EXPRESSION FROM CHIMERIC PROMOTER CONSTRUCTS
DERIVED FROM THE CAULIFLOWER MOSAIC VIRUS 35S GENE
AND THE T-DNA GENE 7

by

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The β-glucuronidase (GUS) reporter gene system was used to investigate transcriptional activity of several promoter constructs in transgenic tobacco plants. The constructs contained the T-DNA gene 7 promoter alone (G7), or combined with one or two copies of the upstream region of the cauliflower mosaic virus (CaMV) 35S promoter (1CAG7 and 2CAG7). The unrearranged 35S promoter (CA1) and its derivative, created by duplication of the upstream region (CA2), were included. Transcriptional fusions of the promoter constructs with the GUS-coding region were introduced into the plant genome by Agrobacterium-mediated leaf disc transformation using a binary vector system. Fluorometric assay revealed very low levels of expression from the gene 7 promoter. However, addition of a single copy of the 35S enhancer resulted in several hundredfold stimulation of GUS expression in all tissues and further increase was observed in roots (but not in stems and leaves) when the enhancer was duplicated. In histochemical assay, GUS expression from gene 7 promoter stayed below the threshold of detection in most transformants. Generally, constructs 1CAG7, 2CAG7, CA1 and CA2 were active in most cell types of all plant organs analyzed, although constructs bearing the duplicated 35S enhancer showed a preference for phloem tissue of the stem and meristematic regions of the stem apex. A comparison of two transcriptional fusions differing in the sequence flanking the GUS initiation codon, suggested that the improved "Kozak" translational initiation context stimulated the GUS expression in transgenic tobacco plants up to 30-fold above the levels obtained with the original GUS gene.
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<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>$A_{600}$</td>
<td>absorbance at 600 nm (or other wavelength)</td>
</tr>
<tr>
<td>as</td>
<td>activation sequence</td>
</tr>
<tr>
<td>ASF</td>
<td>activation sequence factor</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytidine</td>
</tr>
<tr>
<td>CAB</td>
<td>chlorophyll $a/b$ binding protein</td>
</tr>
<tr>
<td>CaMV</td>
<td>cauliflower mosaic virus</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
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<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<tr>
<td>g (mg, $\mu$g)</td>
<td>gram (milligram, microgram)</td>
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G guanosine
GUS β-glucuronidase
HPLC high performance liquid chromatography
IPTG isopropyl-β-D-thiogalactopyranoside
kb kilobases
l (ml, µl) litre (millilitre, microlitre)
LB Luria-Bertani
M (mM, µM) molar (millimolar, micromolar)
min minute(s)
mRNA messenger RNA
MS Murashige-Skoog
4-MU 4-methylumbelliferone
MUG 4-methylumbelliferyl-β-D-glucuronide
N nucleotide or nucleoside
NOS nopaline synthase
NPT neomycin phosphotransferase
OCS octopine synthase
PEG polyethylene glycol
RF replication form
RNA ribonucleic acid
S Svedberg unit of sedimentation
SDS sodium dodecyl sulphate
<table>
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<tr>
<td>T</td>
<td>thymidine</td>
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<tr>
<td>t-DNA</td>
<td>transferred DNA</td>
</tr>
<tr>
<td>T-DNA</td>
<td>transferred segment of the wild-type Ti-plasmid</td>
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<tr>
<td>Ti</td>
<td>tumour inducing</td>
</tr>
<tr>
<td>TL-DNA</td>
<td>left portion of the T-DNA in octopine-type Ti-plasmids</td>
</tr>
<tr>
<td>TMV</td>
<td>tobacco mosaic virus</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl)amino methane</td>
</tr>
<tr>
<td>U</td>
<td>uridine</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>X-gluc</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
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Acknowledgement

I wish to thank Dr. Joan McPherson for providing me the opportunity to work in her lab, and for her encouragement and support. Thanks are also extended to Dr. Carl Douglas, Dr. Brian Ellis and Dr. Victor Runeckles for serving on my committee. I would like to thank Dr. Carl Douglas in particular, for supplying plasmid DNA containing the GUS reporter gene and for the use of specific facilities in his laboratory. I am also grateful to Dr. Michael Shaw for the use of the photomicroscope throughout the project. The support of Hugo E. Meilicke Memorial Fellowship and D.G. Laird and G. Moe Scholarship is gratefully acknowledged. The work was supported by NSERC grant number A2832 to Dr. Joan McPherson.
Chapter 1

INTRODUCTION

1.1 Gene 7 Promoter

Large tumour inducing plasmids of Agrobacterium tumefaciens are responsible for crown gall tumour formation. A defined segment of Ti-plasmid, the T-DNA, is integrated into the plant genome and transcribed by the host RNA polymerase II. Efficient T-DNA transfer and integration require the right border repeat sequence, as well as an "overdrive" sequence outside the right border (in cis). The function of virulence genes within the vir region of the Ti-plasmid and on the bacterial chromosome is required in trans. T-DNA-encoded proteins expressed within the plant cell are involved in oncogenesis and are not necessary for T-DNA transmission (Ream, 1989).

Several independent polyadenylated T-DNA transcripts have been detected in an octopine-type crown gall tissue (Gelvin et al., 1982; Willmitzer et al., 1982) and the T-DNA encoded proteins identified by in vitro translation (McPherson et al., 1980; Schröder and Schröder, 1982). Garfinkel et al. (1981) produced the first detailed map of the T-DNA of an octopine-type Ti-plasmid using transposon insertion mutagenesis. Leemans et al. (1982) constructed a series of Ti-plasmid mutants carrying defined deletions to probe for the functions of T-DNA transcripts. Transcripts that are common
Figure 1. Genetic map of the TL-DNA of an octopine type Ti-plasmid. The bold arrow marks the position of a transcript encoded by gene 7. The gene symbols indicate indoleacetamide hydrolase (iaaH), tryptophan monoxygenase (iaaM), isopentenyl transferase (ipt), opine secretion (ops), tumor morphology large (tml), and octopine synthase (ocs).

to both octopine and nopaline type tumours have been shown to encode products involved in biosynthesis of auxin (Schröder et al., 1984; Tomashow et al., 1986; Van Onckelen et al., 1986) and cytokinin (Akiyoshi et al., 1984; Barry et al., 1984; Buchmann et al., 1985). Other transcripts which are strain-specific code for enzymes participating in the synthesis and secretion of opines (Schröder et al., 1981). These are catabolized by the inciting Agrobacterium as a source of carbon and nitrogen. The genetic map of an octopine type Ti-plasmid T-DNA is presented in Figure 1.

One of the most abundant T-DNA transcripts in octopine-type tumours is 0.7 kb in size (Gelvin et al., 1982) and was identified as transcript 7 (Willmitzer et al., 1982).
The T-DNA region responsible for transcript 7 ("gene 7") is located near the left border and has been isolated and sequenced from both Ti-plasmids and crown gall tumour lines (Dhaese et al., 1983; McPherson, 1984). It contains an open reading frame coding for a polypeptide of 126 amino acids and a molecular weight of 14,400 daltons, in accordance with previous in vitro translation studies. As is the case with other T-DNA genes, its 5' and 3' flanking sequences contain typical eukaryotic expression signals. However, very little is known about the function or regulation of gene 7 expression. Tumours induced by a Ti-plasmid mutagenized within the T-DNA segment from which transcript 7 is transcribed are normal with respect to tumorigenesis and opine synthesis (Garfinkel et al., 1981). Gene 7 is absent in the nopaline-type tumours which are phenotypically different from octopine-type tumours. The sequence of gene 7 is identical in the inducing Ti-plasmid and in the T-DNA of established tumour lines. Except for a few differences in the 5' flanking region, the sequence is also conserved between different octopine-type Ti-plasmid isolates (McPherson, 1984). Although gene 7 is transcribed at barely detectable levels in transgenic plants, its promoter sequences respond remarkably well to the presence of cauliflower mosaic virus 35S enhancer elements (Kay et al., 1987). When measured by the S1 nuclease protection assay, the rate of gene 7 transcription was increased about 40-fold by the presence of an upstream, divergently oriented 35S enhancer and tenfold further when the 35S enhancer was duplicated.
Chapter 1. Introduction

1.2 Cauliflower Mosaic Virus 35S Promoter

Cauliflower mosaic virus (CaMV) is a double-stranded DNA virus that infects members of the Cruciferae. During the virus life cycle, the CaMV minichromosome is transcribed by host RNA polymerase II, producing two major transcripts (commonly referred to as 19S and 35S) from two distinct promoters (Guilley et al., 1982). In addition to serving as a template for translation, the 35S mRNA (which is a complete transcript of the viral genome) functions as an intermediate in virus replication via reverse transcription. The expression of genes from the 35S promoter has been studied extensively in transgenic plants. Several initial studies showed high expression levels from the 35S promoter (Rogers et al., 1985; Sanders et al., 1987) and the apparently constitutive nature of its activity (Nagy et al., 1985; Odell et al., 1985). Others observed differences in the level of expression depending on the cell type (Jefferson et al., 1987; Williamson et al., 1989; Benfey and Chua, 1989) or stage of the cell cycle (Nagata et al., 1987).

Active promoter sequences include the proximal region (containing the transcription initiation site at nucleotide +1 and extending to nucleotide -46) which is sufficient for accurate transcription initiation, and the upstream sequences (-343 to -46) that are responsible for the majority of the 35S promoter strength (Odell et al., 1985; Fang et al., 1989). An analysis carried out with two distinct regions within the promoter demonstrated that domain A (-90 to +8) conferred expression principally in root tissue.
and domain B (-343 to -90) in other tissue types. When both domains were present, expression was detectable in most tissues at all stages of development (Benfey et al., 1989). Several subdomains within domain B have been analyzed and found to confer distinct patterns of expression (Benfey et al., 1990a; 1990b). These studies presented evidence for synergistic interactions among cis-elements within the 35S promoter and enhancer regions. A cis-acting element termed activation sequence 1 (as-1) located between nucleotides -85 and -64 contains a tandem repeat of the TGACG motif which is required for binding of a nuclear factor, ASF-1, and is necessary for expression in root tissue (Lam et al., 1989). This tandem repeat is also a part of the ocs-element first identified in the promoter of octopine synthase (ocs) gene. DNA sequences homologous to the ocs-element were found in other opine synthase genes as well as other viral genes (Bouchez et al., 1989). Similar protein binding properties of ocs- and as-1 elements suggest that transcription of ocs and CaMV 35S genes (and possibly other T-DNA and plant viral genes) is regulated by at least one common trans-acting factor (Fromm et al., 1989). A second cis-element, designated as-2, has been identified in the -100 nucleotide region of the 35S promoter and shown to bind a protein factor, termed ASF-2. ASF-2 binding activity was detected in leaf but not root nuclear extracts (Lam and Chua, 1989).

The upstream region of the 35S promoter acts as a strong enhancer of heterologous promoters and the enhancer duplication simulates transcription up to tenfold (Kay et al., 1987). A specific fragment of upstream sequence (-392 to -55) has been used to raise the level of expression from a low-activity nopaline synthase (nos) promoter up to that observed with the intact 35S promoter (Odell et al., 1989). Recent studies
have demonstrated that the enhancer activity of the 35S promoter resides within the region between nucleotides -287 and -90 (McPherson and Kay, unpublished).

1.3 GUS Reporter Gene System

The regulation of gene expression in plant cells has been explored using "reporter" gene sequences which are expressed under the control of heterologous regulatory elements. The *E. coli* *uidA* gene encoding β-glucuronidase (GUS) has been developed as a reporter gene for the use in transgenic plants (Jefferson *et al.*, 1987). It offers advantages of high sensitivity, versatility and speed of assays, and the possibility of histochemical analysis. Intrinsic GUS-like activities are absent from most higher plants although they have been detected in a few species at certain stages of development (Plegt and Bino, 1989; Hu *et al.*, 1990). Since the structure of the mRNA and its ribosome binding ability should be identical for each chimeric gene construct created by a transcriptional fusion, levels of GUS expression can be used to reflect relative promoter activities. The GUS gene used in this work is a derivative in which the 5' sequences (including the ATG initiation codon) have been removed and replaced with a synthetic oligonucleotide containing the eukaryotic consensus translational initiator sequence (Kozak, 1981; Jefferson, 1988).
1.3.1 Translational Initiation Sequence Context

According to the scanning model for eukaryotic initiation of translation (Kozak, 1981), the small (40S) ribosomal subunit binds to the capped 5' end of mRNA and migrates down the transcript until it encounters the first AUG codon. If the first AUG triplet is in a favourable context for initiating translation the 40S subunit is joined by the 60S subunit and the first peptide bond is formed. The model thus suggests that both proximity to the 5' end and the sequence context of the AUG codon contribute to the selection of the initiation site. In other words, the efficiency with which a 40S subunit recognizes an AUG triplet depends on the flanking sequences. The consensus sequence for initiation in eukaryotes has been identified as (A/G)NNAUGG (Kozak, 1981) and further confirmed and extended as GCCGCC(A/G)CCAUGG (Kozak, 1986; 1987).

Prokaryotic mRNAs, in contrast to eukaryotic systems, usually contain more than one cistron and any exposed AUG codon following an exposed ribosome binding site can be selected to initiate translation (Kozak, 1983). The ribosome binding site is a purine rich sequence that is complementary to the 3' terminus of 16S ribosomal RNA and therefore enables direct internal binding of ribosomes to mRNA (Shine and Dalgarno, 1974).

Differences between initiation mechanisms and structural components of mRNAs in prokaryotes and eukaryotes lead to low efficiencies of translation in the nonhomologous system. The initiator context can have a profound effect on the levels
of product translated by eukaryotic ribosomes from a given amount of mRNA (Kozak, 1981; 1986). A modified initiation context in the GUS gene (isolated from \textit{E. coli}) should result in higher efficiency of the GUS translation start site recognition by the eukaryotic ribosomes, thus increasing the sensitivity of this reporter gene system for the use in transgenic plants.

1.4 Promoter Constructs

Five promoter constructs (G7, 1CAG7, 2CAG7, CA1 and CA2) were used to

![Diagram of promoter constructs](image)

Figure 2. Promoter constructs assessed for their activity.
direct expression of the GUS reporter gene in transgenic tobacco plants (Fig. 2). Construct G7 represents the sequence surrounding the transcription initiation site (+1) of the gene 7 promoter (nucleotides -93 to +15; McPherson and Kingsbury, 1986). 35S B denotes the upstream portion of the CaMV 35S promoter (nucleotides -343 to -90). 35S A is the proximal region of the 35S promoter including the TATA box and the transcription start site at +1, and extending from nucleotide -90 to +8 (Odell et al., 1985). Construct CA1 (the unrearranged 35S promoter from -343 to +8) provided a positive control.

Each promoter construct was fused to the GUS gene derivative containing the eukaryotic translational initiation context (Jefferson et al., 1988). Construct CA1 was also inserted upstream of the GUS gene bearing its native ATG context (Jefferson et al., 1988) and designated proCA1.

1.5 Rationale and Objectives

Different structural units of the regulatory regions of genes can be combined resulting in a pattern of expression which reflects their specific interaction. The use of the reporter gene system based on GUS activity enables levels of the final gene product to be determined. The advantages offered by this system include the ability to detect expression histochemically at the individual cell level. Such cell specific expression thus reflects the cell specificity of the chimeric promoter activity.

The combination of a proximal promoter region from T-DNA gene 7 and a distal
region from the CaMV 35S gene provides such a chimeric regulatory element for investigation. Similarly, the modification of the distal region of the 35S gene to include a duplicated enhancer unit provides a novel gene regulatory element to be analyzed both in the presence of the gene 7 promoter and its homologous promoter. Levels of gene expression are also affected by the structural properties of the transcript. The investigation of gene constructs with sequences characteristic of prokaryotic and eukaryotic translational start sites will enable a comparison of their relative effects on expression in plant cells.

The research plan included:

1. The preparation of transcriptional fusions between described promoter constructs and the GUS reporter gene, and their expression in transgenic tobacco plants.
2. The investigation of transcriptional activity of these promoter constructs by measuring enzymatic activity of the final gene product.
3. A description of spatial distribution of each promoter activity within plant tissues.
4. A comparative analysis of the relative promoter activities and their tissue specificity, based on the levels of GUS expression. This analysis will address some more specific questions concerning: - gene 7 promoter activity,
   - the effect of the 35S enhancer on the gene 7 promoter,
   - the effect of the 35S enhancer duplication on activity of its native as well as another heterologous promoter.
5. An estimation of the effect of the translational initiator context on the levels of GUS expression in transgenic plants.
Chapter 2

MATERIALS AND METHODS

2.1 General Techniques of Molecular Biology

General recombinant DNA techniques were carried out according to Maniatis et al. (1982) unless otherwise specified. Chemicals were obtained from Sigma Chemical Co. and BDH Inc.; restriction and modification enzymes were purchased from Bethesda Research Laboratories, Pharmacia or New England Biolabs.

2.1.1 Bacterial Strains and Growth Media

*Escherichia coli* strain DH5α served as a host for all plasmid constructions. Strain DH5αF’ was used for phage M13 propagation. Unless stated otherwise, all *E. coli* cultures were grown in Luria-Bertani (LB) medium (10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, 5 g/l NaCl, pH 7.5) containing appropriate antibiotics as specified. 15 g/l agar was added for plates and 7.5 g/l top agar used to propagate the phage.
2.1.2 Preparation of Plasmid DNA

Plasmid DNA was isolated by the alkaline lysis procedure described by Maniatis et al. (1982). A single bacterial colony was transferred into 2 ml of LB medium containing the appropriate antibiotic and incubated overnight (37°C with shaking). 1.5 ml of the overnight culture was centrifuged for 2 minutes in an Eppendorf centrifuge and the medium removed with a sterile drawn-out Pasteur pipette. The bacterial pellet was dried briefly under vacuum and resuspended by vortexing in 100 μl of an ice-cold, sterile solution of 50 mM glucose, 10 mM ethylene diamine tetraacetic acid (EDTA), 25 mM tris-(hydroxymethyl)amino methane (Tris)-HCl pH 8.0 and 4 mg/ml lysozyme (added just before use). After leaving the tube at room temperature for 5 minutes, 200 μl of 1% sodium dodecyl sulphate (SDS) and 0.2 N NaOH (freshly prepared) were added to the tube and the contents mixed by inversion. The reaction was stored on ice for 5 minutes and then neutralized by addition of 150 μl of an ice-cold solution of potassium acetate (3 M with respect to potassium, 5 M with respect to acetate). The tube was vortexed gently and incubated on ice for additional 5 minutes. Cell debris and chromosomal DNA were removed by centrifugation (5 minutes in an Eppendorf centrifuge) and the supernatant was extracted twice with an equal volume of phenol/chloroform (1:1) and once with chloroform. DNA was precipitated by addition of two volumes of 95% ethanol and left at -20°C for 30 minutes or overnight. The nucleic acid pellet was recovered by centrifugation, washed in 70% ethanol, dried under vacuum and resuspended in 50 μl
of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Contaminating RNA was digested by addition of DNase-free RNase (20 μg/ml). Aliquots of the sample (5 or 15 μl) were used for subsequent restriction enzyme digestion.

2.1.3 Restriction Endonuclease Digestion of DNA

Restriction enzymes were purchased from Bethesda Research Laboratories (BRL), Pharmacia or New England Biolabs. Restriction digests were carried out according to the supplier's instructions, using BRL React Buffer or One-Phor-All Buffer (Pharmacia) as recommended for the individual enzymes. Reactions were incubated at 37°C for 2 to 5 hours.

2.1.4 Gel Electrophoresis

Prior to electrophoresis, restriction enzyme digests were mixed with 0.1 volume of loading buffer (0.2% bromophenol blue, 0.1 M EDTA pH 8.0, 50% glycerol). DNA samples (0.2-2 μg per lane) were applied to agarose gels (0.7-1.3%) and electrophoresed in 0.5x or 1x TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) at 5-10 V/cm. Electrophoresis in polyacrylamide gels (12%) was carried out in 1x TBE buffer at 5 or 10 V/cm. All gels were stained in a solution of ethidium bromide (0.5-1 μg/ml) for 30 minutes.
2.1.5 Ligations

Restriction digests were heated to 85°C for 30 min to denature restriction enzymes and then ethanol-precipitated. Precipitated DNA fragments were washed in 70% ethanol, dried under vacuum and resuspended in TE buffer at 20 ng/μl. Vector DNA was mixed with 1-3-fold molar excess of insert, warmed at 45°C for 5 minutes to melt any cohesive ends that might have reannealed and chilled on ice. Sticky-end ligations were carried out in 20 μl volume containing 50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 20 mM dithiothreitol (DTT), 50 μg/ml bovine serum albumin (BSA) and 1 mM ATP with 1 unit T4 DNA ligase (Pharmacia). Incubations were done at 15°C overnight. Total concentration of DNA in the ligation reaction was 10-20 ng/μl.

2.1.6 Transformations

_E. coli_ host cells were made competent for DNA transformation by CaCl₂ treatment (Maniatis _et al._, 1982). An overnight culture was diluted 1:100 with fresh LB medium and grown with aeration at 37°C to an optical density of A₅₅₀ = 0.5. After chilling the culture on ice for 10 minutes the cells were pelleted by centrifugation (4000 g for 5 minutes at 4°C). The cell pellet was gently resuspended in half the original volume of an ice-cold sterile solution of 50 mM CaCl₂ and left on ice for 20 minutes. After another 5 minute centrifugation (4000 g, 4°C), the cells were resuspended in 1/10 original volume
of cold, sterile 50 mM CaCl$_2$ and 200 µl aliquots used immediately for transformation. To make frozen stock of competent cells, the pellet was resuspended in 15% glycerol, 50 mM CaCl$_2$, divided in aliquots and stored at -70°C. Competent cells were mixed with 1 or 10 µl of ligation mix, stored on ice for 30 minutes, heat shocked at 42°C for 2 minutes and plated on selective media. Transformation mixtures with recombinant M13 were added to 200 µl overnight bacterial culture, 10 µl of 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 50 µl 2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in N,N-dimethylformamide (DMF), mixed with 3.5 ml of top agar and plated onto an LB plate.

2.2 Oligonucleotide-Directed Mutagenesis

T-DNA gene 7 was isolated and characterized from the T-DNA HindIII restriction fragment Y (McPherson, 1984). In the present study, levels of expression from the gene 7 promoter were determined by use of a reporter gene which was linked to the upstream region of gene 7. To facilitate the linkage, a restriction site was engineered into the 3’ end of the gene 7 promoter region. Oligonucleotide-directed mutagenesis according to Kunkel (1985) was applied to introduce a BamHI restriction site at the location adjacent to the ATG initiation codon, as indicated in Fig. 3.
2.2.1 Oligonucleotide for Mutagenesis

A mutagenic oligodeoxyribonucleotide (5'GCAGTTGGGGGTCCATGGATCCGATGTGAGGTGTGTT 3') was synthesized by T. Atkinson (N.A.P.S. Unit, Biotechnology Laboratory, UBC). The 36mer was designed with 15 bases of homology on either side of the loop carrying the BamHI recognition sequence (Fig. 3). Oligonucleotide purification was carried out by C$_{18}$ column chromatography as described by Atkinson and Smith (1984). The C$_{18}$ SEP-PAK cartridge was prepared for use by washing with 10 ml of HPLC grade acetonitrile followed by 10 ml of distilled sterile water. The crude DNA pellet was dissolved in 1.5 ml 0.5 M ammonium acetate and the oligonucleotide solution was passed through the activated C$_{18}$ cartridge. Contaminants
were washed off the column by 10 ml of sterile distilled water. The oligonucleotide was eluted off the column with 20% HPLC grade acetonitrile/water and the eluant collected in three 1 ml fractions. The yield of oligonucleotide was determined spectrophotometrically. The first fraction of the eluant was evaporated to dryness and the oligonucleotide resuspended in TE buffer at 10 pmoles/μl.

2.2.2 Preparation of M13 Replication Form

The replication form (RF) of M13mp19 was transformed to DH5αF' cells and a single blue plaque was used to inoculate 2 ml of LB medium with 20 μl overnight DH5αF' culture. After 3 hours of growth, 1 ml of the culture was diluted 10x with fresh LB medium and grown for another 3 hours. M13mp19 RF was isolated by the alkaline lysis procedure of medium scale (miniprep scaled up 10x). The DNA was resuspended in 100 μl of TE buffer and 5 μl analyzed by HindIII and SstI digestion and agarose gel electrophoresis.

2.2.3 Construction of Recombinant M13

The promoter region of T-DNA gene 7 linked to upstream regions of the CaMV 35S promoter was available in recombinant clones. Promoter constructs G7, 1CAG7 and 2CAG7 had been previously inserted into pUC18 HindIII and SstI restriction sites to
create pUCg7MT, pUC1Eg7MT and pUC2Eg7MT, respectively (May, unpublished results).

Full length inserts (of 339, 592 and 845 base pairs, respectively) were removed by HindIII and SstI digestion of pUCg7MT, pUC1Eg7MT and pUC2Eg7MT, respectively, and ligated individually into the corresponding sites of M13mp19 RF. After each transformation, 10 colourless plagues were screened by the following procedure: 2 ml of LB medium with 20 μl of overnight DH5αF’culture were inoculated by a single plaque, the culture was grown for 6 hours and the recombinant M13mp19 RF isolated by the alkaline lysis miniprep (as in section 2.1.2.). The RF DNA sample was screened by restriction mapping. The resulting M13mp19 recombinants containing chimeric promoter fragments were used for the preparation of the single-stranded DNA template as described below.

2.2.4 Preparation of Single-Stranded M13 DNA Template

Uracil-containing DNA was produced within an *E. coli dut ung* F’ strain, RZ1032 (Kunkel et al., 1987). *E. coli dut* mutants lack the enzyme dUTPase and the resulting elevated concentration of dUTP effectively competes with dTTP for incorporation into newly synthesized DNA. In addition, *ung* mutants lack uracil N-glycosylase which normally removes uracil from DNA. In the combined *dut ung* mutant (such as RZ1032), uracil residues are incorporated into DNA and are not removed.
RZ1032 host cells were transformed with recombinant M13mp19 RF bearing constructs G7, 1CAG7 and 2CAG7, respectively. All of the following reactions were carried out for each individual construct (i.e. template). A single-stranded, uracil-containing template was prepared by the following method (Sambrook et al., 1989). A single plaque produced by an M13 recombinant was transferred to 1 ml of 2x YT medium (16 g/l Bacto-tryptone, 10 g/l Bacto-yeast extract, 10 g/l NaCl, pH 7.5) and incubated at 60°C for 5 minutes to kill the host bacteria. The tube was vortexed for 30 seconds to release the phage and centrifuged at 12000 g for 2 minutes to eliminate dead bacterial cells and fragments of agar. 50 µl of supernatant were used to inoculate 50 ml of 2x YT medium with 5 ml of a log phase culture of RZ1032. The culture was grown for 6 hours at 37°C with shaking and subsequently centrifuged (5000 g, 4°C) for 30 minutes. Bacteriophage particles were precipitated from the supernatant by adding 0.25 volume of 15% polyethylene glycol (PEG 8000), 2.5 M NaCl and leaving at 4°C overnight. Phage particles were collected by centrifugation (5000 g, 4°C, for 20 minutes) and the well-drained pellet resuspended by vortexing in 6 ml of TE buffer. The suspension was stored on ice for 1 hour, vortexed again and recentrifuged in order to remove bacterial debris. The supernatant was then extracted 4 times with phenol/chloroform (1:1) and twice with chloroform. The DNA was precipitated from the final aqueous phase by 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volumes of 95% ethanol, stored at -20°C overnight and pelleted by centrifugation. The pellet was washed in 70% ethanol, dried under vacuum and resuspended in 100 µl of TE buffer. The
concentration and purity of single-stranded templates were determined by absorbance measurements.

2.2.5 Mutant Phage Preparation

1. Primer Extension Reaction

The mutagenic oligonucleotide (100 pmols) was phosphorylated in 20 µl reaction containing 50 mM Tris-Cl pH 7.5, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 1 mM ATP and 2 units of T4 polynucleotide kinase. The reaction was kept at 37°C for 1 hour, terminated by adding 3 µl 0.1 M EDTA, and heated to 70°C to denature the enzyme. 5 pmols of phosphorylated oligonucleotide were mixed with 0.2 pmols of uracil-containing single stranded M13 template and 1.25 µl of 20x SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7) in a 25 µl total volume. The oligonucleotide primer was annealed to the template by heating the mix to 70°C for 1 minute and cooling down slowly to room temperature. DNA strand elongation was carried out using T4 DNA polymerase (2.5 units) in a reaction (100 µl) containing 0.5 mM each dNTP, 1 mM ATP, 20 mM Tris-Cl pH 8.8, 2 mM DTT, 10 mM MgCl₂, and 2 units of T4 DNA ligase (Pharmacia). The reaction was incubated for 5 minutes on ice, followed by 5 minutes at room temperature and 2 hours at 37°C, and terminated with 3 µl 0.5 M EDTA.
2. Transformation and Product Analysis

The primer extension reaction (10 or 50 µl) was transformed into wild-type (i.e. ung\(^+\)) E. coli DH5α F\(^-\). Strong selection for the newly synthesized strand occurs in the ung\(^+\) E. coli host, where the uracil-containing template strand is rendered biologically inactive by the action of uracil N-glycosylase and subsequent DNA repair (Kunkel et al., 1987). Resulting plaques (carrying the mutant phage) were used to infect 2 ml DH5α F\(^-\) cultures as described above (section 2.2.1.). The cell pellet was used for RF miniprep (as in section 2.1.2.) and the supernatant kept for single-stranded template preparation for DNA sequence analysis. The RF minipreps were screened for the presence of the newly introduced BamHI restriction site by digestion followed by agarose or polyacrylamide gel electrophoresis.

3. DNA Sequence Analysis

Following selection of M13mp19 recombinant clones by restriction mapping, the predicted sequence at the mutagenesis site was confirmed by DNA sequence analysis. Template for sequencing was prepared from cultures infected with mutant phage. Supernatant (1.3 ml) from the culture was mixed with 0.3 ml 20% PEG 8000, 2.5 M ammonium acetate and left overnight at 4°C. The phage was pelleted by centrifugation, the supernatant removed with a drawn-out Pasteur pipette and the pellet resuspended in 0.2 ml of NTE buffer (10 mM Tris-HCl pH 8, 0.25 mM EDTA, 10 mM NaCl). The
Chapter 2. Materials and Methods

Phage suspension was extracted three times with phenol/chloroform (1:1) and once with chloroform. DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol, rinsed with 70% ethanol, dried under vacuum and redissolved in 30 μl TE buffer. Five μl of the template were used per sequencing reaction. All sequencing reactions were performed with Sequenase Version 2.0 sequencing kit (United States Biochemical) which is based on the dideoxynucleotide chain-termination method of Sanger et al. (1977) and uses Sequenase (genetically modified T7 DNA polymerase) in the primer extension reaction. Either [α-32P]-dATP or [α-35S]-dATP was used for autoradiographic detection of DNA fragments that were analyzed on 5% denaturing polyacrylamide-urea gels.

2.3 Construction of GUS Expression Vectors

The GUS coding region bearing the eukaryotic consensus sequence (Kozak, 1981; Jefferson, 1988) around the ATG initiation codon (TCGACCATGGTC) was provided within plasmid pRT99-GUS-JD/Kozak (Hauffe et al., 1991) that was kindly supplied by Dr. C. Douglas (Dept. of Botany, UBC). The plasmid was subjected to BamHI-EcoRI restriction digestion and the fragment bearing the GUS coding region fused to the nopaline synthase (NOS) polyadenylation signal was gel purified (Maniatis et al., 1982). After agarose gel electrophoresis (in 1x TBE), the band was excised from the gel, placed in dialysis tubing and electroeluted in 0.5x TBE at 100 V for 3 hours. The eluate was
purified by phenol/chloroform extraction and ethanol precipitation. The purified fragment was ligated to BamHI and EcoRI sites of the binary vector, BIN 19 (Bevan, 1984), which bears the kanamycin resistance gene (nopaline phosphotransferase II) within t-DNA borders. This modified binary vector (Fig. 4) will be referred to as pBI-K (Moziskova, unpublished results).

Each promoter construct (G7, 1CAG7 and 2CAG7) was excised from recombinant M13 RF at the newly created BamHI sites and upstream at HindIII sites. The resulting HindIII-BamHI fragments (of 110, 363 and 616 bp, respectively) were ligated individually into the corresponding sites in the polylinker of the vector, pBI-K, upstream of the GUS coding sequences (Fig. 4).

Constructs CA1 (351 bp) and CA2 (604 bp) originate from pCa1 and pCa2 plasmids, respectively (Kay et al., 1987). Each of them was transferred as a HindIII-
BamHI fragment into the vector pBI-K polylinker as above.

Construct CA1 was also inserted as a HindIII-BamHI fragment in the polylinker of plasmid pBI101.1 (Jefferson et al., 1987; 1988) which bears the GUS coding region with its original prokaryotic translational initiator sequence context. The CA1 construct fused to this original GUS coding sequence was designated proCA1. The integrity of the binary vectors bearing promoter construct-GUS gene fusions was confirmed by restriction analysis.

2.4 Transformation of Agrobacterium tumefaciens

2.4.1 Freeze-Thaw Method

Binary vectors were directly transformed into A. tumefaciens LBA1404 harbouring the Ti-plasmid pAL4404 (Hoekema et al., 1983) by the freeze-thaw method (An et al., 1988). 50 ml of LB medium were inoculated with 2 ml of an overnight Agrobacterium culture and grown at 26°C with shaking to an A_{600}=0.5. The culture was chilled on ice, centrifuged (3000 g, 4°C for 5 minutes) and the cells resuspended in ice-cold 20 mM CaCl₂ (1 ml), then dispensed in 100 μl aliquots. Each aliquot was mixed with about 1 μg of plasmid DNA, frozen in liquid nitrogen and thawed at 37°C for 5 minutes. After addition of LB medium (1 ml), each tube was incubated for 2 hours at 26°C with gentle shaking. The cells were collected by brief centrifugation, resuspended
in 100 \( \mu l \) of LB medium and plated on LB agar containing 100 \( \mu g/ml \) streptomycin and 50 \( \mu g/ml \) kanamycin. The plate was incubated at 26\(^\circ\)C for 2-3 days until colonies appeared.

2.4.2 Plasmid Preparation

The presence and integrity of binary vectors within *Agrobacterium* were verified by restriction analysis of plasmid preparations (An *et al.*, 1988). A single colony was used to start a 2 ml overnight culture grown at 26\(^\circ\)C in LB medium containing 100 \( \mu g/ml \) streptomycin and 50 \( \mu g/ml \) kanamycin. 1 ml of the culture was centrifuged (30 seconds in Eppendorf centrifuge) and the cell pellet resuspended in 100 \( \mu l \) of an ice-cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 and 4 mg/ml lysozyme (added before use). After 10 minutes at room temperature, 200 \( \mu l \) of freshly prepared 1% SDS, 0.2 N NaOH were added, the tube was mixed by shaking and kept at room temperature for another 10 minutes. The contents was vortexed with 30 \( \mu l \) of alkaline phenol (equilibrated with 2 volumes of 1% SDS, 0.2 N NaOH), neutralized with 150 \( \mu l \) of potassium acetate (3 M potassium, 5 M acetate) and incubated at -20\(^\circ\)C for 15 minutes. The cell debris was removed by 3-minute centrifugation and the supernatant quickly poured into a new tube. The tube was filled with cold 95% ethanol, mixed and left at -70\(^\circ\)C for 15 minutes. The precipitate was pelleted by centrifugation, dried and resuspended in 0.5 ml 0.3 M sodium acetate pH 7. The DNA was precipitated again with
95% ethanol, washed in 70% ethanol, dried under vacuum and resuspended in 25 μl of TE buffer with 10 μg/ml RNase. Restriction analysis was performed with 5 or 15 μl of the preparation depending on the size of fragments to be detected.

2.5 Plant Transformation

Tobacco leaf disc transformation was carried out essentially as described by Rogers et al. (1986). Healthy leaves of Nicotiana tabacum var. Xanthi were sterilized in 10% commercial bleach (0.5% sodium hypochlorite) with detergent for 15 minutes, rinsed 3 times in large volumes of sterile distilled water and cut with a sterile cork borer to generate the leaf discs. The discs were swirled in an overnight culture of Agrobacterium, blotted on a sterile filter paper and placed upside-down on shoot-inducing medium (MS salts, B5 vitamins, 30 g/l sucrose, 1 mg/l benzyladenine, 0.1 mg/l naphthaleneacetic acid, 0.7% agar, pH 5.7). The plates were kept at 26°C under reduced light (15 μEinsteins.m^-2.s^-1). After 3 days, the discs were rinsed in liquid shoot inducing-medium containing 300 μg/ml kanamycin, 500 μg/ml carbenicillin and 400 μg/ml Cefotaxime, blotted on the sterile filter paper and transferred to shoot-inducing plates containing the same concentration of antibiotics. The plates were incubated at 26°C under bright light (16 hr light regime, 320 μEinsteins.m^-2.s^-1) until the shoots were large enough to be removed (3-4 weeks). Excised shoots were placed on rooting medium (MS salts, B5 vitamins, 30 g/l sucrose, 0.7% agar, pH 5.7) containing 50 μg/ml kanamycin and
500 μg/ml carbenicillin. Rooted plantlets were planted in sterile soil (after washing off excess agar with distilled water) and grown under standard plant growth conditions (23°C, 16 hr light regime, 390 μEinsteins.m².s⁻¹).

2.6 Fluorometric GUS Assay

Roots, stems and leaves of plants grown to a height of 20 to 30 cm were used in this assay. Prior to extraction, the root samples were washed thoroughly with distilled water and blotted dry between two sheets of filter paper. Leaf samples were taken from the newest fully expanded leaf, in the middle of the lamina beside the midvein. Stem segments were taken from the internode immediately below the sampled leaf. A single sample of each tissue was taken from each plant. About 0.2 g of tissue was homogenized in 0.5 ml of cold solution of 50 mM sodium phosphate (NaH₂PO₄-Na₂HPO₄) pH 7.0, 1 mM EDTA, 0.1% Triton, 10 mM β-mercaptoethanol (extraction buffer) on ice. The extract was cleared by centrifugation (10000 g, 4°C for 20 minutes). The fluorogenic reaction was carried out with 10 μl of extract in 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) in extraction buffer (total volume 0.5 ml) at 37°C. After an initial 5 minute period, 100 μl aliquots were removed at fixed time points up to 60 minutes (i.e. at 5, 10, 20, 40 and 60 minutes) and added to 1.4 ml 0.2 M Na₂CO₃ to stop the reaction. For low intensity samples, the reaction was extended to 90 minutes. The fluorescence was measured with excitation at 365 nm, emission at 455 nm using a spectrofluorimeter.
calibrated with freshly prepared 4-methylumbelliferone (4-MU) standards (Jefferson et al., 1987). The GUS activity (in picomoles of 4-MU released per minute) was calculated as the slope of the regression line fitted through the measurements at individual time points ($r^2 \geq 0.95$). Protein concentrations of the extracts were determined by the dye-binding method of Bradford (1976) using the Bio-Rad Protein Assay Kit I. Final values of GUS specific activity were expressed in picomoles 4-MU per minute per milligram of total protein. For each construct, mean values of GUS activity in each tissue were calculated from 8 to 12 individual transformants. For each tissue, two means representing GUS expression from two different constructs were analyzed by the one sided t-test for comparing two population means (Mendenhall et al., 1986; p. 408) at 0.05 level of significance.

2.7 Histochemical GUS Assay

Histochemical assay was done according to Jefferson et al. (1987) with some modifications. About 10 to 20 sections of each tissue were generated from each adult plant. The sections were cut by hand and fixed in 0.5% formaldehyde, 100mM sodium phosphate buffer pH 7.0 for 30 minutes on ice, followed by two washes in 100 mM sodium phosphate pH 7.0. All fixatives and the substrate were introduced into the tissue by a brief vacuum infiltration (about 1 minute). Pollen was collected from flowers at anthesis and directly incubated with the substrate. Histochemical reactions were
performed in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), 50 mM sodium phosphate pH 7.0 and incubated at 37°C overnight. After staining, the sections were rinsed in 25%, 40% and 55% ethanol (5 minutes each) and left in 70% ethanol at 4°C for several hours or overnight. The sections were subsequently rinsed in distilled water and mounted in 50% glycerol on microscope slides.
Chapter 3

RESULTS

3.1 Mutant Recovery

To establish the linkage between the gene 7 promoter and the GUS reporter gene, the BamHI restriction site was introduced at the 3' end of constructs G7, 1CAG7 and 2CAG7 by oligonucleotide-directed mutagenesis (Kunkel, 1985). The mutagenesis protocol was designed to favour recovery of phage DNA from the oligonucleotide-initiated strand rather than the uracil-containing template (i.e. more than 50% recovery of mutants was expected). However, only about 30-40% of plaques screened after the final transformation step carried the BamHI restriction site (Table 1). Furthermore, some sequence rearrangements (one base deletions) were observed in several BamHI

<table>
<thead>
<tr>
<th>Template</th>
<th>Clones screened</th>
<th>Clones carrying BamHI site</th>
<th>Clones sequenced</th>
<th>Clones carrying correct target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>G7</td>
<td>16</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1CAG7</td>
<td>10</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>2CAG7</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
site carrying isolates. This low efficiency could be attributed either to low purity of reagents and enzymes or, most likely, to the fact that the mutagenic primer was not gel-purified to eliminate shorter oligonucleotide contaminants that arise from incomplete DNA synthesis. Purification by polyacrylamide gel electrophoresis is often recommended for oligonucleotides more than 30-40 nucleotides in length and those used for less efficient "loop-in" or "loop-out" mutagenesis (Kunkel et al., 1987; Sambrook et al., 1989).

3.2 GUS Activity Measured by Fluorometric Assay

Transcriptional fusions between the five promoter constructs (G7, 1CAG7, 2CAG7, CA1 and CA2) and the GUS coding region were designed to encode mRNAs that have similar structure and affinity for eukaryotic ribosomes. Thus, measurements of GUS expression can be used to evaluate relative activity of individual promoter constructs (see section 1.3). However, the mRNAs transcribed from gene 7 promoter-bearing constructs contain 15 nucleotides of gene 7 untranslated leader, whereas those transcribed from constructs CA1 and CA2 include a small fragment of the 35S untranslated leader sequence (8 nucleotides long).

The levels of GUS enzymatic activity in tissues of primary transformants were measured by enzymatic conversion of a fluorogenic substrate (MUG) to a fluorescent dye (4-MU). Mean values of GUS activity are presented in Table 2 and measurements on individual plants are given in Figs. 5-11.
Table 2. GUS expression from different promoter constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>n</th>
<th>ROOT (pmol·min⁻¹·mg⁻¹)</th>
<th>STEM (pmol·min⁻¹·mg⁻¹)</th>
<th>LEAF (pmol·min⁻¹·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>8</td>
<td>5 (± 1.5)</td>
<td>2 (± 0.9)</td>
<td>1 (± 0.6)</td>
</tr>
<tr>
<td>G7</td>
<td>10</td>
<td>61 (± 7)</td>
<td>26 (± 5)</td>
<td>33 (± 19)</td>
</tr>
<tr>
<td>1CAG7</td>
<td>12</td>
<td>25 800 (± 9 110)</td>
<td>16 300 (± 7 240)</td>
<td>31 400 (± 6 070)</td>
</tr>
<tr>
<td>2CAG7</td>
<td>11</td>
<td>72 300 (± 17 100)</td>
<td>69 000 (± 64 900)</td>
<td>14 900 (± 5 900)</td>
</tr>
<tr>
<td>CA2</td>
<td>11</td>
<td>610 000 (± 96 500)</td>
<td>114 000 (± 84 800)</td>
<td>590 000 (± 110 000)</td>
</tr>
<tr>
<td>CA1</td>
<td>8</td>
<td>475 000 (± 59 700)</td>
<td>566 000 (± 124 000)</td>
<td>403 000 (± 84 400)</td>
</tr>
<tr>
<td>proCA1</td>
<td>8</td>
<td>15 400 (± 4 390)</td>
<td>20 100 (± 8 140)</td>
<td>20 500 (± 6 160)</td>
</tr>
</tbody>
</table>

The values represent GUS activity of protein extracts expressed as picomoles 4-MU produced per minute per milligram of protein. Extracts from tissues were prepared and assayed as described in Materials and Methods (section 2.6). Data are mean values calculated from measurements on n individual transformants (± standard error of the mean). For each tissue type, two means representing GUS expression from two different promoter constructs were compared by the one-sided t-test. Means designated by the same letter are not significantly different at 0.05 level of significance.
Figure 5. GUS activity in individual non-transformed plants.

Figures 5 to 11:
The points on the figures represent levels of GUS activity in samples of specific tissue types of individual transgenic plants and wild-type controls. The points are joined to represent the trends of relative expression levels observed between different tissues for each individual plant.
Figure 6. GUS activity in individual plants transformed with the G7-GUS fusion.
Figure 7. GUS activity in individual plants transformed with the 1CAG7-GUS fusion.
Figure 8. GUS activity in individual plants transformed with the 2CAG7-GUS fusion.
Figure 9. GUS activity in individual plants transformed with the CA1-GUS fusion.
Figure 10. GUS activity in individual plants transformed with the CA2-GUS fusion.
Figure 11. GUS activity in individual plants transformed with the proCA1-GUS fusion.
A considerable plant-to-plant variation in the levels of GUS activity was observed within each group of plants carrying the same construct. However, most plants within each group showed a similar trend in the relative levels of GUS expression in different tissues (Figs. 5-11). High standard errors detected occasionally (e.g. with constructs 2CAG7 and CA2 in the stem; Table 2) could be attributed to the extremely unusual expression level observed in an individual transgenic plant (one out of eleven; Figs. 8, 10). This may be a result of transgene integration at a unique site, providing a chromatin environment which resulted in excessive stimulation or suppression of transcriptional activity. The trends observed for relative root:stem:leaf expression levels (Figs. 5-11) indicate a relatively predictable regulatory pattern for the individual transgenes examined.

Non-transformed, wild-type plants (WT) were regenerated from leaf discs that had not been treated with Agrobacterium, and were propagated and grown under the same conditions as transformed individuals. Measurements of GUS activity in non-transformed plants are presented in Fig. 5.

3.2.1 Constructs CA1 and CA2

Plants transformed with the CA1-GUS fusion exhibited similar high levels of GUS activity in the roots, leaves and stems (Table 2). Addition of a second copy of the 35S enhancer (construct CA2) did not result in a significant increase in GUS expression.
Furthermore, stem tissue containing the CA2-GUS fusion showed considerably less activity than that bearing construct CA1: about a 5-fold drop in GUS expression was observed in the CA2-GUS transformants, and a relatively high variation detected among individual transformants (Table 2) was a result of an unusually high GUS activity in a single plant (Fig. 10).

3.2.2 Constructs G7, 1CAG7 and 2CAG7

The expression conferred by construct G7 was detected at very low levels, the highest being found in the roots. Fusion of the 35S enhancer to the gene 7 promoter (construct 1CAG7) caused a several hundredfold increase in GUS expression (about 400-fold in roots, 600-fold in stems and almost 1000-fold in leaves; Table 2). The GUS expression was further stimulated only in the root (about 3-fold) by the enhancer duplication. However, the expression stayed unexpectedly low in leaf tissue of plants carrying the 2CAG7-GUS fusion. Also, GUS expression levels from construct 2CAG7 in the stem were generally low except for one transformant which showed GUS activity comparable to that from constructs CA1 and CA2 (718 nmoles/min.mg; Fig. 8). The overall expression from 1CAG7 and 2CAG7 chimeric constructs was well below that from the unrearranged 35S promoter (Table 2).
3.2.3 Constructs CA1 and proCA1

As explained above, all five constructs were fused to the GUS gene version containing the improved eukaryotic ("Kozak") sequence at the translation start site. The functional significance of the translational initiation context for the GUS reporter gene expression has not been quantitated in transgenic plants. A direct comparison between two transcriptional fusions, which differ solely in the translational initiator context, was made. The 35S promoter (nucleotides -343 to +8) was fused to: 1. the GUS gene with the ATG context resembling the "Kozak" consensus sequence which reads TCGACCATGGTC (construct CA1), and 2. the GUS gene with its original ATG context from \textit{E. coli} which reads TCCCTTATGTTA (construct proCA1). Results of the fluorometric assay revealed that GUS levels were 20-fold higher in leaves and about 30-fold higher in stems and roots of tobacco plants transformed with the CA1-GUS fusion.
3.3 Histochemical Localization of GUS Activity

To examine the pattern of expression conferred by individual promoter constructs, the GUS activity within plant tissues was identified by the presence of a blue precipitate formed by GUS-catalyzed conversion of an indigogenic substrate (X-gluc). Under the conditions used, no GUS activity was observed in any tissues examined from wild-type tobacco plants.

3.3.1 Expression in Root

Analysis of GUS expression was carried out in longitudinal and cross sections of roots, and in root tips (Fig. 12).

Expression from construct G7 was not detectable in most plants (data not shown). However, two plants out of ten showed a light staining in the procambium (provascular tissue) of the root tip (Fig. 12B).

Construct 1CAG7 gave expression throughout the root, which was generally stronger in the vascular tissue and epidermis (Fig. 12C). Variation in this pattern included only one plant (out of twelve analyzed) in which the stain was absent from the root tip but detectable in all differentiated tissues further from the tip.

Nine transformants containing the 2CAG7-GUS fusion exhibited strong expression
Figure 12. Histochemical localization of GUS activity in root tips of representative plants. (A) WT control; (B) construct G7; (C) 1CAG7; (x45).
Figure 12. (continued). (D) construct 2CAG7; (E) CA1; (F) CA2; (x45).
in all cell types (Fig. 12D). In two individuals, staining in the root was confined to the vascular cylinder.

With constructs CA1 and CA2, staining of variable intensity was observed throughout the root tissues in all plants analyzed (Fig. 12E,F).

### 3.3.2 Expression in Stem

GUS expression was analyzed in longitudinal sections through the stem apex (Fig. 13) and transverse sections of a stem (Fig. 14).

In longitudinal sections of the stem apex, weak expression from construct G7 was detected in the same individuals that showed detectable expression in the root tip. One showed light vascular staining in the petiole of an emerging leaf and the other in the differentiating cortex tissue and axillary bud just behind the apex (data not shown). No expression was detected in transverse sections of the stem (Fig. 14B,J).

All transformants carrying the 1CAG7-GUS fusion stained well, with no tissue specificity, at the stem apex (Fig. 13C) as well as in the stem cross section (Fig. 14C,K), where the stain was most intense in the vascular tissue and weakest in the stem pith.

Construct 2CAG7 showed a greater variation of expression patterns. At the stem apex, staining was found in apical and axillary meristems, leaf primordia, the youngest cortex cells and differentiated phloem (seven out of eleven plants - Fig. 15A). Two individuals showed the same expression pattern but lacked the stain in apical meristems.
Figure 13. Histochemical localization of GUS activity in longitudinal sections of the stem apex of representative plants. (A) WT control; (B) construct G7; (C) 1CAG7; (x45).
Figure 13. (continued). (D) construct 2CAG7; (E) CA1; (F) CA2; (x45).
Figure 14. Histochemical localization of GUS activity in stems of representative plants. Transverse section of the stem (x45): (A) WT control; (B) construct G7; (C) 1CAG7; (D) 2CAG7.
Figure 14. (continued). Transverse section of the stem (x45): (E) construct CA1; (F) CA2. Detail of the vascular tissue (x180): (G) construct CA1; (H) CA2.
Figure 14. (continued). Detail of the vascular tissue (x180): (I) WT control; (J) construct G7; (K) 1CAG7; (L) 2CAG7.
Figure 15. An example of a tissue-specific GUS expression from construct 2CAG7. (A) longitudinal section of the stem apex (x36); (B) detail of the vascular tissue (x230); (C) transverse section through the vascular tissue (x230).
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In transverse sections of a stem, the expression was most frequently limited to phloem (Fig. 15B,C), vascular cambium, a few cortex cells and the epidermis. Constitutive expression throughout the stem and stem apex occurred in only two transformants (Fig. 13D; 14D,L).

Construct CA1 conferred high levels of expression in every tissue at the apex (Fig. 13E) and throughout the stem (Fig. 14E,G). The staining was generally weaker in the pith. In one exception, the expression was confined to the vascular tissue and meristematic regions of the apex.

The staining appeared to be uniform also in stems of most CA2-GUS transformants (seven out of eleven total - Fig. 13F; 14F,H). Variations in the staining pattern occurred in four individuals: the stain was concentrated in apical and axillary meristems, epidermis and young vascular tissue at the stem apex, and in phloem and epidermis of a differentiated stem.

3.3.3 Expression in Leaf

GUS expression was studied in sections of petioles, midribs and mesophyll of developing leaves (about 3 cm in length - Figs. 16-17) and mature leaves (at least 14 cm in length - Figs. 18-19).

In the leaf, no GUS staining was detected in any of the plants transformed with the G7-GUS fusion (Figs. 16-19B).
Figure 16. Histochemical localization of GUS activity in developing leaves of representative plants - transverse section of the petiole. (A) WT control; (B) construct G7; (C) 1CAG7; (x45).
Figure 16. (continued). (D) construct 2CAG7; (E) CA1; (F) CA2; (x45).
Figure 17. Histochemical localization of GUS activity in developing leaves of representative plants - transverse section of the lamina. (A) WT control; (B) construct G7; (C) 1CAG7; (x45).
Figure 17. (continued). (D) construct 2CAG7; (E) CA1; (F) CA2; (x45).
Figure 18. Histochemical localization of GUS activity in mature leaves of representative plants - transverse section of the midrib. (A) WT control; (B) construct G7; (C) 1CAG7; (x45).
Figure 18. (continued). (D) construct 2CAG7; (E) CA1; (F) CA2; (x45).
Figure 19. Histochemical localization of GUS activity in mature leaf mesophyll of representative plants. (A) WT control; (B) construct G7; (C) 1CAG7; (D) 2CAG7; (E) CA1; (F) CA2; (x45).
With construct 1CAG7, the same pattern of expression was observed in developing and mature leaves. The stain was present in all cell types, and was strongest in the mesophyll, and in vascular tissue of midribs and petioles (Figs. 16-19C).

The expression from construct 2CAG7 was apparent throughout the mature leaves: staining was most consistent in the mesophyll (Fig. 19D) and generally weaker in large parenchyma cells of midribs and petioles (Fig. 18D). Eight plants showed similar patterns of expression in developing leaves (Figs. 16-17D), three plants, however, showed no staining in midribs and veins except in the phloem.

Construct CA1 conferred expression throughout the leaf tissues in all transformants (Figs. 16-19E). The overall expression was weaker in some cases but it stayed constitutive in terms of spacial distribution.

Expression from construct CA2 was detected in all cell types in both developing and mature leaves (Figs. 16-19F). In most plants, the staining was rather sporadic in parenchyma of midribs and petioles (data not shown).

3.3.4 Expression in Flowers

A histochemical assay was performed using sections from various reproductive tissues including the ovary (Fig. 20), stigma (Fig. 21), anther (Fig. 22) and pollen (Fig. 23), as well as from sterile flower parts (petals, sepals). Unless otherwise specified the same staining pattern was observed in flowers sampled before and just after opening.
Figure 20. Histochemical localization of GUS activity in flowers of representative plants - transverse section of the ovary. (A) WT control; (B) construct G7; (C) 1CAG7; (x45).
Figure 20. (continued). (D) construct 2CAG7; (E) CA1; (F) CA2; (x45).
Figure 21. Histochemical localization of GUS activity in flowers of representative plants - longitudinal section of the stigma. (A) WT control; (B) construct G7; (C) 1CAG7; (x45).
Figure 21. (continued). (D) construct 2CAG7; (E) CA1; (F) CA2; (x45).
Figure 22. Histochemical localization of GUS activity in flowers of representative plants - transverse section of the anther. (A) WT control; (B) construct G7; (C) 1CAG7; (x45).
Figure 22. (continued). (D) construct 2CAG7; (E) CA1; (F) CA2; (x45).
Figure 23. Histochemical localization of GUS activity in pollen of representative plants. (A) WT control (x180); (B) construct G7 (x230); (C) 1CAG7 (x180); (D) 2CAG7 (x230); (E) CA1 (x230); (F) CA2 (x180).
Most plants containing construct G7 showed no detectable GUS expression in any tissues (Figs. 20-22B). In two out of ten transformants, moderate staining was evident in pollen grains (Fig. 23B).

Construct 1CAG7 conferred expression in most floral tissues. Within the ovary, staining was well distributed throughout the ovary walls, placenta and the ovules, and was most conspicuous in vascular bundles (Fig. 20C). Variation in this pattern included one plant in which only the vascular tissue stained. The expression was also relatively strong in the stigma and the upper part of style (Fig. 21C). Although the stain in the stigma was harder to see through a film of secreted material over the epidermis, it was easily detectable in the papillae - hair-like extensions of epidermal cells. Six individuals showed weak staining throughout the lower part of style and the other six stained only in vascular tissue and trichomes. In anthers, most stain was concentrated in connectivum and vascular cylinder but weak expression was apparent in all other cell types (Fig. 22C). Filaments showed light staining throughout the sections but in some the stain was limited to the vascular tissue and hairs at the base, in a manner similar to the expression in the style. In eight transformants, pollen stained with an apparent 1:1 segregation ratio (Fig. 23C). In one plant, the proportion of stained pollen was about 70% and in the other three no expression in pollen was observed. The expression from construct 1CAG7 was also found throughout the sepals where vascular tissue and epidermis (namely trichomes and stomatal guard cells) stained with preference. Staining in petals was observed in all cell types but tended to be stronger in well-pigmented regions of the upper part, and in
vascular tissue throughout the petal and the lower flower tube where the overall staining was extremely light.

In transgenic plants carrying the 2CAG7-GUS fusion, the expression patterns were somewhat more variable. Most plants (seven out of eleven) showed uniform expression in the ovary (Fig. 20D). In four individuals the stain was limited to the phloem and random areas within the ovary wall. Consistent staining in the stigma was observed most frequently (Fig. 21D), with variations occurring in three plants that stained only in the region below the stigma. When detectable in the lower part of the style, light stain was present either in all cells (four plants) or in vascular tissue and trichomes only (three plants). Expression in the anthers appeared to be rather irregular but in most transformants it involved every type of tissue (Fig. 22D). Variations in staining pattern were represented by one example of staining in the connective tissue and one example of staining in the anther wall. Filaments showed the same kind of expression as in the style described above. GUS staining in pollen (with ratio 1:1) was evident in eight individuals (Fig. 23D). In one plant, ratio of stained to unstained pollen was at least 2:1 and in the other two, no pollen staining was detected. In the sepals, six plants showed uniform staining while three plants stained only in the phloem and epidermis (particularly in trichomes and guard cells of stomata). The staining in petals was detected either in all tissues (five plants) or in upper and lower epidermis and vascular tissue (four plants). Most transformants showed a very weak expression throughout the lower part of the floral tube. In petals, the pattern of expression from construct 2CAG7
resembled that from construct 1CAG7 in that the stain appeared to be stronger or more uniform in the pigmented upper edges of petals. The overall expression in petals of 2CAG7-GUS transformed plants was weaker than in 1CAG7 transformants.

The expression from construct CA1 in transgenic flowers was most consistent and showed little variation. Strong, uniform staining was observed in sections through the ovary (Fig. 20E). In seven out of eight plants, expression was high throughout the stigma (Fig. 21E); in one individual it was not detectable in the upper epidermis. The lower part of the style showed clear vascular staining. GUS activity was also relatively high in the anthers; although irregular in a few cases, it was present in every tissue and strongest in the vascular cylinder, connectivum and stomium (Fig. 22E). Half of the pollen grains were stained in all plants (Fig. 23E). Weak expression was found in the vascular bundles, parenchyma and the epidermis of filaments. All tissues within the sepals stained, with slight preference for the vascular tissue and epidermis. Expression in petals was quite uniform and was strongest in young tissue sampled before anthesis. In the lower part of the floral tube, staining was most obvious in the vascular tissue and very light in all other cell types.

With construct CA2, GUS expression in the flowers was generally at lower levels. Uniform staining was detected in the ovary as well as in the stigma (Fig. 20-21F). Vascular staining was observed in the lower part of the style (in six out of eleven plants). In anthers (Fig. 22F), expression was most concentrated in the connective tissue and adjacent inner tapetal cells, and in the wall, particularly around stomium (a slit-like
opening in the anther wall). Variation in this pattern was represented by staining restricted to the connective tissue (in three individuals). In eight transgenic plants, light and fairly uniform staining occurred in the filaments; in three plants stain was absent from most tissues but detectable in the trichomes. Pollen showed a relatively light blue staining in 10 transformants (with 1:1 segregation ratio - Fig. 23F)). Weak expression throughout the sepals was prevalent, with variations occurring as epidermis-limited stain in two individuals. Stain was also detectable in the petals of most plants analyzed; in one exception, it was confined to the upper epidermis and trichomes on the lower epidermis. The lower flower tube stained either in all types of cells (six plants) or in the vascular tissue and epidermis only (trichomes in particular - five plants).

3.3.5 Summary of the Histochemical Data

In the histochemical assay, GUS expression from the G7 construct remained below the threshold of detection in most tissues of plants analyzed. Only in a few individuals did construct G7 show a detectable staining: in meristems of root tips and stem apices, and in pollen grains. These findings (if correlated with specific GUS activity levels measured by fluorometric assay) indicated that levels of activity obtained for the G7 construct (less than 100 pmoles per min per mg of protein) were not detectable in the histochemical assay.

Constructs 1CAG7, 2CAG7, CA1 and CA2 were active in most cell types of all
plant organs. No striking differences between expression patterns conferred by these constructs were observed. However, individual transformants bearing construct 2CAG7 showed a greater variation of expression patterns, particularly in the stem tissue. Most plants exhibited rather strong preference for the phloem tissue in the stem and meristematic regions of stem apex. To a lesser extent this pattern was observed in stems of plants transformed with the CA2-GUS fusion.
4.1 The Effect of the Translational Initiator Context on the Efficiency of Expression from Chimeric Genes Containing the CaMV 35S Promoter Fused to GUS Coding Sequences

Although the nucleotides flanking the initiation codon of the GUS gene were altered to conform to eukaryotic expression systems (Jefferson, 1988), no detailed study of GUS gene expression in relation to the translational initiation context was carried out in stably transformed plant cells. We decided to take advantage of enhanced GUS expression by the use of the "improved" GUS gene construct for the transcriptional activation studies, as well as to analyze the differences between the levels of GUS expression conferred by the two GUS gene types. Two transcriptional fusions bearing the same 35S promoter sequences, GUS coding region and NOS polyadenylation signal, but differing in sequence flanking the ATG initiation codon, were constructed in an attempt to evaluate the effect of the ATG context on translational efficiency in the GUS reporter gene system. In transgenic tobacco plants, the GUS expression levels obtained with the optimized "Kozak" sequence (ACCATGG) were up to 30-fold higher than those with GUS gene bearing its natural 5' flanking region from E. coli (CTTATGT). The
stimulation observed in this study is much more profound than previously reported for plant expression systems. However, it is comparable to results obtained in other eukaryotic systems (Kozak, 1986), where a similar sequence change from TTTATGT to ACCATGG resulted in about 25-fold increase in protein expression in transfected mammalian cells.

The modified version of the GUS reporter gene has been used in several studies to investigate tissue-specific expression from various promoters in transgenic plants (e.g. Bevan et al., 1989; Hauffe et al., 1991) or transient expression at early stages of transformation with Agrobacterium tumefaciens (Janssen and Gardner, 1989). Some comparative studies were carried out in vitro using the two derivatives of the GUS coding region fused to the tobacco mosaic virus (TMV) 5' untranslated leader sequence (Sleat et al., 1987). The consensus start codon context itself allowed only a 1.3- to 2.0-fold increase in expression of uncapped mRNAs in two cell-free translation systems (either in presence or in absence of the TMV leader). Several capped TMV leader-GUS mRNAs, bearing either the eukaryotic or the native initiation context, were introduced and expressed in tobacco protoplasts (Gallie et al., 1987; 1988). Relative levels of GUS specific activity showed that a maximum 5-fold stimulation of expression can be attributed to the improved AUG context.

Based on a survey of large number of mRNA sequences and on the in vitro binding experiments (Kozak, 1981), the sequence (A/G)NNAUGG has been identified as the optimal context for translational initiation by eukaryotic ribosomes. A more recent
survey of vertebrate mRNAs has revealed that GCCGCC(A/G)CCAUGG is a consensus sequence for translational initiation (Kozak, 1987). A purine (usually A) in position -3 (3 nucleotides upstream of the AUG triplet) is the most highly conserved nucleotide in eukaryotic mRNAs, and a mutation in that position affects translation in mammalian cells more profoundly than a point mutation anywhere else (Kozak, 1986). In the absence of a purine in position -3, G at +4 (immediately following the AUG codon) is essential for efficient translation, and contributions of nucleotides in positions -1 and -2 can also be detected (Kozak, 1986). Lütcke et al. (1987) pointed out that plant mRNAs have a slightly different consensus sequence (AACAAUGGC); furthermore, the nucleotide at -3 position does not modulate the efficiency of translation by wheat germ ribosomes \textit{in vitro}.

Our results suggest that the enhancement of GUS expression in transgenic plants due to the improved sequence flanking the initiation codon is remarkably strong, and that modification of the translational initiation context should prove very useful in stimulating the expression of other foreign genes in transgenic plants, particularly those originating from a heterologous source.
4.2 CaMV 35S Enhancer in Combination with the Gene 7 Promoter

In this study, GUS activity provides an indication of transcriptional activity of different promoter constructs, although, aspects of overall gene expression, including mRNA and protein stability, could modulate the level of GUS activity detected. The promoter constructs were fused to the GUS reporter gene in a similar fashion so that the resulting gene fusions are transcribed into RNAs of similar structure and affinity for the ribosomes, which are presumably processed in an identical manner during subsequent steps of gene expression.

As reported earlier (Kay et al., 1987), the presence of a divergently oriented unrearranged 35S enhancer resulted in an estimated 40-fold increase in the rate of gene 7 transcription and a further 10-fold increase in transcript accumulation was observed when the upstream 35S enhancer region was duplicated. Transcription of gene 7 in the presence of the unrearranged 35S enhancer produced RNA levels similar to those generated by the 35S promoter itself and both were simultaneously activated by the enhancer duplication.

Fluorometric assay revealed very low levels of GUS expression from the gene 7 promoter. A single copy of the 35S enhancer region ("domain B") fused to the gene 7 promoter stimulated expression of GUS several hundredfold in all plant organs analyzed. However, with a duplicated enhancer a significant increase of GUS enzyme activity
occurred only in the root tissue. Furthermore, GUS expression levels from constructs 1CAG7 and 2CAG7 were only up to 15% of those obtained with the intact 35S promoter region (construct CA1), which presumably reflects the natural regulatory characteristics of the different promoter types.

Since the previous studies were carried out using the entire gene 7 including its own 5' and 3' regulatory sequences, it can be argued that the native gene 7 transcript is innately more stable than the chimeric RNA obtained with GUS transcriptional fusions. In the latter case, the 5' untranslated region of gene 7 (15 nucleotides long) is fused to the 5' untranslated region of GUS via the introduced restriction site, thus increasing its total length and possibly creating an interference between the two sequences. While studying expression of fusions between the promoter of the chlorophyll \(a/b\) binding protein (\(cab\)) gene and octopine synthase (\(ocs\)) gene sequences (Jones et al., 1985), it was noticed that transcriptional \(cab/ocs\) fusions in which a linker sequence interrupted the 5' untranslated region gave rise to less chimeric mRNA than a translational fusion bearing the unrearranged \(cab\) promoter region. Likewise, the amount of mRNA from the chimeric \(cab/ocs\) genes was only a fraction of that obtained from the endogenous wild-type \(cab\) gene.

Another possible cause of differential transcriptional regulation may be the orientation of the 35S enhancer relative to the gene 7 promoter. Divergent orientation (as in Kay et al., 1987) may, in this specific case, prove to be more advantageous for the interaction of the two regions. Also, the absence of the 35S domain A which was, in this
work, replaced by the gene 7 promoter may have a negative effect on the overall transcriptional stimulation. Domain A may interact synergistically with other cis-regulatory elements from the 35S promoter (Benfey et al., 1990b) and play an accessory role in increasing transcriptional activity of upstream sequences within domain B (Fang et al., 1989). Thus, differences in expression levels from the chimeric constructs and the 35S promoter fragment (domain A + B) may also reflect the difference between the natural strengths of the gene 7 promoter versus the 35S domain A.

Since the overall gene expression relies on both transcriptional and translational efficiencies, subsequent steps of post-transcriptional and translational regulation may modify the final levels of GUS enzyme expressed from the G7 chimeric transcripts. The sequences between the 5' end of mRNA and the AUG initiation codon and a possible interaction between the gene 7 and GUS 5' untranslated regions may affect the migration of 40S ribosomal subunits and thus the efficiency of recognition and translation of mRNA.

The gene 7 promoter, however, does not seem to have a significant negative effect on the relative root/leaf expression ratios when it replaces domain A of the 35S promoter, which was preferentially active in root tissue of transgenic tobacco seedlings (Benfey et al., 1989). It was estimated that, in tobacco seedlings, domain B alone conferred about 10-fold higher expression levels in the leaf than in the root (Benfey et al., 1989). In mature plants, analyzed in this study, expression levels from the gene 7 promoter fused to domain B (construct 1CAG7) did not differ significantly between the
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root and the leaf. In addition, the gene 7 promoter in combination with two copies of domain B (construct 2CAG7) appeared to be almost 5-fold as active in the root than in the leaf.

4.3 The Effect of CaMV 35S Enhancer Duplication on Its Native Promoter

The results of fluorogenic GUS assay showed no significant increase in the levels of GUS expression following the 35S enhancer duplication. Since GUS expression from the duplicated 35S enhancer construct (CA2) was extremely high, a series of diluted protein extracts from CA2-GUS transformants was assayed in fluorogenic reactions (data not shown). The GUS specific activity was the same as with the extracts analyzed by the standard procedure (as described in section 2.6). Previous studies (Kay et al., 1987) showed that, in leaf, tandem duplication of the upstream region of the 35S promoter (nucleotides -343 to -90) resulted in a transcriptional increase of up to an order of magnitude. The discrepancy between relative levels of transcript accumulation observed by Kay et al. (1987) and levels of protein expression detected in this work can be explained by a possible translational threshold for specific mRNA. It may also reflect an interference between this specific RNA-coding region and the promoter activity at higher multiplicity of units in the upstream region. These levels of GUS activity (for constructs CA1 and CA2) are significantly higher than any reported elsewhere for expression from constructs carrying the 35S upstream region. The unexpectedly low amount of GUS
activity in stem obtained with construct CA2 may be a result of its preference for specific types of tissue (as discussed below - section 4.5.2).

4.4 Variation between Independent Transgenic Plants

Variation in expression of the introduced genes observed between individual transformants is often seen in transgenic plants generated by Agrobacterium-mediated transformation. Several factors may be involved in this inter-transformant variability. Copy number of the transgene may vary among individual transformants, but no positive correlation has been determined between the copy number and the level of expression observed (Jones et al., 1985 and 1987; Odell et al., 1987; Shirsat et al., 1989). However, some studies indicate that low expression levels and higher methylation of the introduced genes occur in plants carrying multiple T-DNA insertions (Jones et al., 1987; Hobbs et al., 1990). The activity of the introduced gene may be influenced by the site of the integration on the chromosome ("position effect"). Cis-elements within adjacent plant DNA can have a positive or negative effect on the expression of the transgene (Odell et al., 1987; Sanders et al., 1987). Also, the interaction between trans-factors and cis-elements within the introduced DNA can be altered. Transformation can produce insertions of truncated or rearranged T-DNA but a direct correlation has not been detected between low expression and deletions or rearrangements (Jones et al., 1987). Differences in gene expression can occur in physiologically different plant material, at
different developmental stages and in plants grown under different environmental conditions. However, Hobbs et al. (1990) have shown that expression of CaMV 35S-GUS chimeric gene in tobacco plants grown in the field and in a controlled environment (under low or high light) were very similar. In histochemical assay, the staining intensity can vary with cell size, the number of cells per unit area and metabolic activity. Another possible variable is the non-uniform penetration of the histochemical substrate into different cell types (Jefferson et al., 1987). Thus, GUS expression in certain cells can stay below the limit of detection.

Plants described in this work were grown under identical environmental conditions and analyzed at the same developmental stages. At least eight independent transgenic plants were examined for each construct and both positive and negative controls were included. The position effect is most likely the most important although not exclusive cause of variable expression. The principal expression pattern as well as the variations that occurred in histochemical analysis are reported.

Blue staining of approximately 50% of the pollen grains in almost all plants that showed a detectable pollen expression suggests a 1:1 segregation of a single transcriptionally active transgene locus. GUS expression in pollen can thus indicate whether the transgene is integrated and expressed at a single or multiple sites in the plant genome.
4.5 Interpretation of Histochemical Data

The fluorometric assay provides a highly sensitive method of quantitation of the gene expression over a large range of values through the use of the reporter enzyme kinetics. However, it does not reflect the differences between individual types of cells. Histochemistry permits a direct localization of the reporter enzyme activity at the level of a single cell although its sensitivity is limited. Nevertheless, these two types of assays together represent one of the best means presently available for studies of gene expression in plant tissues.

4.5.1 Expression from Gene 7 Promoter and Intrinsic GUS-like Activities

Previous histochemical assays indicated that vegetative organs of seedlings and mature tobacco plants, as well as the female reproductive organs and seeds lack intrinsic GUS-like activities (Jefferson et al., 1987; Hu et al., 1990). However, during later stages of male gametogenesis, consistently high levels of GUS activity were found in tapetal cells and microspores in tobacco, potato and tomato plants (Plegt and Bino, 1989).

Under the conditions used in this work, no endogenous GUS-like activity was observed in any tissue analyzed in the histochemical assay. Thus, detection of very low levels of GUS expression was possible, such as those observed in G7-GUS transformants.
The GUS activity found occasionally in the meristematic regions and pollen was expressed by the introduced gene under control of the gene 7 promoter. This tissue-specific staining may be in part a consequence of higher metabolic activity of these cell types. However, in most transformants, GUS expression from the gene 7 promoter stayed below the threshold of detection.

The promoter region of another T-DNA gene with unknown function (gene 5) has been studied in transcriptional fusion with nopaline synthase coding sequences (Koncz and Schell, 1986). Expression from the gene 5 promoter was regulated in a tissue specific fashion and showed dependency on the levels of endogenous plant growth factors.

4.5.2 Expression from the Chimeric CaMV 35S-Gene 7 Promoter Constructs

The pattern of expression from construct 1CAG7 was very similar to that observed with the intact 35S promoter fragment. In this case, the gene 7 promoter (in combination with the 35S domain B) did not appear to modify the transcriptional activity in terms of its spatial distribution in transgenic tissues.

An example of a positive interaction between the gene 7 promoter and the 35S domain B was a consistent, uniform expression in all root tissues conferred by constructs 1CAG7 and 2CAG7. In roots of mature tobacco plants, domain B alone gives a detectable expression only in the phloem, part of the root cap and occasionally in the cortex cells (Benfey et al., 1990b). Expression from the gene 7 promoter alone was
detected only in the provascular tissue of the root. Thus, a synergistic interaction between the cis-elements of domain B and those within gene 7 promoter may mediate expression in other cell types of the root.

Construct 2CAG7 was able to confer expression in all cell types of most plant organs. In most plants, however, it showed a preference for the phloem tissue of the stem and meristematic regions of the stem apex. This tissue specific staining occurred in 2CAG7-GUS transformants which showed lower expression levels as determined by the fluorometric assay. This could suggest that GUS expression in other cell types remained below the detection threshold. Also, staining in the phloem elements and meristematic cells of the stem may be more easily detectable due to higher metabolic activity and absence of a large vacuole in these cell types. The same type of tissue specific staining was, to a lesser extent, apparent in stems of plants transformed with CA2-GUS fusion where the overall expression levels were relatively high.

Previous studies showed that, in young tobacco seedlings, expression from domain B appeared to be restricted to the vascular tissue of the hypocotyl but no staining was observed in the region below the apical meristem (Benfey et al., 1989). In mature plants, however, domain B conferred strong expression in nearly all cells of the stem and stem apex (Benfey et al., 1989; 1990b). The 35S enhancer (-343 to -90) appears to be made up of multiple cis-elements, each of which confers expression in specific cell types but also acts synergistically with the 35S domain A to activate expression in more tissues
(Benfey et al., 1990ab). A higher concentration of these cis-elements in the duplicated enhancer may modify its activity by determining specificity for certain types of cells, particularly in the absence of domain A. These results suggest that duplication of the upstream region of the 35S promoter creates a promoter element with a greater degree of specificity that may target the expression to particular cell types, such as meristematic cells and the phloem tissue of the stem.

4.5.3 Expression from the CaMV 35S Promoter

In vegetative parts of transgenic plants, the CaMV 35S promoter (construct CA1) appeared to be active in all cell types. In accordance with previous results (Benfey et al., 1989), the 35S promoter (nucleotides -343 to +8) directed expression in epidermis, cortex, pith and vascular tissue of the stem. In stem, GUS expression from the 800 bp long fragment of the 35S promoter was concentrated in phloem as observed by Jefferson et al. (1987). The same promoter fragment has been used to direct GUS expression in anthers of transgenic tobacco flowers (Plegt and Bino, 1989). Prior to anther dehiscence, staining was manifest in the vascular cylinder, the connectivum, the stomium and the microspores. In our study, a shorter version of the 35S promoter (-343 to +8) produced additional staining in anther walls.

In petals and sepals, similar expression patterns have been observed using the entire 35S upstream region (nucleotides -941 to +8; Benfey and Chua, 1989) and the
combination of domain A and B (nucleotides -343 to +8; Benfey and Chua 1990). Here, in addition to the vascular staining in the lower flower tube (observed previously), weak expression was detected in the mesophyll and epidermis, and more consistent staining occurred in the mesophyll of mature petals.

Using the 35S promoter linked to the GUS coding region with the modified ATG context, expression was observed in more cell types than previously reported with constructs bearing the native ATG flanking sequence. This may be a result of the improved AUG recognition and translational efficiency resulting from the presence of a typical eukaryotic translational initiator. Also the presence of the shorter promoter fragment may account for differences in expression seen in individual cell types, such as the anther walls.
Summary

The enhancement of expression produced from the proximal promoter region of gene 7 when combined with the domain B of the CaMV 35S gene was several hundredfold. The gene 7 promoter region alone showed very low levels of activity in the sensitive fluorometric assay, but it was undetectable in the histochemical assay, with the exception of low activity in pollen and meristematic cells. Thus the presence of the 35S enhancer region allowed the interactive expression of the 35S B-gene 7 promoter combination to be visualized in transgenic tissues. The contribution of tissue or cell-specific activity attributed to the gene 7 promoter region with the upstream 35S region was determined by comparison to the distribution of activity of the homologous combination of 35S domains A and B. The pattern was similar in both cases, suggesting that the proximal promoter of gene 7 did not exert a strong specific modulation effect on the 35S promoter qualitative expression.

The levels of expression obtained with the 35S domain B in combination with the gene 7 promoter were at least an order of magnitude lower than with the homologous 35S domain A (of approximately the same length as the gene 7 promoter fragment). This may reflect the natural promoter strengths of the gene 7 promoter and the 35S domain A. It also suggests that the proximal region of the gene 7 promoter shows some degree of non-productive interaction with domain B compared to that with domain A. The combination of the promoter domains A and B of the 35S gene may represent a specific
synergistic interaction developed under the control of evolutionary forces on the CaMV genome.

With an upstream duplicated enhancer unit, the pattern of expression changed somewhat both in the presence of the proximal domain A and with the proximal gene 7 promoter fragment. A preferential expression in phloem and actively dividing meristematic cells was observed in plant stems. Such changes in the pattern of transgene activity presumably reflect the precise interactions of individual regulatory gene elements.

Sequences involved in ribosome recognition had a strong impact on the overall levels of expression observed from structurally related constructs. Comparison of the glucuronidase levels from constructs with a wild-type sequence (of uidA locus of E. coli) flanking the initiation codon, with those having a modified eukaryotic translational start site, indicated greater than 20-fold differences in each of the tissues examined. Although primary regulation of expression levels is dependent on promoter related characteristics, other factors, such as mRNA structure have a significant contribution to the levels of the final gene product. The differential effects resulting from both transcriptional and translational regulation are important considerations for effective expression from chimeric transgenes.
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