEERHVTGAWKVVQEHTDAILGALGEDVCI*  518
TYDC6  ::::::::::::::::::::::::::::::::::::DGKTTTIHEILD*  525
TYDC5  :::::::::::::::::::SM::::I::::::::::TVDDS:VA*  523
TYDC4  ::::::::::::::::H:::::::::::::::::T::S::DA-TT-APEIVG  525
TYDC2  :::::::::S::::::::::IY::::L:::A:L:::KFS:ADFSS*  531
TYDC7  :::::::::S::::::::::IH::::L:::A:L:::KFS:ADFSS*  531
TYDC3  :::::::::S::::::::::IH::::L:::A:L::SKFD:ANFSS*  350
```

Figure 14: Comparison of predicted amino acid sequences of all cloned *TYDC* genes. Adapted from Maldonado-Mendoza *et al.*, 1996 by P.J. Facchini (Facchini and DeLuca, 1994; Maldonado-Mendoza *et al.*, 1996, Facchini *et al.*, 1997 - unpublished).

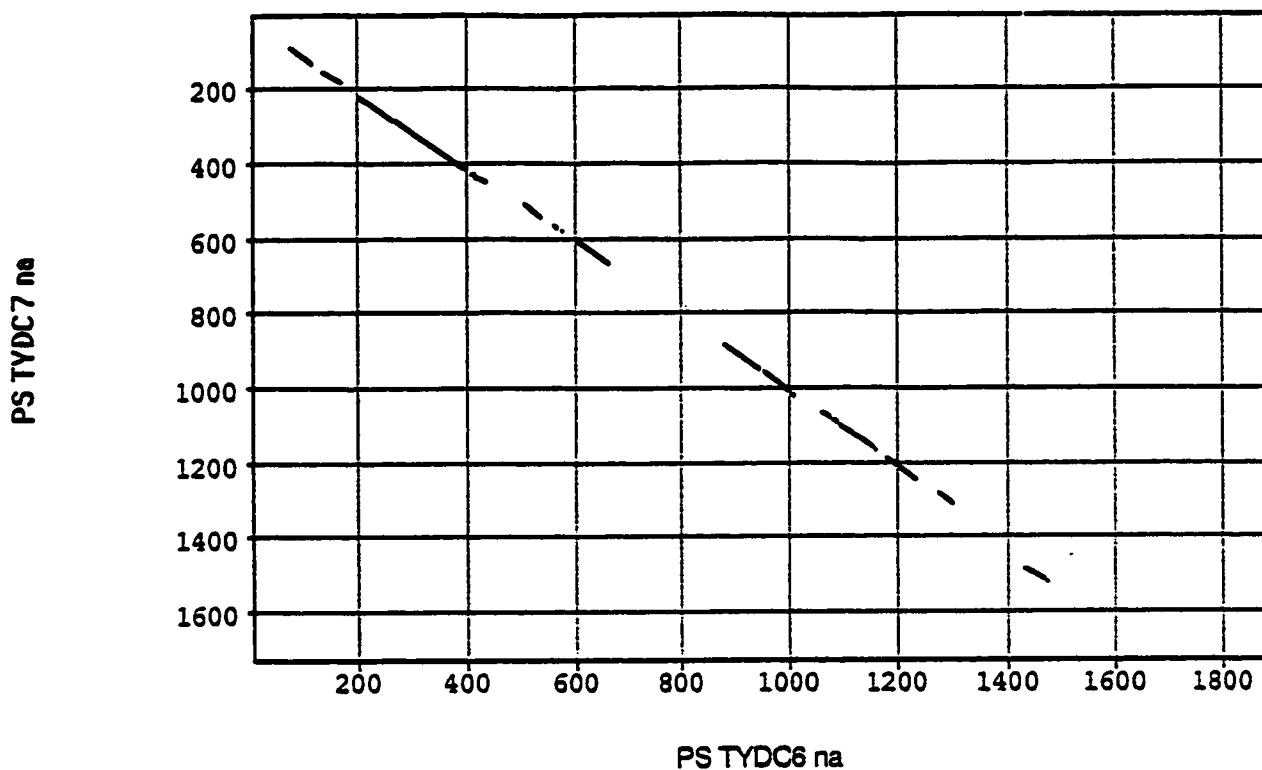


Figure 15: Pustell DNA matrix (MacVector 6.0) showing the nucleotide homology through the coding region for the clones *TYDC6* and *TYDC7* (minimum 65% identity over 30 bp window).

3.2 Creation of nested deletion series'

In order to determine the location of the promoter elements responsible for the inducible regulation of the *TYDC* genes and *BBE1*, a nested deletion series of each promoter was created. The promoters in the pUC-TYDC6::GUS, pTYDC7::GUS, and pUC-BBE1::GUS constructs were progressively shortened from the 5′ end by treatment with exonuclease III and mung bean nuclease. The size of the promoter in the resulting constructs was analyzed by PCR. *TYDC6* promoter deletions were amplified with the primers TYDC6-11 and reverse (Figure 16); *TYDC7* used TYDC7-13 and T7 (Figure 17); and *BBE1* promoter lengths were determined using primers 8-2D-8 and reverse (Figure 18).

3.3 Location of transcription start sites

Transcription start sites were mapped via primer extension with total RNA isolated from cell cultures which were collected two hours after being elicited. Antisense primers, called TYDC6-PE, TYDC7-PE, and BBE1-PE, specific for sequences approximately 50 bp downstream of the translation start site were designed and annealed to the RNA. Genomic clones of *TYDC6* (pTYDC6g), *TYDC7* (pTYDC7g), and *BBE1* (8-2D-5) were then sequenced with these primers. The primer extension products were run along side the sequencing reactions to determine the first base pair transcribed (Figure 19). There is a tendency for the first base of mRNA to be an A, flanked on either side by pyrimidines (Lewin, 1990). The transcription start sites of *BBE1* and *TYDC7* conform to this established initiator sequence, and are located 23 bp and 103 bp upstream of the translation start site, respectively. *TYDC6* transcription appears to begin with an unconventional T residue, and is 83 bp upstream of the first methionine codon. By designating the transcription start site as +1, individual base pairs in the promoter constructs can be assigned a number relative to this base pair. For example, the *TYDC6* promoter consists of base pairs -3000 to -1, oriented 5′ - 3′ respectively.

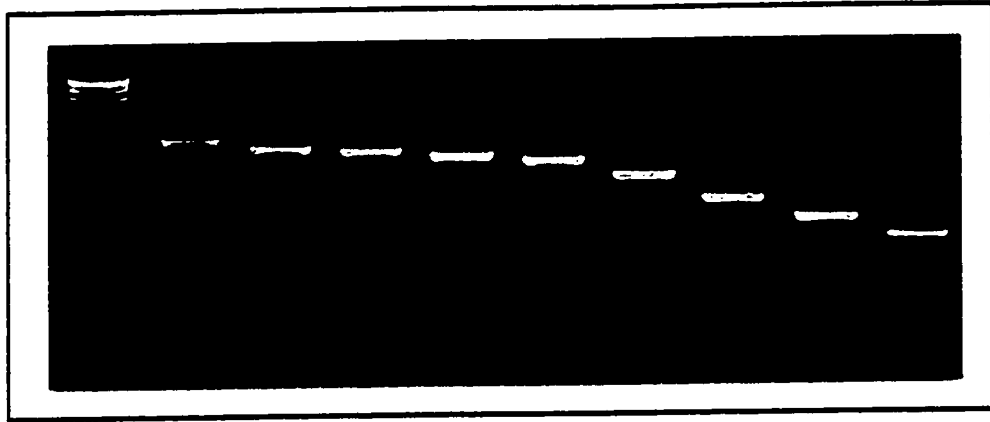


Figure 16: 1.0% agarose gel showing *TYDC6* promoter deletion lengths following exonuclease III and mung bean nuclease treatment.

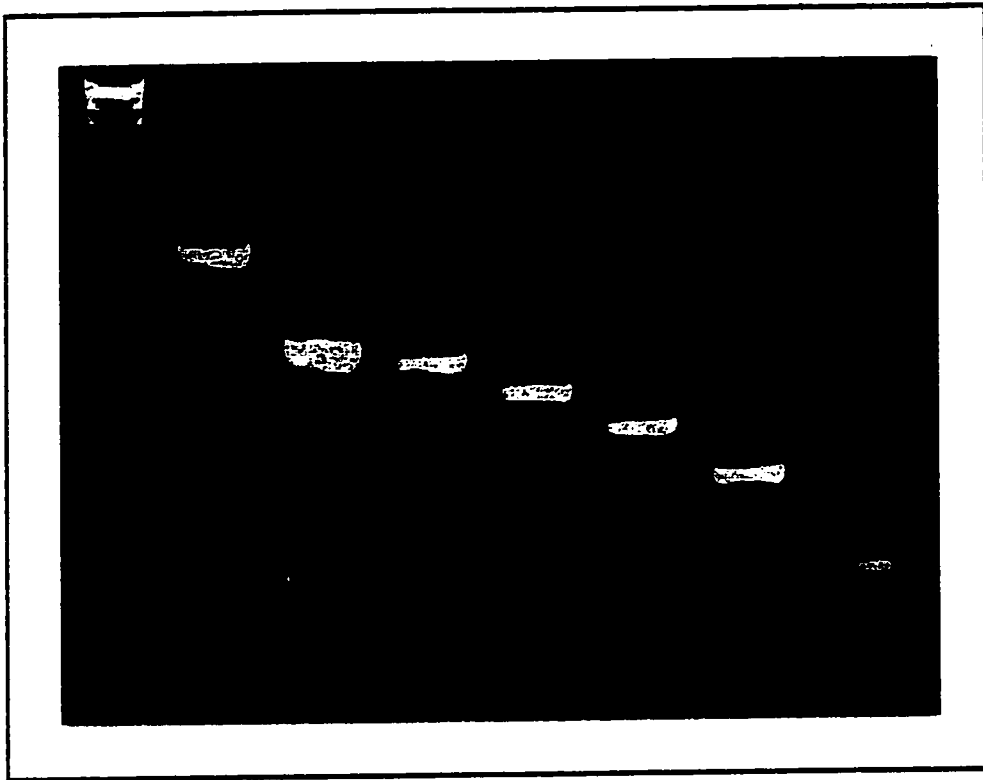


Figure 17: 1.0% agarose gel showing *TYDC7* promoter deletion lengths following exonuclease III and mung bean nuclease treatment.

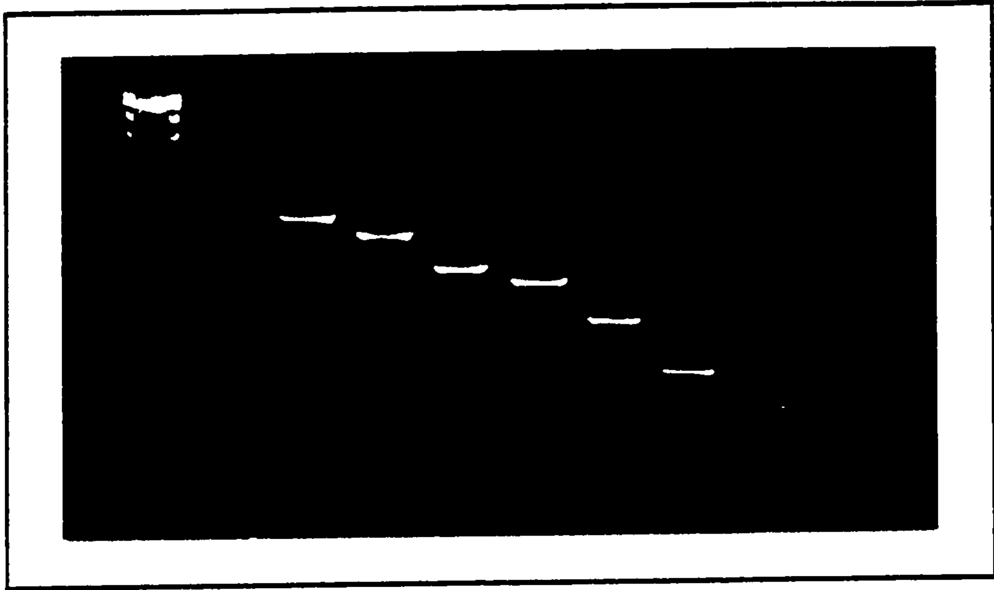


Figure 18: 1.0% agarose gel showing *BBE1* promoter deletion lengths following exonuclease III and mung bean nuclease treatment.

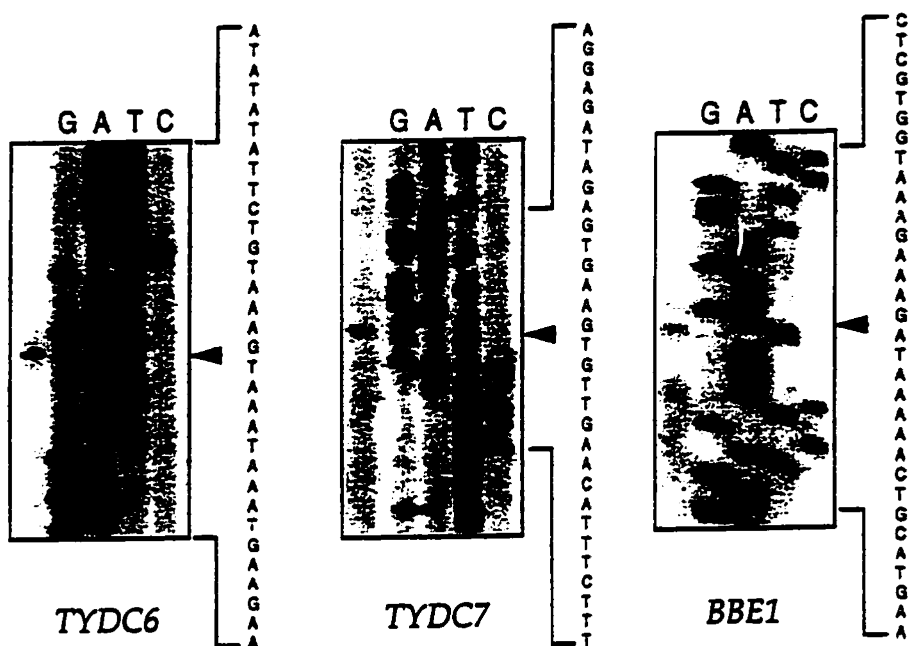


Figure 19: Primer extension products and genomic sequencing reactions electrophoresed on a 6.0% polyacrylamide gel to determine transcription initiation sites for the *TYDC6*, *TYDC7*, and *BBE1* promoters.

3.4 Sequence of promoters

Promoter deletion constructs were used as templates for the Sanger dideoxynucleotide chain termination sequencing method (Sanger, *et al.*, 1977). Primers used were a modified reverse primer for pUC-202 constructs, and T7 primer for the pBluescript constructs. When overlaps could not be achieved between one deletion and the next, specific primers were designed. The promoter sequences of *TYDC6*, *TYDC7*, and *BBE1* are presented (Figures 20, 21, 22) and the putative TATA and CAAT boxes are underlined. A near perfect 40 bp direct repeat almost 2000 bp upstream of the transcription start site in the *BBE1* promoter is also underlined, as is a smaller direct repeat (18 bp) at approximately -1200 bp. The *TYDC7* promoter also contains sequences which are directly repeated and are also underlined. The promoter for *TYDC6* doesn't contain any of these obvious repeated elements, but it does contain interesting sequence domains such as a string of 18 'A' residues between -873 and -856 and an open reading frame of almost 1.0 kb located between -1900 and -919. This ORF was compared to sequences available in Genbank, and did align with portions of 9 clones including a *Drosophila* G-3-P dehydrogenase and an unidentified *C. elegans* sequence, but no significant homology was found. As well, all three promoters were searched for putative regulatory sequences identified in other elicitor-, MeJA-, and/or wound-inducible promoters. In both the *TYDC6* and *TYDC7* promoters there are regions which correspond at 11 of 13 positions to the consensus sequence (TGAAGTTGAAATT) of a wound and elicitor responsive element of the potato *PR-10a* gene (Matton *et al.*, 1993). Also in these promoters, there are additional sequences which resemble the elicitor responsive element (AATTGACC) from the maize *PRms* promoter (Raventos *et al.*, 1995). All three promoters have many G-box (consensus CACGTG), C-box (consensus GACGTC), and A-box (consensus TACGTA) sequences which have been implicated in a number of promoter studies to be

ttgactggtgatggtaagttaacaaagttttaagacaatctcttggaaaa	-1986
gagtttacacactatatgaggaaggtggtcttggcatcaaaagattgaaa	-1936
actattaacaaataattattaataaagatgatgtggaaaatcttaacctc	-1886
agataaagaatgggcattatttatctctgcaaagtttaaagacaaaaatg	-1836
cgcaatggacctgtaattggaaacagacttcagtttggaagggtttaaaa	-1786
tgggcatggaataatttaaaagaagatatgagatgggatgctgggaatgg	-1736
ctcaaagatttctgtatggtttgacatttggttaggtgaatgtgctat***aa***	-1686
ttgaagagactgggttcacagactatgtgaaaagtcacattggaattaaa	-1636
gtacagtatttgatcctagaaggaaattggtgtgttcctactgaattaca	-1586
acagatcattccagtgaattctttgccagtaactttgggtggaactgatc	-1536
acatggcgtggactgctagtaatgatggaaatttctacactgatattgca	-1486
gttgagaagataaggcataaagaatcaatacttccatggcctaagtatat	-1436
atggcagaattttctgcatactagcatagccagtaacatctggaaaatac	-1386
aacaagaagtatatgttgatgatgaggtaatgaggaagaa***tgaatttgaa***	-1336
atggtttccatgtgttgcatatgtttggcagctcaagacaccatgaatca	-1286
cactctgtgggaatgtgctttcagtaatgcagtttgggactggttgaaca	-1236
gggtcttttgttttgcaaatccaaaatcatttgatgaagtttgtacatta	-1186
gcaaagaataagagtcttcttgtcaggcaagtttggatgattgcagcttg	-1136
tgcaaccatgacagaattgtggtttcagaaaaatgcaaaaaaaattgatg	-1086
agaagaaacccaatctgaatggatttaaatgcagaattatacagctggtt	-1036
catgaaggtggttatagattgaatggggttagttggcagcaaccttatga	-986
atctcaggtgagaaattttcaaagtttaagaaggatttgtgtctttcttt	-936
ggaactggttgtaatgatacaaaggcacagttttcttaggtattgggctt	-886
aggatcattttgggccatctacattattgtctttgcttttccttctttag	-836
tctcttgggtttttaagttcattttgggtcagtcctgtaaactgtttctt	-786
tagtttttcaatcaataaaaaatgtgattcagcaaaaaaaaaaaaaaaaa	-736
actcggacgccgacaccccatgtatatttttaatcagattgcagaaatca	-686
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gaaagaaatcaagtagcggacgctctggcaaatcaagcgatatatgattc	-586
gtctcaggggaaagctcacgacaacaatatgggacaatgtaactcccccca	-536
ttgatagttctattttaatagtagactctatggtatacatatccccgtgt	-486
tatttcagcttaatctcataaaatacatgcttcaaaaaaaaagtgatatc	-436
tagctcaaatagcactaaaatcataaatatgaacggaatcacatctcaaa	-386
tcggatataaaaataatgaaaacacaaacatccttacatctaatttaagt	-336
cgagtcggattcaaattatgagcccctaaacaggcatggtgtaagtcaat	-286
aagtgtacccttaaaaaaaagcgaacc*cacgt*ttgtaaactagtttcttc	-236
ttgtcactttcttgcatcagccactgagtgtattatcagtgcagcactat	-186
atggatcatataaaatgtgtttattaggtttaaagtgaactt<u>ccactt</u>ca	-136
gtcttcatatcaaggaaacacaaaatgcattccctttttgactaactac	-86
cttgaagccaccttg*aacgtg*tcttaattaaagctctttccatgtgcgtt	-36
cat<u>tatata</u>tctatatatatatataagacatttcatttatttacttcttc	+15
ttcactaaactcaataccaattctctctatctccctcactaatcagcaaa	+65
actgatagtaacaATGGGAAGTCTTCCAGCTAATAACTTTGAAAGCATGT	+115
M G S L P A N N F E S M	

Figure 20: Promoter sequence of *TYDC6* gene. Putative CAAT and TATA boxes are underlined, 5′-ACGT-3′ core sequences are italicized, and consensus sequences for MeJA-, elicitor-, and/or wound-responsiveness are bolded and italicized.

Sequence	Position
aagcttttatccacacccaactcatcattcaaaacattgaacttattagg	-1144
agaaaccacgatagaagtttcagcaacagatccaacaactgtatttgtgg	-1094
cagtggtaggtatagcagttgtaggtgtaggagttgtagtatctgaaagc	-1044
gagtcttaagtccttgtag*aacgtc*tttatctacaaggacagttgaggca	-994
ttgctttgcacacaaagctggctaaggaacgaatacaacttgatcttcta	-944
tctgattacataccagatga***tgatgttgaaattgaag***aaggtaacatatt	-894
acattattacttgtgtttggcagtaatagatatagtctaatggaaactaa	-844
ctgctattattatttttttgcagcgttacttggtcctatcaacaatgatg	-794
acaccgcaagtgctgctgacatggattagaagatcaatattcaaggctac	-744
tgcagcactgctagactgctagttgttcttttatcagattttctcatttt	-694
caagacttagtgtgtgtctgtgactactgctacttctttttttaagatttt	-644
caagacattgtttgtttggtttgctattaatattgctacttattagttgt	-594
tgctttgagatattgaaaaatagataaaaaaacaggtaaaaaaataaaaa	-544
acagggcagtagatttctgtttttttttcccccctgaaatggaaccggaacc	-494
ggtaccggtaccgaccgaaccgcccggtcccgaatggaaccggaatcgag	-444
gtactcggtaccggtgaccggtccatttttttggtaccgaaggtgtaagg	-394
tacaggtactcggtctcggcaagaaccgagccgaaccgtaccgtgtgcac	-344
ccttagtcaaactctatcattcaggattactgtaataactcttctaatct	-294
ggctagggatcctcttggagctatgattagcccagttgattctcaaatta	-244
ctgttgtaattttggagtaagttgctctttccagagcttctgttctcttt	-194
ttattattcaaaaaaaaaaaaaaaagacttaaattcagtagtgccagatg	-144
aatatacaataataattaaaaaaatgttt***cacgtggg***tggaactggaacc	-94
actaaatttcctcattatcaaaatttccatagcaaactctctccgg***tcat***	-44
caaactccccctatataaatcccccatttcctctatctcacttcacaactt	+7
gtaaagaaaagaaaaacaaaaccctttcgttcttgcttgcaaaccaatca	+57
ccttctttaacctgaaaaccacctctctttaattttctatctagaaATGG	+107
M	

Figure 21: Promoter sequence of *TYDC7* gene. Direct repeats, putative CAAT and TATA boxes are underlined. 5′-ACGT-3′ core sequences are italicized, and consensus sequences for MeJA-, elicitor-, and/or wound-responsiveness are bolded and italicized.

gaattcagaatgggttagtcctttcttttacgatccagtccaaattaaat -2578
ttttctagtgacaaatctaaccttcctatgaaaacctcgggtactcattc -2528
atttagctgctctcttcctcttctcaaacggcgaatcttcgatgaaagaa -2478
aaaccaaagatatcttcatttcatctttttctagtttgacgaaaatttca -2428
tcgattatcttcttcatctccatctccatctctcaaatcatagggtaagg -2378
gttcgaatttattcttactttatttcatctcttttttcatccccatctag -2328
atctgagctcaaaattagtttcaaatatgtctttagggtattcttttctt -2278
ttcattttgatatatcttgtacagattgtcgtaggttttgagtttgtta -2228
gttgttacttgtttgaaatactacaatggtcgtatttctagagaatctat -2178
ctctgttattagagattatttatggttttggtctgaactgtcgtccatat -2128
tcacaattttattaagacaataataaattttttgttcctttgctacatagt -2078
attggctttgaagaaagtagatcaggattttttgtatcaatttgggaaaga -2028
tactgacatttatttgtaaatttattgattgatgtgtttgactaggacaa -1978
aaatatttatttgtcaatttattgattgatgtgtttgattaggacaaaag -1928
cagagtttccaaaccaatagtttacttgggtactattgaatcacccttgg -1878
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tttttcttcccaaacattttccaactgctacaaaagatgataagattttc -1778
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aactttctagctcttacattggctattagataggtttttccgttaatctc -1678
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tgacctgcgactaaataatggtgtta*tacgtt*cattaattttgata*cacg* -1578
*tag*cgtcatgatgtgtccttatggagatatatcaaccagcttcaggtagg -1528
catatgaattgggtgaaacacataatgctttttctttccaccaactatac -1478
gtaaacatgtattttgttaataccaggcatgtgcataatgaaacattgcc -1428
acaaaagtatattatgaatgttgtcagtactgtttcagttgacttcccta -1378
tctttctttagtatagtttttttaatgctattacaatatatttgcatgaca -1328
tctagaaatcttcaatcagcagttggtagtctaaaactttttttctatatt -1278
gaatgcggacattagagaagcctaatggtcttacttgacaatatgacata -1228
gaaatgtgtttcagcagaatgagagaaagtagaaaatggtcttacatgtc -1178
aagtttggcaacttaaatatgaacattcattctgagtacgtgtactcatt -1128
atgtatctctctttcttcttcgtatatttttttattcatgttaactgtcg -1078
attttctttttcagtgtcttggcatcataggatgaccgcatggggctccc -1028
atggatattaactcactgccttcattcaagaagacaaatgaacgttccta -978
taaatgtggcttacagaagtcttgactcttgactgatgacaccaatgaca -928
atcccgagaagttatctaaatgatctcgactgcaatcaagagcaaatttt -878
gaataccacagtaaaattaagtagaagaaatagaaaaaatagagcccgag -828
aaccttactgatttgaacaatattgtggttctatctttccgatttgttat -778
ttcatagctttttttattatgttattcaacttgatggatcttttttctcat -728
atagtggaaagctaaattacacaggagcatgaccgactgcttaccagttt -678
gatgtccactcccaaaaaatctcgacttcgaacccaccacaaaatatcca -628
tccctaataacggtcgacaataaaggtttaacatcatgtcttccttaatc -578
cattgccacagtaaaatctttttttacaacatttattttctgaaagaaaac -528
atttacacagtgagcacttcaacaaatgacaccctagagagagtgccaaa -478
aggcataaaactagagatccgcggagggctgtggagtgcgaacaagacat -428
ctcgacacttcattatctgtcaaaatatgatgattagctaccacaagata -378
cccaatcaaggataaaaagagttaaataaatatcatattcgtttcatgaa -328
aagaaatattatcactattttattttaatttaaaaatatgtaaattggaa -278
aaatgatagccactttttctacggaaacagagggaacataatccgaatct -228
aagtgttagtttgtctatctccatctttgatgaccattgaaatgcaatgt -178
ccaatcctaacgaaactggaatggcccgtgacatgtagcacagctgcaca -128
gcgcatttgagaaagtcagacgcgtttactcc*cacgtg*catcgcgtttac -78
atctaataaaatgttagagttg*cacgtg*cctgcgggttattaaaaccagc -28
actatattttgagcaccatttctttctattttttgacgtacttctccaaca +23
ATGATGTGCAGAAGCTTAACATTACGTTTCTTCTTATTCATTGTTTTATT +123

Figure 22: BBE1 promoter sequence. Direct repeats, putative CAAT and TATA boxes are underlined. 5′-ACGT-3′ core sequences are italicized.

responsible for confering an inducible response to elicitor or MeJA treatments, or to wounding (Kim *et al.*, 1993; Kim *et al.*, 1994; Arias *et al.*, 1993; Mason *et al.*, 1993; Ceci *et al.*, 1995). Finally, an element found to be responsive to both MeJA treatment and wounding was identified in the *TYDC7* promoter at position -187 (Kim *et al.*, 1992; Kawaoka *et al.*, 1994). It should be noted that the promoter sequences were searched for many other reported regulatory motifs known to be involved in inducible regulation which are not present (Fukuda and Shinshi, 1994; Meier *et al.*, 1991; Tymowska-Lalanne *et al.*, 1996). Also indicated on these figures are the putative transcription initiation sites which appear as a single bolded base pair.

Sequencing the promoters did not just reveal interesting repeats and putative regulatory sequences. It also revealed the complete restriction enzyme maps of each promoter (Figure 23), and these were used to generate some additional deletion constructs. The exo III/mung bean nuclease treatment produced a relatively continuous deletion series for all three promoters, but there were some obvious gaps. In the *TYDC6* promoter, for example, the next shortest deletion after -447 was only 10 bp upstream of the transcription start site. Two additional deletions were needed; one which would produce a promoter construct ~250 bp in length, and one which could represent a minimal promoter, containing the putative TATA box but little sequence upstream of that.

For the *TYDC7* promoter, four additional deletion constructs needed to be made to create a relatively continuous series. The longest product of the exoIII/mung bean nuclease treatment was only 634 bp, so an additional construct was needed between the full length promoter at -1194 and this first deletion. The two shortest promoter constructs were -393 and -53, so

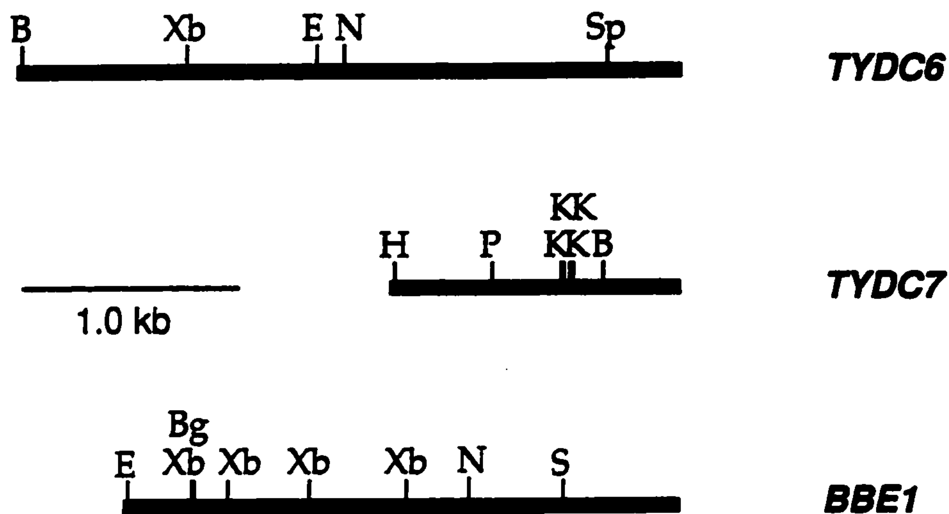


Figure 23: Restriction enzyme maps of the promoters from *TYDC6*, *TYDC7*, and *BBE1*. Only the unique sites are indicated. B - *Bam*HI, Bg - *Bgl*II, E - *Eco*RI, H - *Hind*III, K - *Kpn*I, N - *Nco*I, P - *Pst*I, S - *Sal*I, Sp - *Spe*I, Xb - *Xba*I.

additional constructs were made at -287 and -165. One more construct, which was lacking the putative TATA box, was created as a negative control.

The *BBE1* promoter deletion series only required the construction of one new plasmid in order to have a continuous truncation ranging from full length to a minimal promoter. This complete cloning stategy has been described in Chapter 2, Section 2.3.5.

3.5 Functional analysis of deletion constructs

One of the major topics molecular biology endeavors to understand is how gene expression is regulated. Using transcriptional fusions, in which a promoter is fused to the coding region of a reporter gene, one can determine the influence this promoter has on controlling transcription. Further, it is possible to identify the specific regions within the promoter which confer this regulation by creating constructs with progressively shorter promoter sequences.

In an effort to identify and localize the regulatory regions of the *TYDC6*, *TYDC7*, and *BBE1* promoters, a 5′ deletion series was created for each (Figure 24). These constructs were then transiently expressed in cultured opium poppy cells using particle bombardment, or biolistics. This approach has become widely used in the the past decade to deliver DNA fusion constructs directly into the nucleus of a living plant cell where the gene product is subsequently expressed. After incubating in the dark at room temperature for 48 hours, the 'shot' poppy cells were either histochemically stained (Figure 25), or homogenized and fluorometrically assayed for GUS activity. Each construct was shot in replicates of three along with an internal control (luciferase), and the entire experiment for each promoter was repeated twice with consistent results. Error bars are indicative of standard deviation between the three replicates of one experiment.

3.5.1 Functional analysis of *TYDC6* promoter

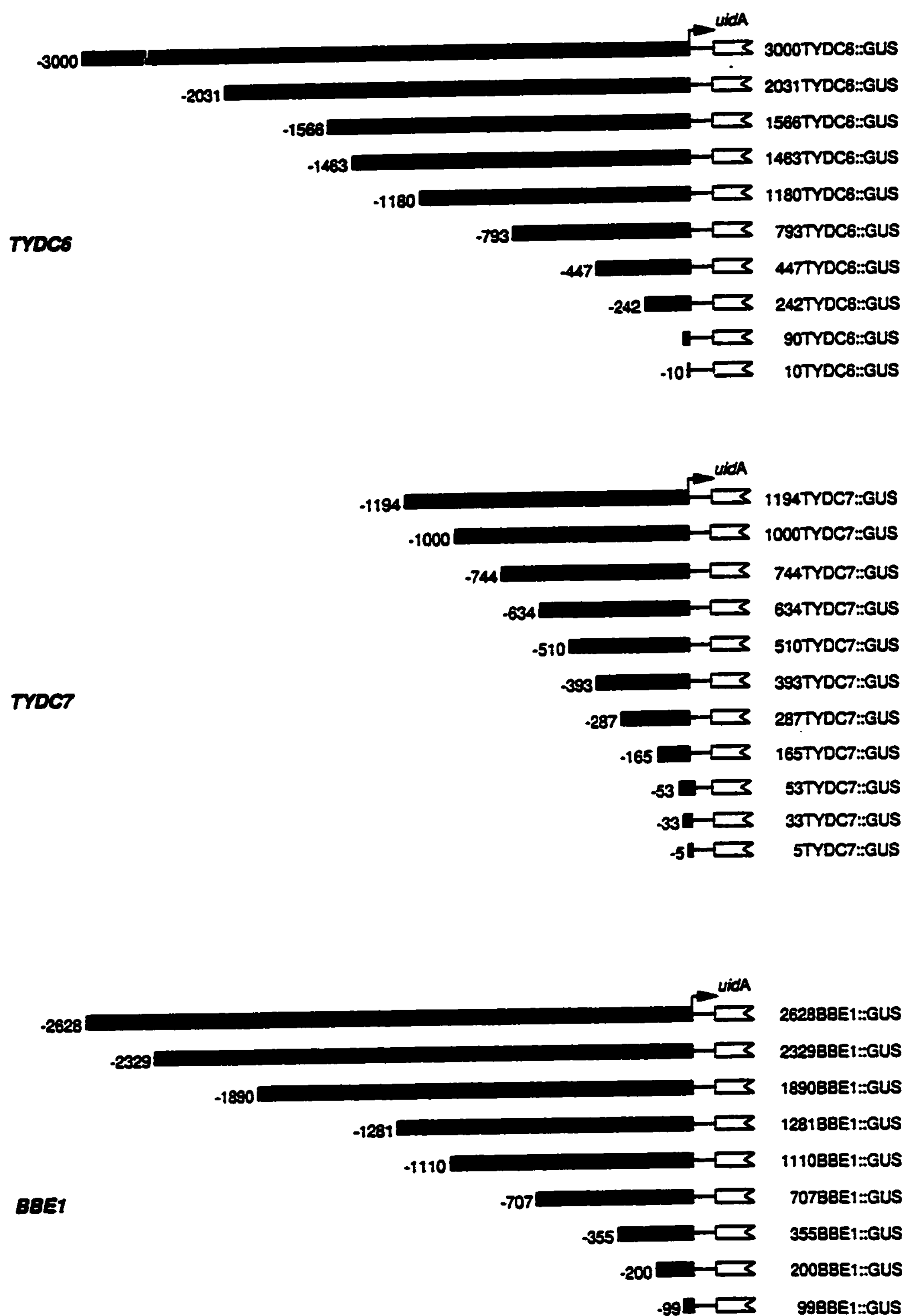


Figure 24: Deletion constructs used to functionally analyze promoters.

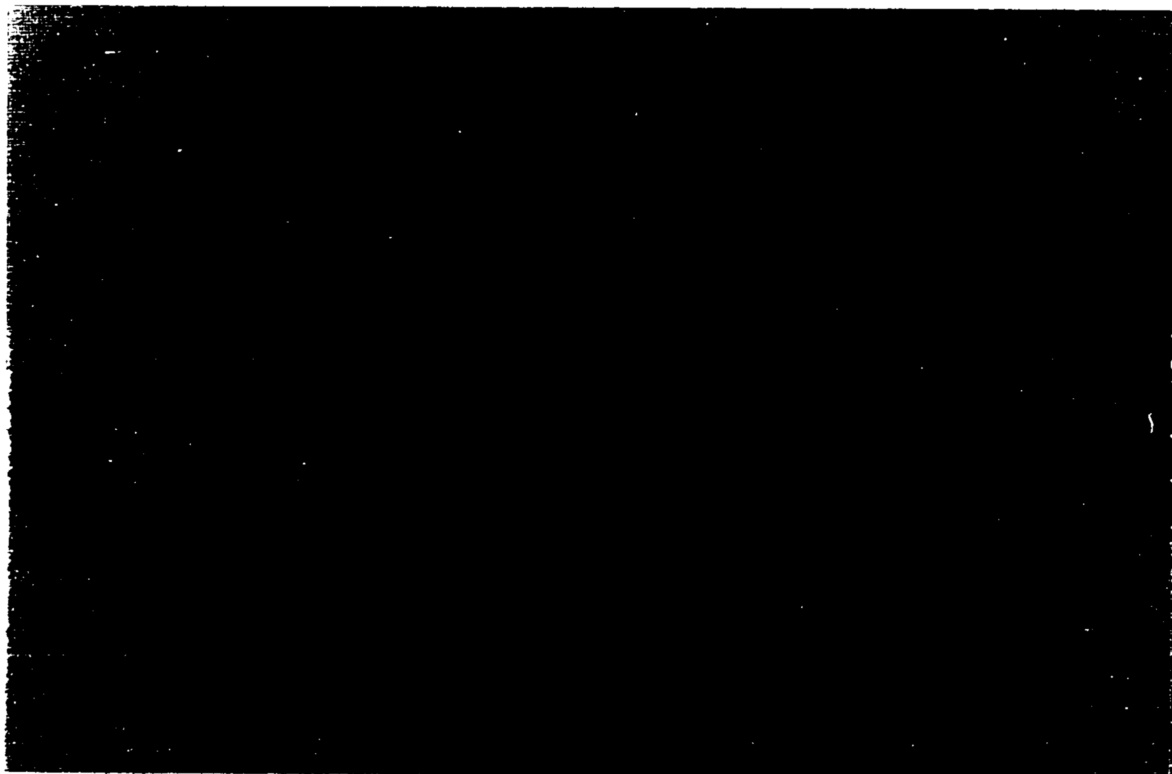
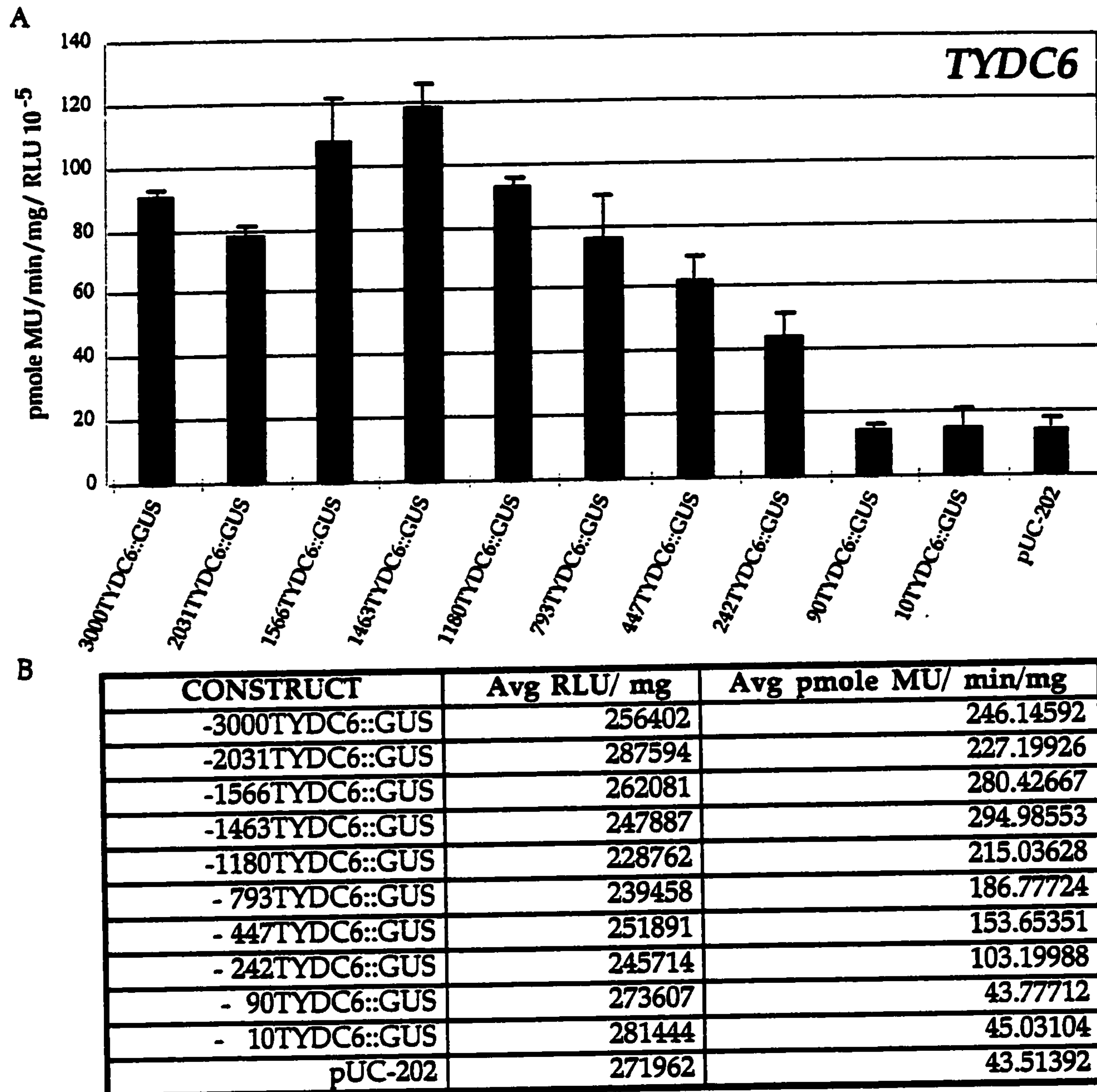


Figure 25: Histochemical staining of cultured opium poppy cells which are transiently expressing the GUS reporter gene under the control of the full length *BBE1* promoter.

When the full length construct pUC-TYDC6::GUS, with 3000 bp of promoter sequence controlling GUS expression, was transiently expressed in 2-4 day old opium poppy cell cultures, there was ≈ 4x as much GUS activity compared to the promoterless control (Figure 26). When the promoter was deleted to half of its original length so that it contained only 1463 bp, the GUS activity levels peaked, but they were not significantly different from the activity achieved with the full length construct upon subsequent repetitons of the entire experiment. When the promoter was deleted beyond -1463, there was a steady decline in the activity of the reporter gene. The most significant loss of activity was observed when the promoter was deleted from -242 to -90. When the promoter was 242 bp long, it still resulted in 2x more GUS activity relative to the control, but when the promoter was reduced to only 90 bp, the GUS activity level was indistinguishable from that of the control. So, this region was selected for further analysis since it seemed to contain an element necessary for transcriptional activation.

3.5.2 Functional analysis of *TYDC7* promoter

The full length *TYDC7* promoter construct contained nearly 1200 bp and the activity level was almost 20x that of the control construct which had no promoter (Figure 27). When the promoter was deleted from 744 to 634, the activity of the promoter always increased. Although the statistical significance of this increase is questionable, (it appeared to be significant in some experiments but not in others), this region was selected for further testing because sequence analysis revealed that it contained a direct repeat of ≈30 bp. The activity of the promoter continued to increase as additional sequence was deleted until it was only 393 bp. This deletion construct resulted in a GUS activity level 30x higher than that of the control. If the promoter was further deleted to 287 bp, there was a substantial

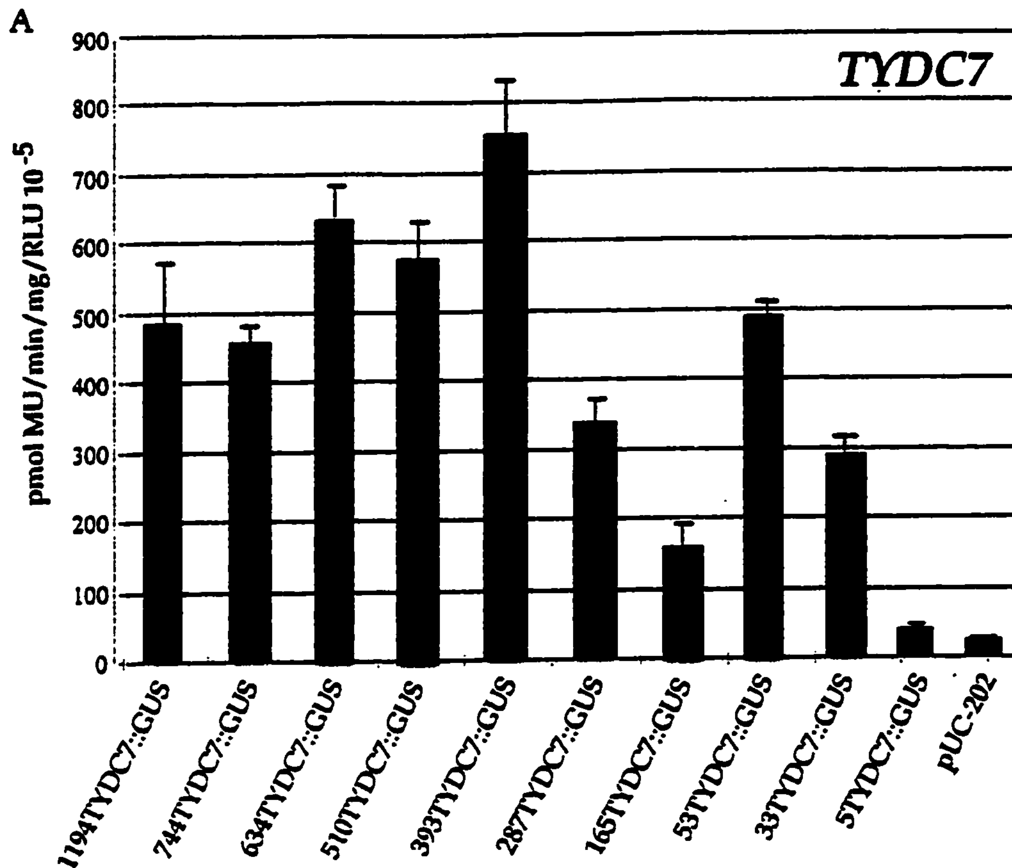


CONSTRUCT	Avg RLU/ mg	Avg pmole MU/ min/mg
-3000TYDC6::GUS	256402	246.14592
-2031TYDC6::GUS	287594	227.19926
-1566TYDC6::GUS	262081	280.42667
-1463TYDC6::GUS	247887	294.98553
-1180TYDC6::GUS	228762	215.03628
- 793TYDC6::GUS	239458	186.77724
- 447TYDC6::GUS	251891	153.65351
- 242TYDC6::GUS	245714	103.19988
- 90TYDC6::GUS	273607	43.77712
- 10TYDC6::GUS	281444	45.03104
pUC-202	271962	43.51392

Figure 26: A) Functional analysis of *TYDC6* promoter. Data shown represents one experiment in which each construct was assayed three times and normalized against an internal control. Error bars indicated standard deviation.

B) Average measurment of GUS activity generated by each promoter construct without being normalized against luciferase.

A



B

CONSTRUCT	Avg RLU/ mg	Avg pmole MU/ min/mg
-1194TYDC7::GUS	53688	264.14496
- 744TYDC7::GUS	53389	242.38606
- 634TYDC7::GUS	54974	343.58750
- 510TYDC7::GUS	52614	305.16120
- 393TYDC7::GUS	56881	426.61329
- 287TYDC7::GUS	59023	200.67820
- 165TYDC7::GUS	53712	85.93920
- 53TYDC7::GUS	56051	277.45245
- 33TYDC7::GUS	54267	160.63032
- 5TYDC7::GUS	53774	21.50960
pUC-202	54234	14.64318

Figure 27: A) Functional analysis of *TYDC7* promoter. Data shown represents one experiment in which each construct was assayed three times and normalized against an internal control. Error bars indicated standard deviation.

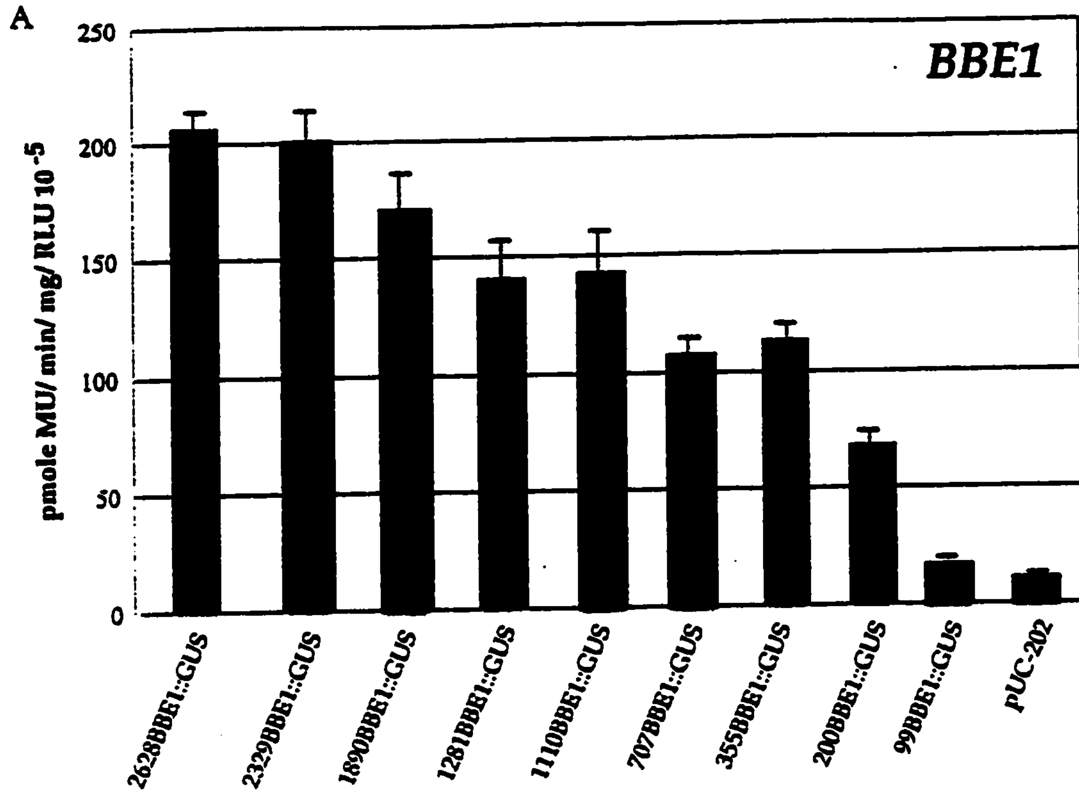
B) Average measurment of GUS activity generated by each promoter construct without being normalized against luciferase.

loss of activity. Despite being only 106 bp shorter than the 393 bp construct, the 287 bp promoter could only generate half as much GUS activity. This region was therefore believed to contain an element, or elements, necessary for optimal promoter function, and as such was selected for continued study. The activity of the promoter was again halved with the removal of the next 122 bp. The 165 bp promoter had an activity level ≈5x as high as the control. Perhaps the most interesting observation of all was made when the promoter was deleted to only 53 bp. The activity of this construct was comparable to that of the full length construct, which had almost 1200 bp of promoter sequence. When the sequences from 165 to 53 were removed, the activity of the reporter gene increased ≈ 3-fold. When a further deletion construct with only 33 bp of sequence before the putative transcription start site was analyzed to verify this finding, it was also found to drive GUS expression at 12x the background level. As a further control, a construct was designed with only 5 bp of DNA upstream of the transcription start site. The TATA sequence believed to be the RNA polymerase II binding site was removed in this construct, and, as expected, this promoter could not mediate GUS expression. The third and final region of the TYDC7 promoter selected for additional analysis then, was this region between -287 and -53, which seemed to contain two regulatory elements; one between -287 and -165 which seemed to be capable of activating inducible expression, and a second element between -165 and -53 which repressed transcription.

3.5.3 Functional analysis of the *BBE1* promoter

The full length *BBE1* construct contained 2628 bp of sequence upstream of the putative transcription start site. When this full length construct was tested for its ability to drive GUS expression, levels measured were ≈20x greater than those of the promoterless control (Figure 28). When the promoter was further deleted from -2329 to -1890, the activity

A



B

CONSTRUCT	Avg RLU/ mg	Avg pmole MU/ min/mg
-2628BBE1::GUS	296188	601.26164
-2329BBE1::GUS	287901	575.80200
-1890BBE1::GUS	276424	480.97776
-1281BBE1::GUS	243138	354.98148
-1110BBE1::GUS	229771	337.76337
- 707BBE1::GUS	251852	264.4446
- 355BBE1::GUS	261790	293.20480
- 200BBE1::GUS	257847	162.44046
- 99BBE1::GUS	271212	51.53028
pUC-202	280380	33.64560

Figure 28: A) Functional analysis of *BBE1* promoter. Data shown represents one experiment in which each construct was assayed three times and normalized against an internal control. Error bars indicated standard deviation.
B) Average measurment of GUS activity generated by each promoter construct without being normalized against luciferase.

level consistently fell. Although this decrease was deemed insignificant from a statistical perspective, we selected this region for continued study because the sequence between -2329 and -1890 contained an unusual, almost perfect 40 bp repeat. As the promoter deletions continued toward the 3′ end, the measured levels of GUS activity steadily decreased. The most substantial loss of activity was observed when the 355 bp promoter construct, which was ~11x as active as the negative control, was truncated to 99 bp. With only 99 bp of upstream sequence, the *BBE1* promoter was essentially incapable of activating transcription of the reporter gene. The sequences between -355 and -99 must therefore contain elements which are required for regulating inducible expression.

3.6 Wound responsiveness of *TYDC6, TYDC7,* and *BBE1*

When these experiments were originally performed, the cells were incubated in the presence of the fungal elicitor after being bombarded with the full-length promoter-GUS fusions for *TYDC6, TYDC7,* and *BBE1*. The unelicited negative controls, however, were expressing GUS at comparable levels to the elicited samples. The wound caused by the bombardment seemed to be sufficient to induce the promoters of the genes we were analyzing. Northern analysis of RNA extracted from wounded tissue determined that this was indeed the case (Figure 29). Cultured opium poppy cells were either mechanically wounded (squashed with a sterile spatula), or treated with a fungal elicitor, and RNA was extracted 10h - 24h later. RNA from these two samples were electrophoresed on formaldehyde agarose gels along side RNA extracted from untreated control cells. The gels were blotted onto nylon membrane, and then probed with the full length coding regions of *TYDC6, TYDC7,* or *BBE1*.

3.7 Creation of internal deletion constructs

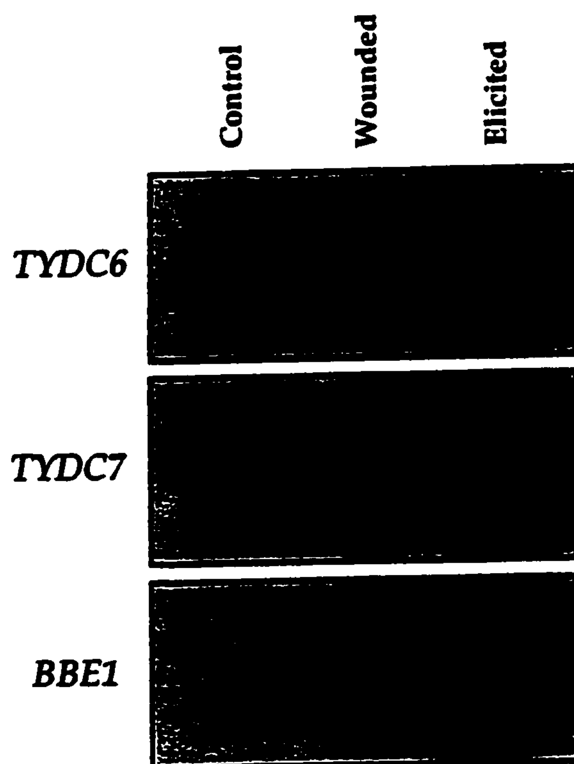


Figure 29: Northern blot which confirms the induction of *TYDC6*, *TYDC7*, and *BBE1* in cultured opium poppy cells in response to being wounded or elicited.

As described above, deletion analysis revealed regions in each promoter which seemed to be responsible for regulating inducible expression. By specifically removing these regions, it would be possible to determine whether the regulatory elements contained within were necessary and/or sufficient for the inducible expression of the corresponding genes (Figure 30). The Δ1TYDC6 construct contained the entire 3000 bp of the *TYDC6* promoter except for the sequence between -242 and -90.

The three *TYDC7* internal deletion constructs, designated Δ1, Δ2, and Δ3, were also complete *TYDC7* promoters which were missing the sequences between -744 and -634, -393 and -287, or -287 and -53 respectively.

The Δ1BBE1 construct had the sequence between -2068 and -1890 removed, while Δ2BBE1 was missing base pairs -366 to -108.

3.8 Analysis of internal deletions

These seven internal deletion constructs, which were created with the assistance of Dr. Peter Facchini, were then transiently expressed in opium poppy cell suspension cultures and the ability of the altered promoters' to drive expression was measured as a function of reporter gene activity. Original deletion constructs were also assayed to allow for the accurate comparison of the GUS measurements.

3.8.1 Analysis of Δ1TYDC6

When the sequence between -242 and -90 of the pUC-TYDC6::GUS construct was specifically removed, the promoter could no longer induce GUS expression over background levels (Figure 31).

3.8.2 Analysis of Δ1TYDC7, Δ2TYDC7, and Δ3TYDC7

Deleting the sequences between -744 and -634 of the *TYDC7* promoter was expected to remove an element which repressed the maximal promoter activity. When the GUS activity of Δ1TYDC7 was

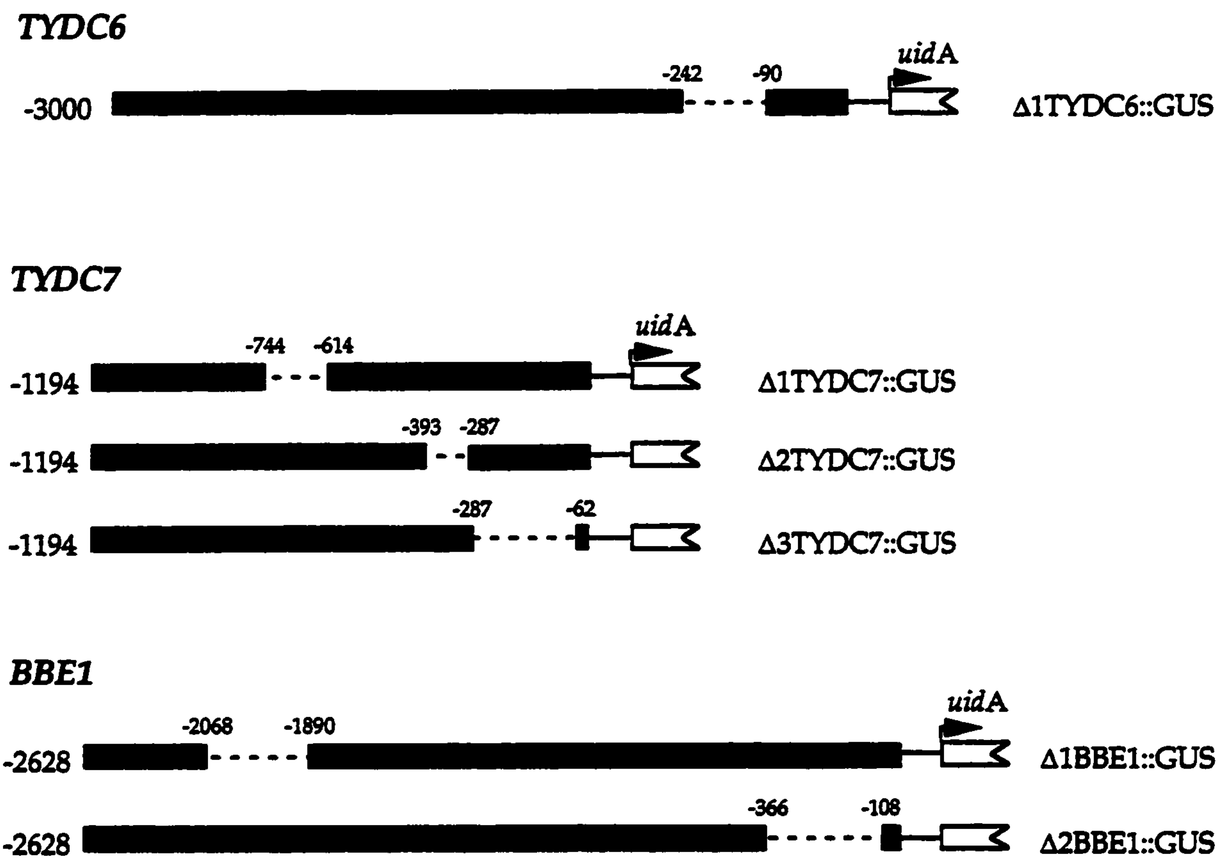
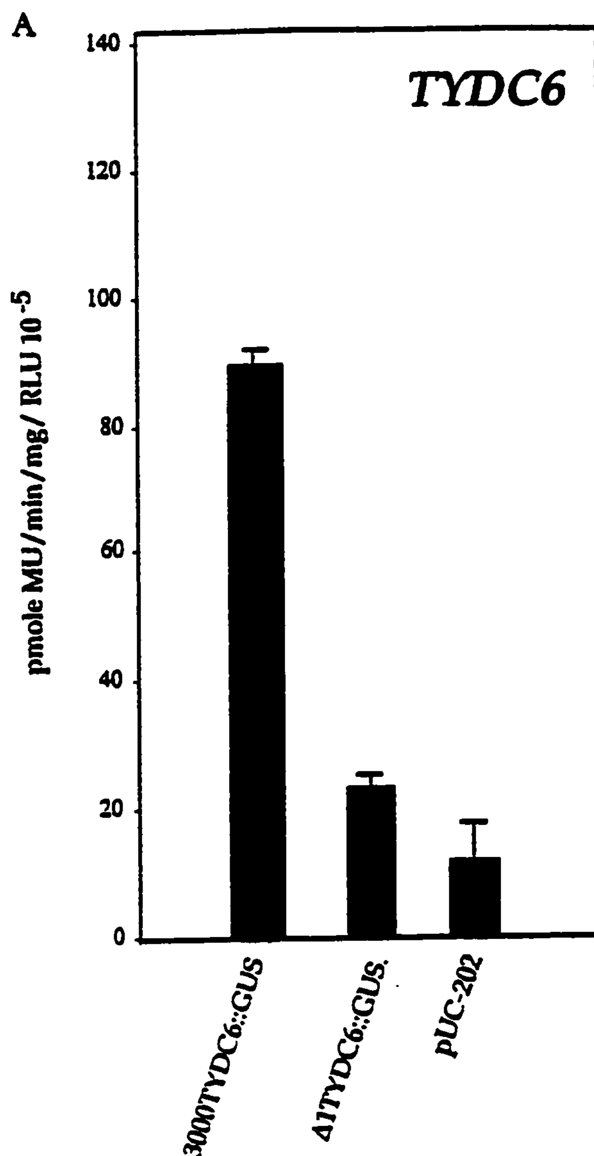


Figure 30: Internal deletion constructs created to test sufficiency of functionally identified regulatory regions

A



B

CONSTRUCT	Avg RLU/ mg	Avg pmole MU/ min/mg
-3000TYDC6::GUS	282215	270.92640
Δ1TYDC6::GUS	251186	52.74906
pUC-202	260849	28.69339

Figure 31: A) Functional analysis of *TYDC6* internal deletions. Error bars indicate standard deviation of three replicates normalized against and internal control.
B) Average measurment of GUS activity generated by each promoter construct without being normalized against luciferase.

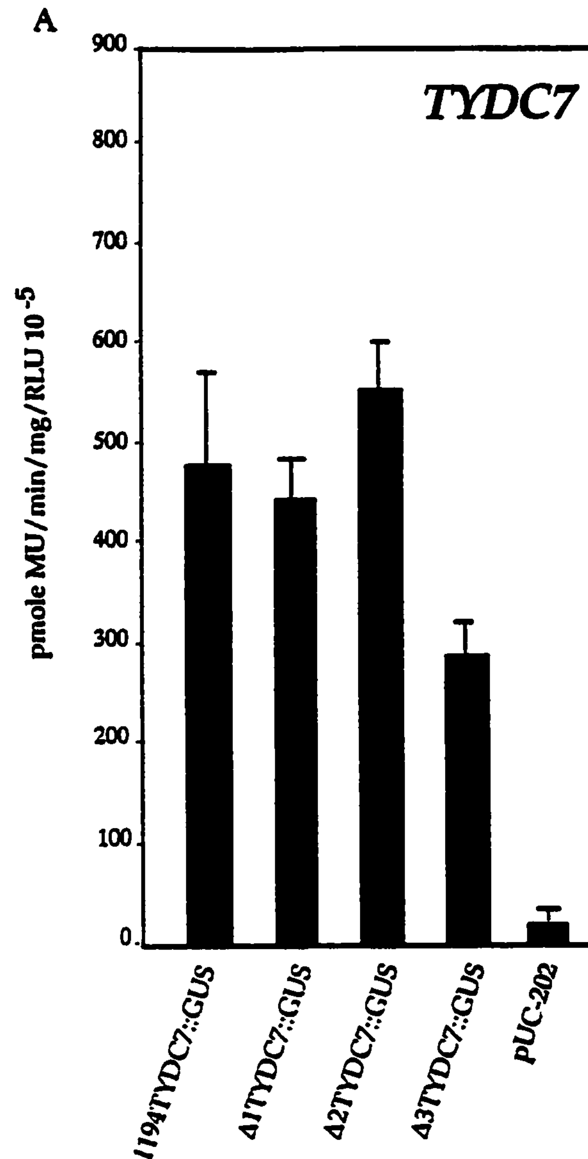
compared to that of the full length promoter construct however, there was no significant difference in the measurements (Figure 32). There was also no discernible difference between the GUS activity levels of the full length promoter and the Δ2TYDC7 construct, even though the sequence between -393 and -287, which was missing from the latter, seemed to be very important in the deletion analysis. The construct Δ3TYDC7 (missing -287 to -53), was expected to generate higher GUS levels than the full length promoter, since the missing region was thought to contain an element which repressed the activity of the promoter. However, as was the case with both the Δ1TYDC7 and the Δ2TYDC7 constructs, the expected outcome was not observed. In fact, removing the sequences between -287 and -53 actually decreased the activity of the promoter by ≈1.5x.

3.8.3 Analysis of Δ1BBE1 and Δ2BBE1

The Δ1BBE1 construct contained all of the promoter sequence except for a 178 bp region between -2068 and -1890 which contained an interesting 40 bp direct repeat. Compared to the activity of the full length promoter, this internal deletion construct, which was designed to attempt to determine the function of this repeated sequence, did not significantly alter the expression of the reporter gene (Figure 33). Δ2BBE1 was missing the region of the promoter which was earlier identified as being necessary for the inducible regulation of a downstream gene. Without the 258 bp between -366 and -108, the promoter could only generate 25% as much GUS activity as was observed with the construct containing the full length 5′ flanking region.

Table 4 summarizes the effect each internal deletion had on the ability of the promoters to mediate GUS expression.

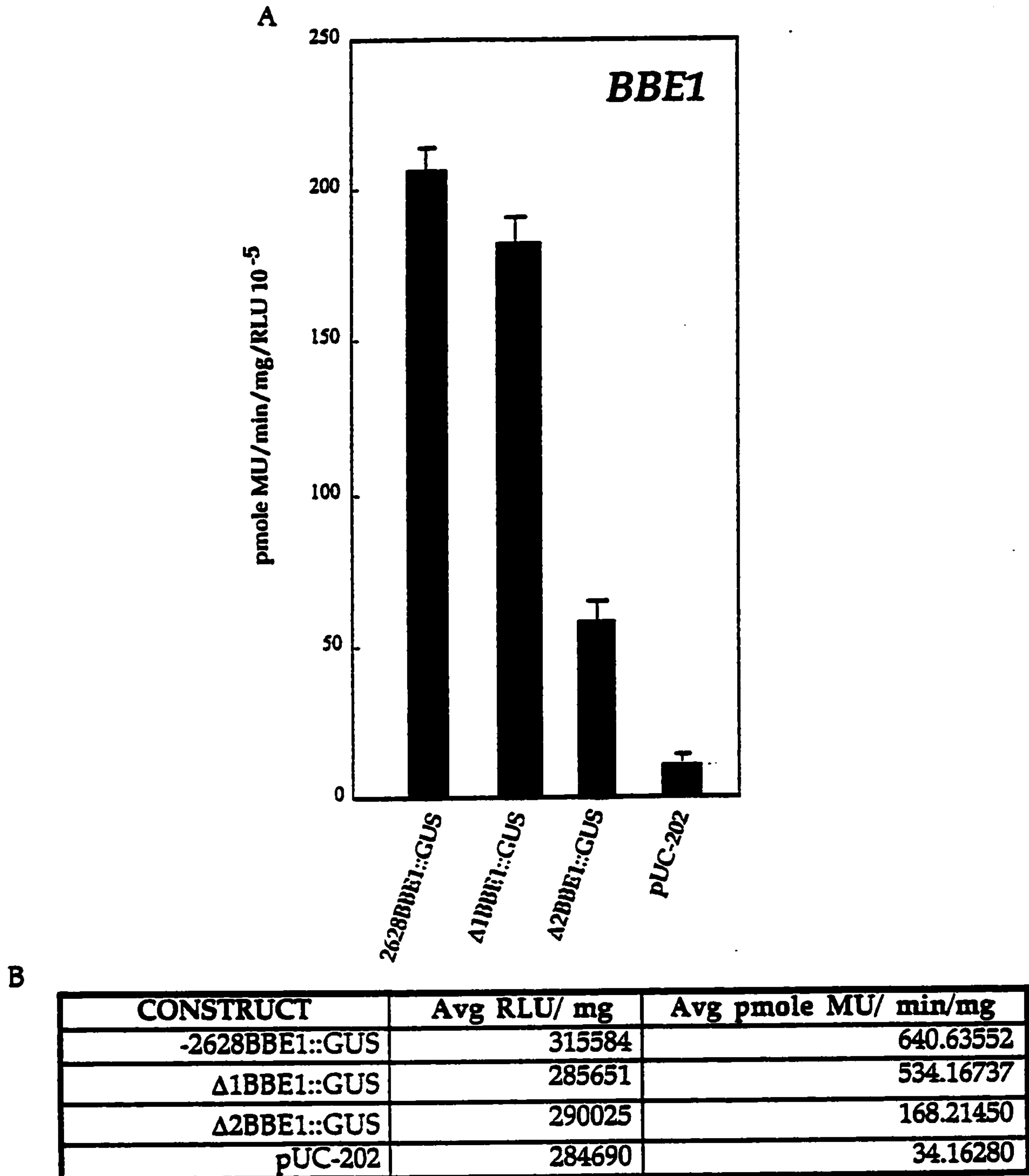
A



B

CONSTRUCT	Avg RLU/ mg	Avg pmole MU/ min/mg
-1194TYDC7::GUS	61295	301.57140
Δ1TYDC7::GUS	58743	257.29434
Δ2TYDC7::GUS	57756	324.58872
Δ3TYDC7::GUS	56233	159.70172
pUC-202	58016	6.96192

Figure 32: A) Functional analysis of *TYDC7* internal deletions. Error bars indicate standard deviation of three replicates normalized against and internal control.
B) Average measurment of GUS activity generated by each promoter construct without being normalized against luciferase.



CONSTRUCT	Avg RLU/ mg	Avg pmole MU/ min/mg
-2628BBE1::GUS	315584	640.63552
Δ1BBE1::GUS	285651	534.16737
Δ2BBE1::GUS	290025	168.21450
pUC-202	284690	34.16280

Figure 33: A) Functional analysis of *BBE1* internal deletions. Error bars indicate standard deviation of three replicates normalized against and internal control.
B) Average measurment of GUS activity generated by each promoter construct without being normalized against luciferase.

Table 4: Effects of deleting the regions which contain putative *cis*-acting elements from the promoters of *TYDC6*, *TYDC7*, and *BBE1*.

Name of Construct.	Location of deleted putative cis-acting element.	Expected effect of deletion on expression of GUS.	Observed effect of deletion on expression of GUS.
Δ1TYDC6	Between -242 and -90	Decrease	Decrease
Δ1TYDC7	Between -744 and -634	Increase	No effect
Δ2TYDC7	Between -393 and -287	Decrease	No effect
Δ3TYDC7	Between -287 and -53	Increase	Decrease
Δ1BBE1	Between -2068 and -1890	Decrease	No effect
Δ2BBE1	Between -355 and -99	Decrease	Decrease

4.0 Discussion

Secondary metabolites are considered to play an active and integral role in the defense of a plant against attack by pathogenic microbes and herbivorous animals. Plant cell cultures are often unable to produce significant amounts of these secondary products, but can be induced to substantially increase the synthesis of these compounds if presented with an elicitor (Kutchan *et al.*, 1991). Elicitors are substances capable of stimulating a defense response, presumably by interacting with a receptor on the plant cell membrane and then triggering an unknown signal cascade which results in the activation of defense related genes. The opium poppy suspension cell culture used to carry out this research is a model system for studying the molecular aspects of inducible regulation because it responds readily to the addition of a fungal elicitor (Eilert *et al.*, 1985). Sanguinarine, an orange coloured antimicrobial phytoalexin, can be detected in this cell culture 10 hours after being treated with a *Botrytis* preparation, and levels remain elevated even after 80 hours (Facchini *et al.*, 1996a). When genes encoding two of the enzymes of the sanguinarine biosynthetic pathway were cloned from opium poppy (Facchini and DeLuca, 1994; Facchini *et al*, 1996b) it became possible to study the induction kinetics of their respective mRNA.

Sanguinarine is the end product of a well characterized enzymatic pathway, involving 15 conversions (Figure 1), the first of which is catalyzed by tyrosine/dopa decarboxylase. Four of the estimated fourteen genes encoding this enzyme were cloned in 1994, and based on sequence homology, were divided into two, functionally identical isoform classes. Both of these isoforms, represented by the clones *TYDC1* and *TYDC2*, are transcriptionally activated when the cultured poppy cells are challenged with the elicitor, and are regulated in a differential-, and temporal specific manner (Facchini and DeLuca, 1994; Facchini *et al.*, 1996a). Northern analysis showed that *TYDC1*-

like transcript levels increase rapidly, peak approximately 2 hours after elicitation, and are not detectable at 50 hours. In contrast, *TYDC2*-like transcripts accumulate much more slowly, (peaking at 5 hours post-treatment), and then remain elevated, even after 80 hours have elapsed. In 1996, a second sanguinarine biosynthetic enzyme gene was cloned from opium poppy (Facchini *et al.*, 1996b). This clone, designated *BBE1*, encodes the berberine bridge enzyme which catalyzes the reaction committing the alkaloid intermediate (S)-reticuline to sanguinarine biosynthesis and away from morphine production. When the expression pattern for *BBE1* was characterized, it was found to be remarkably similar to that of the *TYDC2*-like genes. Upon addition of a fungal elicitor, *BBE1* transcript levels did not peak until approximately 10 hours had elapsed, and returned to base line levels slowly.

In addition to being induced by the *Botrytis* elicitor, *TYDC1*-like, *TYDC2*-like and *BBE1* mRNA levels could all be increased when methyl jasmonate (MeJA) was added to the cell cultures, and the induction patterns mimicked those observed with the elicitor (Facchini *et al.*, 1996a,b). It has been suggested that jasmonic acid (JA), or one of its precursors, could be involved in the signal transduction pathway regulating inducible defense genes, since addition of synthetic JA induced *de novo* defense protein synthesis in tomato (Farmer and Ryan, 1990). Recently, similar findings have been reported which have relevant implications to our research. All of the enzymes in the sanguinarine biosynthetic pathway were analyzed for induction in MeJA treated *E. californica* cell suspension cultures, and it was determined that none of the enzymes prior to BBE, and only four of the six enzymes following this step were significantly induced (Blechert *et al.*, 1995). As well, these authors report no significant difference in sanguinarine accumulation when *E. californica* cultures were treated with MeJA or a yeast cell wall elicitor. This is in contrast to what is observed in the opium poppy

system, where MeJA treatment results in an induction of the *TYDC* genes as well as *BBE1*. Additionally, MeJA treatment does not result in sanguinarine production (Facchini *et al.*, 1996 a,b), suggesting that the signal transduction pathways in the two systems must be uncoupled. This demonstrates that the regulation of the benzylisoquinoline alkaloid biosynthetic pathway is very complex. In an effort to begin to elucidate the defense response signal transduction pathway in opium poppy, we initiated research to identify and characterize *cis* elements necessary for the inducible transcription of the genes which have been cloned to date.

Screening an opium poppy genomic DNA library with the full length coding regions of *TYDC1*, *TYDC2* and *BBE1*, resulted in the isolation of the genomic clones for *TYDC6*, *TYDC7*, and *BBE1*, which were then subcloned into pBluescript and mapped to locate the gene. Based on the sequence homology of the open reading frames, it was determined that *TYDC6* was a representative member of the *TYDC1*-like gene family, and that *TYDC7* belonged to the *TYDC2*-like family. The genomic subclones for *TYDC6*, *TYDC7* and *BBE1* all included putative promoter sequences 5′ to the open reading frame which were 3.0 kb, 1.2 kb, and 2.6 kb respectively. Using available restriction sites or designing primers which incorporated restriction sites, the full length promoter regions were subcloned into GUS expression vectors (Figure 23). Putative transcription start sites were identified for the *TYDC6*, *TYDC7*, and *BBE1* clones using a primer extension protocol (Wu *et al.*, 1988) and were mapped 82 bp, 103 bp, and 22 bp upstream of the ATG start codon respectively (Figure 19). From the final 'A' in the putative TATA boxes to the transcription start sites there were 21 to 33 intervening base pairs. Additional bands were visible when the primer extension products were run on the sequencing gel indicating that there may be alternative transcription initiation sites. In the *PAL5* promoter of tomato it was recently discovered that transcription start sites changed in response to different conditions (Lee

et al., 1994). When presented with an environmental stress, transcripts were preferentially initiated from a site close to the translation start site. Under normal conditions, these shorter transcripts were present at low levels, while a longer transcript seemed to represent constitutive expression. The residues designated as the initiation sites for *TYDC6*, *TYDC7*, and *BBE1* were the bands with the strongest intensity.

The full length promoter::GUS constructs were progressively deleted from the 5′ end to generate a continuous series of promoters ranging from full length to less than one hundred base pairs. These deletion constructs were then used as templates to obtain the sequence for the full length promoters. A number of interesting sequence motifs were identified, including two direct repeats in the *BBE1* promoter, (an almost perfect 40 bp direct repeat located between -2020 and -1928 and a second 18 bp repeat in the region between -1254 and -1176), two direct repeats in the *TYDC7* promoter, (one which is located between -705 and -627, and the second between -508 and -432), and an open reading frame in the *TYDC6* promoter which extends from -1900 to -919. In addition, a number of consensus sequence domains previously reported to have regulatory functions were identified (Figures 20, 21, 22). These include putative elicitor responsive elements (ERE), wound-responsive elements (WRE), and MeJA-responsive elements (MJRE) which have been identified from a variety of plant gene promoters (Kawaoka *et al.*, 1994; Matton *et al.*, 1993; Raventos *et al.*, 1995; Logemann *et al.*, 1995; Kim *et al.*, 1992; Arias *et al.*, 1993).

To functionally determine whether or not these consensus *cis* elements were involved in the inducible regulation of the *TYDC6*, *TYDC7*, and *BBE1* promoters, the 5′ promoter deletion::GUS fusion constructs were transiently expressed in cultured poppy cells via particle bombardment. At this time it was discovered that these promoters are wound inducible. The penetration of the DNA coated gold particles into the cultured cells was

capable of inducing GUS expression without the addition of an elicitor. This finding was confirmed by Northern analysis (Figure 29). Although there appears to be a greater induction of the *TYDC6*, *TYDC7*, and *BBE1* genes in elicited cells compared to wounded cells, this may be due to an inability to mechanically wound as many individual cells as the elicitor can contact.

The ability of each promoter deletion construct to transiently express the GUS reporter gene was determined. This approach allowed for the functional identification of regions which appeared to positively or negatively regulate expression levels. In the *TYDC6* promoter, the greatest GUS activity was achieved with the deletion construct which had only 1463 bp of promoter sequence (Figure 26). Constructs with more than 1463 bp of 5′ flanking sequence may have been hindered in their ability to maximally drive GUS expression as a result of the open reading frame between -1900 and -919. Additional deletions, which further shortened the promoter, resulted in a steady corresponding decrease in GUS activity. The most significant loss of activity occurred when the promoter was deleted from -242 to -90, suggesting that this region contained a positive regulatory domain. An internal deletion construct, Δ1TYDC6, was designed to remove this region to determine whether the promoter was capable of activating GUS expression without it. When the GUS activity levels generated with this construct were compared to those of the full length promoter construct and the promoterless control, it was concluded that this region is absolutely necessary for a functional *TYDC6* promoter (Figure 31). The sequence between -242 and -90 does not contain any of the reported *cis*-elements mentioned earlier, but it does contain the putative CAAT box and removal of this may account for the loss of activity (Rieping and Schoffl, 1992).

The *TYDC7* promoter analysis revealed three regions with putative regulatory function. Although the full length promoter was capable of

directing high levels of reporter gene expression, the GUS activity doubled when the 5′ flanking sequence was deleted to only 634 bp (Figure 27). This implied that a sequence capable of repressing transcriptional activation was removed when the promoter was deleted from 744 bp to 634 bp. A positive regulatory domain was localized in this promoter between -393 and -287 since GUS activity levels decreased approximately 5x when this region was removed. Two additional regulatory elements exist in this promoter between -287 and -53. One of these is located in the region between -287 and -165 and exerts a positive influence on transcriptional activation. The second element must be a negative regulatory element, since deleting the 165 bp promoter to only 53 bp results in an increase in GUS activity. Although this is the only functionally important region in the *TYDC7* promoter which contains a previously reported *cis*-acting element (at -114), there are interesting sequence motifs in the others. For example, the region between -744 and -634 contains a 29 bp direct repeat, and there is a 16 bp string of A residues beginning at position -184. Perhaps these rare sequences represent previously unreported *cis*-acting elements. Additional analysis will have to be performed to confirm or refute this possibility. When these regions which seem to play a role in regulating the transcriptional activation of the *TYDC7* gene were removed from the full length promoter, the results were not as clear as they were with the *TYDC6* promoter. The Δ1TYDC7 construct, which was missing the sequences between -744 and -614, was expected to generate GUS levels which were significantly higher than those generated with the full length promoter since a negative regulatory domain was missing, but this was not observed (Figure 32). Deleting this region had no discernible effect on the function of the promoter compared to the full length promoter construct. Similarly, when the Δ2TYDC7 construct (missing -393 to -287) was analyzed, the results were different from those expected. The consequence of specifically removing

an activating element should have been a loss of activity, but again, the internal deletion construct had no significant effect on the level of GUS activity. The final TYDC7 internal deletion construct, Δ3TYDC7, was missing the sequences between -287 and -62, and should have resulted in an increase in GUS expression. When the GUS activity levels mediated by this altered promoter were compared to the controls, a decrease was observed. Since the original functional analysis suggested that there was even less activity with the 165 bp promoter than the 287 bp promoter, perhaps this internal deletion construct removed both a positive and a negative regulating element. Additional experiments need to be performed with constructs which are missing the sequences either upstream of -165, or downstream of -165 to more accurately define the regulatory capablities of this region. Alternatively, the decrease in activity could be attributed to the loss of the CAAT box and/or the G-box sequence at position -114 (Kawaoka *et al.*, 1994; Kim *et al.*, 1992: Rieping and Schoffl, 1992).

The internal deletions for the *TYDC7* promoter revealed that the specific regions which were identified by functional analysis were necessary but not sufficient for controlling the inducible regulation of the downstream gene on their own. A similar situation was uncovered when the promoters of the *PAL* genes in parsley were analyzed. Researchers found three regions common to all parsley *PAL* genes that, if removed, resulted in a loss of elicitor responsiveness, but they also discovered that no one of these regions alone could confer elicitor responsiveness to a reporter gene in transient expression assays (Logemann *et al.*, 1995). This suggests that transcriptional activation of an inducible gene may depend on interactions between a number of separate regulatory elements. Removing any one of these regions may not be sufficient to significantly reduce the function of the promoter (as in the case of the internal deletions), but the removal of a combination of *cis*-

acting elements may substantially impair optimal activity (as observed in the progressive deletions). Alternatively, the failure to observe what was expected with the *TYDC7* internal deletions may be the result of experimental design. All of the internal deletion constructs were created in two steps. The promoter sequences on either side of the region to be removed were amplified by PCR and then joined by means of an engineered *PstI* site. This resulted in the insertion of foreign nucleotides which possibly interfered with the integrity of the promoter. As well, removing internal sequences alters the spatial organization of the promoter. In many instances, the length of the intervening sequence between regulatory elements is critical for maximal promoter function (Gilmartin and Chua, 1990; Block *et al.*, 1990; Olive *et al.*, 1990).

The functional analysis of the *BBE1* promoter revealed that although the full length construct directed the highest levels of GUS activity, the strongest activating element was present in the sequence between -355 and -99 (Figure 28). As well, the region containing the near perfect 40 bp repeat seemed to confer some kind of positive influence, although removing this seq'ence did not result in activity levels which were obviously different from those obtained with constructs containing it. When the region containing this interesting and unusual repeated sequence was specifically removed and the resulting construct, Δ1BBE1, was transiently expressed, it was clear that this region was not necessary for regulating the inducible expression of the *BBE1* gene (Figure 33). This does not imply that this repeat is without function. Perhaps it plays a role in mediating the transcription levels in a developmental or tissue-specific manner. In contrast to the Δ1BBE1 construct, the Δ2BBE1 did significantly alter the expression of the reporter gene compared to the construct with the full length promoter (Figure 33). Without the sequence between -366 and -108, the promoter is only able to

generate approximately one-fourth of the activity of the full length construct, so within this relatively short sequence elements must exist which are required for the inducible regulation of the *BBE1* gene. As suggested with the *TYDC6* promoter, this critical sequence may again be the CAAT box since it is located at -179. It is interesting to note that this region between -366 and -108 is extremely rich in A and T residues. In fact, almost 68% of the 258 bp which are missing from the promoter in the Δ2BBE1 construct are either A or T, and out of the first 94 bp only 14 are a G or a C. Previously, an A/T rich area in the wound-inducible *At-beta-fruct1* gene in *Arabidopsis* was found to positively regulate transcription levels (Tymowska-Lalanne *et al.*, 1996). Additional upstream elements which influence transcription levels must also exist in the *BBE1* promoter, since the Δ2BBE1 construct still generated GUS activity levels which were ≈3x higher than that of the 99 bp minimal promoter.

The analysis of promoter-reporter gene fusions has been widely used in recent years to identify and characterize sequences responsible for mediating transcriptional regulation. With the advent of microprojectile bombardment technology, this approach has become even more accessible. Using biolistics, it is possible to determine the functional ability of a promoter in a matter of days, and the promoter can be transiently expressed in a homologous environment, thereby ensuring the existence of critical cellular factors. Additionally, since this technique does not integrate the promoter-reporter gene fusion into the host's genome, one does not need to be concerned with the complications of multiple gene insertions or position effects. Transient expression studies do have limitations, however. Removing an isolated fragment of DNA from its native surroundings, and then analyzing its function introduces many unnatural conditions. *In vivo*, DNA exists as a molecule with an intricate secondary structure complexed with proteins and

the cellular matrix. Many regions of the promoter which would normally be inaccessible to transcription factors, would be free to associate with these factors in a transient system (Frisch et al., 1995). As well, intragenic sequences in the coding region and the 3' untranslated region have been shown to play an important role in regulating gene expression, so their absence could result in artifactual expression patterns (Sieburth and Meyerowitz, 1997).

By functionally analyzing the promoters of *TYDC6*, *TYDC7*, and *BBE1*, we have identified regions in each which contain some of the *cis*-acting elements responsible for regulating the inducible expression of these genes. This study has also allowed us to conclude that there are no apparent homologies in either the sequence or location of regulatory regions within these promoters. This suggests that these genes do not bind common *trans*-acting factors and therefore, that the regulation of these genes is not coordinated at the level of DNA binding proteins. The similar induction patterns of *BBE1* and *TYDC7* in response to wounding or to elicitor treatment may be the result of events which occur earlier in the signal transduction pathway.

5.0 References

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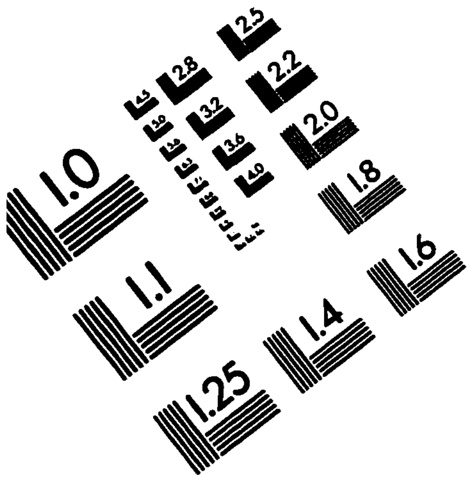

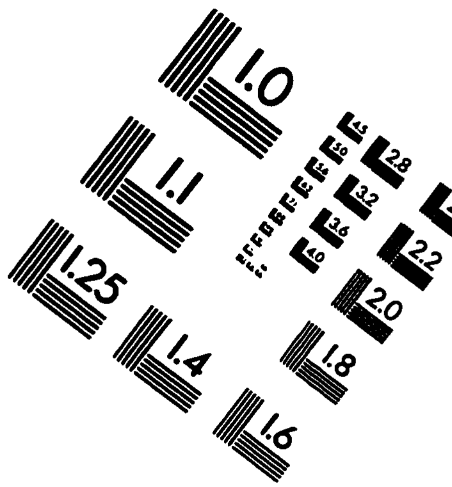

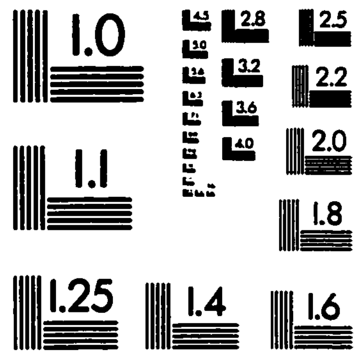

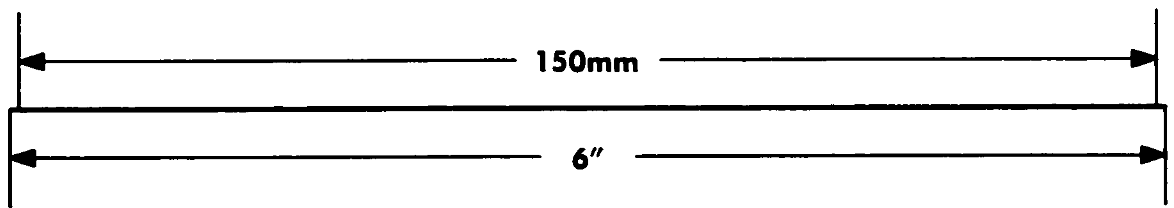

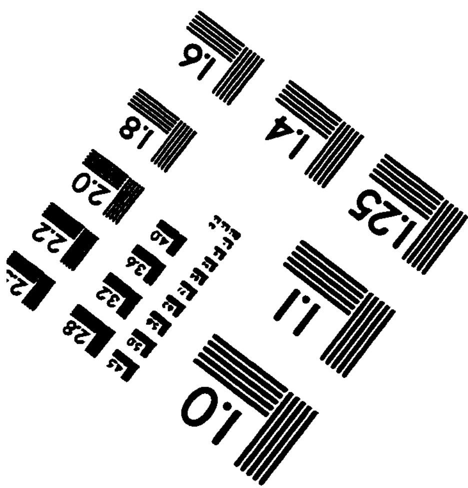

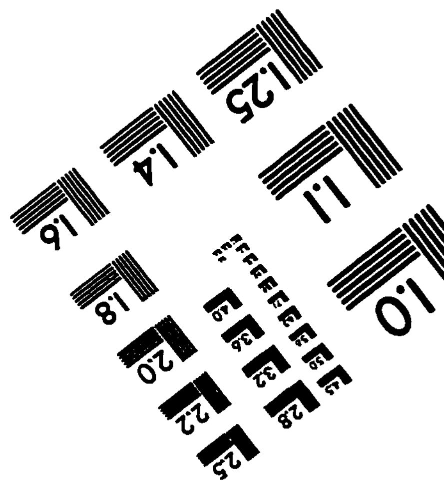

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