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**EXPRESSION OF AN ARABIDOPSIS THALIANA 2S ALBUMIN
STORAGE PROTEIN GENE IN TRANSGENIC
NICOTIANA TABACUM AND BRASSICA NAPUS**

by

JANICE MARIE DEMOOR, B.Sc., M.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario

February 21, 1992

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JANICE MARIE DEMOOR, B.Sc., M.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis Supervisors

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Date: May 5, 1994

ABSTRACT

An Arabidopsis thaliana Landsberg erecta 2S albumin storage protein gene (atar1) was isolated and sequenced. It differed from the homologous gene in A. thaliana C24 (at2S1) (Krebbers et al., 1988) by 1 basepair. Transformation vectors were constructed containing 5' regulatory sequences from the arabin gene fused to the coding region of the gene encoding the β -glucuronidase (GUS) reporter molecule. Expression of the constructs was studied in transgenic Nicotiana tabacum and Brassica napus. A comparison of GUS activity levels in transgenic N. tabacum plants transformed with different atar1-GUS constructs indicated that the arabin gene 5' sequences include an enhancer-like element.

Seed-specific fluorogenic GUS enzyme activity was first detected in transgenic N. tabacum embryos at the late globular/early heart stage. In transgenic B. napus seeds, fluorogenic GUS activity was not detected until the early cotyledon stage of development. Spatial localization of GUS activity was determined by histochemical staining. The GUS activity first appeared in the ground meristem cells of the upper axis of N. tabacum embryos and in the lower axis of E. napus microsporic embryos. In both kinds of embryos, the GUS

staining radiated out from these initial sites as the embryos developed and matured. These results are discussed in the thesis with respect to possible regulatory mechanisms affecting storage protein gene expression.

The mRNA levels for endogenous storage proteins (1.7S napin and 12S cruciferin) were compared in B. napus microsporic and zygotic embryos over a range of developmental stages. The temporal patterns of napin and cruciferin gene expression were found to be similar in the two types of embryos but storage protein mRNA levels were lower in microsporic embryos. Unexpectedly, napin mRNA was detected in microspores after the 4-day 32.5°C treatment used to induce embryogenesis. Napin expression appeared to be correlated with the potential of a sample to form embryos.

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To my family

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LIST OF ABBREVIATIONS

A	adenine
ABA	abscisic acid
BA	6-benzylaminopurine
bp	base pairs
BPB	bromophenol blue
BSA	bovine serum albumin
C	cytosine
CaMV	Cauliflower Mosaic Virus
CAT	chloramphenicol acetyltransferase
Cb	carbenicillin
Cb ^R	carbenicillin-resistant
Cef	cefotaxime
cm	centimetre
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphosphate
DEAE	diethylaminoethyl
DH ₂ O	distilled water
DDH ₂ O	double distilled water
DNA	deoxyribonucleic acid

dpa	days post anthesis
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
G	guanine
g	grams
Gent	gentamycin
Gent ^R	gentamycin-resistant
GUS	β -glucuronidase
hr	hour(s)
IPTG	isopropyl- β -D-thiogalactopyranoside
kb	kilobases
kbp	kilobase pairs
kD	kilodaltons
Km	kanamycin
Km ^R	kanamycin-resistant
Km ^S	kanamycin-sensitive
L	litre
M	molar
m ²	square metre
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
MU	4-methylumbelliferone
MUG	4-methylumbelliferyl- β -D-glucuronide

N	normal
NAA	1-naphthaleneacetic acid
ng	nanogram(s)
nM	nanomolar
nmol	nanomoles
NPT	neomycin phosphotransferase
NOS	nopaline synthase
O.D.550	optical density measured at wavelength 550 nm
O.D.600	optical density measured at wavelength 600 nm
PEG	polyethylene glycol
pfu	plaque-forming units
pmol	picomoles
PVP	polyvinylpyrrolidone
rbcS	small subunit of ribulose-1,5- bisphosphate carboxylase
Rif	rifampicin
Rif ^R	rifampicin-resistant
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec	second(s)
T	thymine
tRNA	transfer ribonucleic acid
UV	ultraviolet

V	volt(s)
Vhr	volt hours
v/v	volume per volume
wk	week(s)
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
X-gluc	5-bromo-4-chloro-3-indolyl- β -D-glucuronide
μ Ci	microCurie(s)
μ E	microEinstein(s)
μ g	microgram(s)
μ l	microlitre(s)
μ m	micrometre(s)
2,4-D	2,4-dichlorophenoxy acetic acid

CHAPTER 1

GENERAL INTRODUCTION

Development of an organism is governed by complex genetic regulatory processes which can be influenced by numerous internal and external factors. The sum of gene expression in an organism determines the outcome of the developmental program. Due to the complexity of developmental processes, scientific investigations are often limited to the study of a specific gene and its role in development. The ability to create transgenic plants and animals has allowed scientists to study the expression of a cloned gene in vivo, and has also opened the door to genetic engineering.

The objective of this thesis project was to study the expression of a seed storage protein gene during seed development. Storage proteins as well as storage oils are deposited within the seed in large quantities as the plant embryo matures, and act as nutritional reserves during seed germination.

Isolation of a storage protein gene was the first step in the project. A gene encoding a 2S albumin storage protein (arabin) was isolated from the genome of Arabidopsis thaliana. The small A. thaliana genome size (7×10^7 bp),

the low level of repetitive DNA, and the predominance of gene families with relatively few members simplify the process of genomic cloning (Meyerowitz, 1989). The gene was sequenced and transformation vectors were constructed containing sections of the regulatory region of the arabin gene fused to the coding region of the β -glucuronidase (GUS) reporter gene. GUS was selected because of its sensitivity and versatility. GUS activity can be quantitated using a fluorometric method. A histochemical assay is also available for localization of enzyme activity within tissues. Chapter 3 outlines the gene isolation, sequencing and analysis of the promoter and upstream regions of the arabin gene, as well as the construction of transformation vectors.

Both Nicotiana tabacum (tobacco) and Brassica napus were transformed with the atar1-GUS constructs via Agrobacterium tumefaciens - mediated transformation. Seed-specific gene expression is of particular interest in Brassica species due to the commercial value of the oilseeds. N. tabacum is often used as a gene recipient due to the relative ease of transformation and regeneration of this species. Chapter 4 documents the results of the N. tabacum transformation experiments. Expression of the reporter enzyme was traced quantitatively, spatially, and temporally in the seeds and somatic tissues of transgenic plants.

In B. napus, haploid embryos derived from microspores can be obtained using a tissue culture system. Large numbers of staged embryos can be isolated from these cultures which allows for the analysis of embryonic gene expression even at the early stages of development. Expression of endogenous storage protein genes was investigated in the developing B. napus microsporic embryos using Northern blot analysis. The results are presented in Chapter 5.

Chapter 6 contains the results of the B. napus transformation experiments. GUS activity was studied in both zygotic and microsporic embryos of transgenic plants.

The final chapter summarizes the thesis work and discusses the similarities and differences in expression of the atar1-GUS constructs in transgenic N. tabacum and B. napus embryos. The significance of these results and the possible role of 2S albumin gene expression in embryo development are also discussed.

In the survey of the literature that follows, relevant background information is reviewed. In some sections, particular emphasis has been placed on the three plant species used in this study, N. tabacum, B. napus, and A. thaliana.

1.I. LITERATURE REVIEW

1.I.1. Storage Proteins

Seed storage proteins accumulate to significant amounts during seed development. They are then rapidly degraded after germination to provide the seedling with reduced nitrogen (Higgins, 1984). Storage proteins are divided into four major classes, based on solubility (Osborne, 1924). Albumins are soluble in water, globulins are soluble in saline solutions, prolamins are extractable in aqueous alcohol and glutelins can be extracted in acids or alkalis.

The major seed proteins of dicotyledonous plants (dicots) are globulins and albumins. Globulins contain large amounts of arginine, glutamine, glutamate, asparagine, and aspartate which leads to a high nitrogen content. The 11-12S globulins (eg. legumin in Pisum sativum [peas], glycinin in Glycine max [soybean] [Casey et al., 1986], cruciferin in A. thaliana and B. napus [Heath et al., 1986; Crouch and Sussex, 1981]), are usually composed of six subunits, each consisting of an acidic (α) and a basic (β) polypeptide which are derived from the same precursor (Barton et al., 1982; Maplestone et al., 1985; Casey et al., 1986; Simon et al., 1985; Meinke et al., 1981). The signal sequence is removed cotranslationally from the precursor and the α and β

subunits are cleaved post-translationally but remain disulfide-linked (Simon et al., 1985).

The 7-8S globulins form the vicilin group, which is more heterogeneous than the legumin group. Conglycinin (soybean) consists of several minor components and three major subunits, α' , α , and β , each consisting of a single polypeptide (76 kD, 72 kD, and 53 kD, respectively). Pea vicilin contains more than 10 polypeptides (12 kD-75 kD) (Shotwell and Larkins, 1989). As with legumins, leader sequences are removed co-translationally from the precursors. No vicilins have been found in the Brassicaceae or in the sunflower (Borroto and Dure, 1987).

Members of the 2S albumin class of storage proteins (eg. 1.7S napin in B. napus [Crouch and Sussex, 1981], 2S arabin in A. thaliana [Heath et al., 1986]) are found in the oilseeds of diverse species. Youle and Huang (1981) analysed seeds from 11 different families which included sunflower, mustard, brazil nut, castor bean and cotton. Albumins constituted 20-60% of total seed protein. In addition to a high nitrogen content, 2S albumins contain relatively large amounts of cysteine and methionine and therefore act as sulfur as well as nitrogen reserves. A list of cloned 2S albumin storage protein genes for B. napus and A thaliana is presented in Table 1.1.

The major storage proteins of monocotyledonous plants (monocots) are prolamins and glutelins. Prolamins contain a

Table 1.1 Cloned 2S Albumin Storage Protein Genes
from Arabidopsis thaliana and Brassica sp.

The A. thaliana 2S albumin gene family consists of 4 members which are arranged in a tandem array (Krebbers et al., 1988).

It has been estimated that the multigene family encoding napin is comprised of 10 to 16 genes in B. napus (Scofield and Crouch, 1987; Josefsson et al., 1987).

** No sequence information was available for the 1-2 gene.

GENE	PROTEIN	PLANT SPECIES	TYPE OF CLONE	REFERENCE
<u>at2S1</u>	2S arabin	<u>A. thaliana</u>	genomic	Krebbers <u>et al.</u> , 1988
<u>at2S2</u>	2S arabin	<u>A. thaliana</u>	genomic	Krebbers <u>et al.</u> , 1988
<u>at2S3</u>	2S arabin	<u>A. thaliana</u>	genomic	Krebbers <u>et al.</u> , 1988
<u>at2S4</u>	2S arabin	<u>A. thaliana</u>	genomic	Krebbers <u>et al.</u> , 1988
<u>napA</u>	1.7S napin	<u>B. napus</u>	genomic	Josefsson <u>et al.</u> , 1987
<u>gNa</u>	1.7S napin	<u>B. napus</u>	genomic	Scofield and Crouch, 1987
<u>Bng NAP1</u>	1.7S napin	<u>B. napus</u>	genomic	Baszczynski and Fallis, 1990
<u>napB</u>	1.7S napin	<u>B. napus</u>	genomic	Ericson <u>et al.</u> , 1991
<u>1-2</u>	1.7S napin	<u>B. campestris</u>	genomic**	Radke <u>et al.</u> , 1990
<u>pN1</u>	1.7S napin	<u>B. napus</u>	cDNA	Crouch <u>et al.</u> , 1983
<u>pN2</u>	1.7S napin	<u>B. napus</u>	cDNA	Crouch <u>et al.</u> , 1983
<u>pNAP1</u>	1.7S napin	<u>B. napus</u>	cDNA	Ericson <u>et al.</u> , 1986

high proportion of proline and glutamine but very little lysine. The group includes the barley hordeins, wheat gliadins and the zeins of maize (Ingversen, 1983; Rafalski et al., 1986; Shotwell and Larkins, 1989). Glutelins are found in large amounts in rice (80% of total endosperm protein) (Ramachandran and Raghavan, 1990) and wheat (50% of the seed storage proteins) (Payne et al., 1984). The major site of storage protein deposition in cereals is the endosperm, although accumulation does occur in the embryos (Higgins, 1984; Robert et al., 1989; Ramachandran and Raghavan, 1990).

Minor seed proteins such as trypsin inhibitors, lectins, lipoxxygenases, ureases and amylases may also act as storage proteins, but due to their amount rather than their intrinsic properties (Casey et al., 1986; Kreis et al., 1986; Ryan, 1988; Kreis et al., 1987).

1.1.2. Seed and Embryo Development

Embryogenesis begins with fertilization. The pollen tube from a germinated pollen grain grows through the transmitting tissue of the style and enters the ovule through the micropyle. Two sperm nuclei are released. One fuses with the ovum (egg) to form the zygote (2n). The other sperm nucleus fuses with the polar nuclei of the central

cell to give rise to the $3n$ endosperm (Crouch, 1988). During seed development, the outer layers of the ovule thicken and harden and form the seed coat (Browder, 1984).

The seed coat may synthesize a variety of substances, including pigments, enzymes and structural compounds. It may also play a role in germination inhibition through physical constraints, regulation of the amount of water and light reaching the embryo, and synthesis of chemical inhibitors (Crouch, 1988).

The seed endosperm usually develops more rapidly than the embryo. It is believed to contribute nutrition and hormones to the developing embryo but it is not known if the endosperm is the source or merely a conducting tissue for these substances. The endosperm is usually coenocytic at first but becomes cellular as development proceeds. The endosperm cells differentiate and may perform different functions according to their position in the seed. In most angiosperms, the endosperm surrounds the early embryo everywhere except where the suspensor attaches (Crouch, 1988).

The first division of the zygote gives rise to two cells; one goes on to form the embryo, the other forms the suspensor. The suspensor attaches the embryo to the ovule wall through its basal cell, and is believed to be involved in the transfer of nutrients and other molecules to the young embryo (Crouch, 1988; Earle, 1982).

As the embryo develops, the first tissue that becomes evident is the epidermis. The next phase in development is the formation of the shoot-root axis. In dicotyledonous plants, cells divide in two ridges opposite the suspensor and begin to form the cotyledons resulting in a heart-shaped embryo with bilateral symmetry. Prevascular tissue can be detected as a central cylinder of cells at the heart-stage. The root apical meristem forms near the suspensor and often includes the first suspensor cell, or hypophysis (Browder, 1984). Cell differentiation continues as the embryo elongates to the torpedo stage. As the embryo grows, the cotyledons begin to curve to fit into the embryo sac. In B. napus and A. thaliana, the cotyledons continue to grow and bend until they are packed tightly against the embryo axis in the mature seed (Figure 1.1, B,C).

The initial phase of cell differentiation in the developing seed is followed by a maturation phase during which the cells expand and accumulate storage reserves. In B. napus, embryogenesis consists of four stages: (i) a cell division stage, (ii) a stage where mostly storage oil is synthesized, (iii) a stage where mostly storage proteins (napin and cruciferin) are synthesized, (iv) synthesis of mostly apolipoprotein (oleosin) (Murphy and Cummins, 1989a). Cell division occurs for approximately three weeks after anthesis, depending on conditions and cultivar, by which time the cotyledons have started to form (Figure 1.1, C).

Figure 1.1 Stages of Embryo Development

Embryo development is compared in three plant species: A, Nicotiana tabacum; B, Arabidopsis thaliana; C, Brassica napus.

dpa: days post anthesis.

Adapted from Chen et al. (1988), Meinke and Sussex (1979), Haughn and Somerville (1988), Raven et al. (1981), and Crouch and Sussex (1981).

EMBRYO DEVELOPMENT

A. *Nicotiana tabacum*

B. *Arabidopsis thaliana*

C. *Brassica napus*

Starch and sugars accumulate during this phase but levels decline as storage oils and proteins are produced (Tykarska, 1982; Norton and Harris, 1975). When cell division ceases, growth continues through cell expansion. Storage triacylglycerols can be detected in field-grown B. napus before the embryo reaches two weeks of age but the most rapid phase of deposition is between 4 and 6 weeks. The oil accumulates as large oil bodies (2 to 4 μm diameter). Napin and cruciferin can first be detected anywhere from 3.5 to 5 weeks after anthesis (Crouch and Sussex, 1981; Murphy and Cummins, 1989a). Rates of synthesis for the two proteins are similar, with a maximum between 5 and 7 weeks, but napin accumulation stops after 42 dpa while cruciferin continues to accumulate until seed maturity. The amount of cruciferin in the dry seed is 2 to 4 times the amount of napin (dry weight) (Crouch and Sussex, 1981; Murphy and Cummins, 1989a). The third major seed protein in B. napus is oleosin, a 19 kD lipoprotein thought to be directly inserted into oil bodies by ribosomes (Murphy et al., 1989) and degraded at the time of storage oil mobilization following germination (Murphy and Cummins, 1989b). Oleosin is synthesized between 7 and 10 weeks after anthesis, during which time oil bodies are reduced to 0.3 to 1.0 μm in diameter. Proposed functions for oleosin include the prevention of coalescence of the oil bodies, stabilization and protection of oil bodies from degradative enzymes during dehydration and germination, and

attachment sites for lipases after germination (Murphy and Cummins, 1989a; Murphy *et al.*, 1989).

Mature B. napus seeds contain approximately 45-55% (w/w) oil and 25-30% (w/w) protein. The protein consists of approximately 50% cruciferin, 20% napin, 20% oleosin (Murphy and Cummins, 1989a; Murphy *et al.*, 1989). Triacylglycerols make up 96% of the total lipids of the mature seed, with polar lipids contributing 3% (Murphy and Cummins, 1989a). Water content begins to decrease after 42 dpa. The B. napus embryo requires approximately 8 weeks to mature but the seed does not reach its final water content of approximately 8-9% (w/w) until about 12 weeks (Crouch and Sussex, 1981; Murphy and Cummins, 1989a). The endosperm in B. napus is reduced to only a single layer in the mature seed (Stanley *et al.*, 1976).

Like B. napus, A. thaliana embryos develop in a pattern similar to the dicot Capsella bursa-pastoris, but the time period is much reduced (Figure 1.1, B). Embryonic cell division is complete by 7 dpa and embryo maturation and preparation for dormancy occurs during the second week (Haughn and Somerville, 1988; Patton and Meinke, 1990). Curled cotyledon stage embryos contain immature protein bodies in the hypocotyl and cotyledons. Lipid bodies can be found around the periphery of the cells. Many protein bodies filled with storage proteins can be seen in mature embryos (Patton and Meinke, 1990). The two storage proteins in

A. thaliana are a 12S globulin, cruciferin, and a 2S albumin, arabin (Heath et al., 1986). The liquid endosperm is abundant at the embryo globular and heart stages then decreases until essentially no endosperm remains in the mature seed. Embryos are mature by approximately 12 dpa. Dessication of the seed requires several more days (Meinke and Sussex, 1979).

N. tabacum seeds mature in 24 to 33 days, depending on conditions (Sano and Kawashima, 1982). The embryo is somewhat kidney-shaped (Figure 1.1, A) and, unlike B. napus and A. thaliana, comprises only about one quarter of the volume of the mature seed (McCormick, 1932). The endosperm starts to develop almost immediately after fertilization but the division of the embryo is delayed until the formation of sufficient endosperm tissue (Jos and Singh, 1968). Three zones form in the endosperm. An external layer consists of small cells with few vacuoles. A central zone contains cells which accumulate reserve material. The embryo develops within the inner zone. No reserve proteins accumulate in these inner endospermal cells, which eventually separate and disintegrate (Erdelská, 1985). The N. tabacum endosperm completely surrounds the embryo but is thinner over the embryo's radicle end (McCormick, 1932). Endosperm in N. tabacum remains throughout development. Non-embryonic tissues comprise approximately 70-75% of the seed at maturity (Sengupta-Gopalan et al., 1985; McCormick, 1932).

Protein accumulates in N. tabacum seeds from 7 to 21 dpa with 70% of the accumulation occurring from 15 to 21 dpa (Sano and Kawashima, 1983). Both embryo and endosperm store protein and lipid. Starch appears transiently in the ovule of the young seed but disappears before the embryo is mature (McCormick, 1932). The major storage protein in N. tabacum is an 11.7S globulin (Vickery et al., 1932; Sano and Kawashima, 1983). A minor 7.4S globulin can also be detected (Sano and Kawashima, 1983). Mature seeds contain 42-50% fat, 19-20% protein, and attain a moisture content of 3.5-7% (Vickery et al., 1932).

1.I.3. Embryogenic Systems

The study of plant embryo development is often hindered by the difficulty of obtaining sufficient zygotic embryonic material to work with. Fortunately, alternative embryogenic systems exist.

As the name suggests, somatic embryos arise from somatic cells. They occur naturally (in vivo) in many species, usually from ovule-associated tissues, but can also arise in culture from additional tissues such as the epidermis or hypocotyls (Ammirato, 1983). Perhaps the best characterized somatic embryogenesis system is that of the carrot (Daucus carota). Approximately 85-90% of manually

selected cells or Percoll-fractionated cell clusters can become embryos upon the removal of exogenous auxin from carrot suspension cultures (Nomura and Komamine, 1985). The carrot somatic embryos pass through the same morphological stages as zygotic embryos, and the stages can be separated using nylon filters of various pore sizes (Giuliano et al., 1983). There are several experimental advantages to using the carrot system to look at embryo development. Particular stages can be studied through the use of mutants (Breton and Sung, 1982; Lo Schiavo et al., 1988; Lo Schiavo et al., 1990) or chemicals which can block embryo development (Sung et al., 1984). Carrot proembryogenic suspension culture cells have been transformed by Agrobacterium tumefaciens, and plants have been regenerated from transformed material by somatic embryogenesis in the presence of a selective agent (Scott and Draper, 1987).

Haploid embryos can be obtained by culturing immature pollen grains. Since this discovery in 1964 (Guha and Maheswari), haploid embryos derived from anthers or isolated microspores have been reported in over 200 species (Dunwell, 1986; Harada et al., 1988; Keller et al., 1987a). Anther culture has some experimental disadvantages. Plants can originate from other parts of the anther besides the pollen. This results in a mixture of somatic and microspore-derived embryos covering a range of ploidy. Developing embryos may have to compete for space and nutrients. Substances released

from the anther wall are known to affect microspore development, often negatively (Ammirato, 1983). These problems can be avoided by isolating the microspores from the anthers before culture.

Embryos have been obtained from cultured isolated microspores in several plant species, including Nicotiana tabacum (Harada et al., 1988; Rashid and Reinert, 1983), N. rustica (Kyo and Harada, 1985), Hordeum vulgare (Köhler and Wenzel, 1985; Wei et al., 1986), Zea mays (Coumans et al., 1989; Pescitelli et al., 1989), and various Brassica species (Lichter, 1989; Fan et al., 1988; Chuong and Beversdorf, 1985).

The frequency of embryogenesis can be influenced by donor genotype, bud size, hormone levels, donor plant growth conditions, medium composition, and incubation temperature (Lichter, 1982; Lichter, 1985; Gland et al., 1988; Charne and Beversdorf, 1988; Chuong and Beversdorf, 1985; Chuong et al., 1988). It has been suggested that reduced levels of embryogenesis could be partially due to the rapid depletion of nutrients or the generation of inhibitory compounds in B. napus microspore cultures. Potential solutions include the replacement or dilution of culture media, and the addition of activated charcoal (Mathias, 1988; Huang et al., 1990; Kott et al., 1988). Despite the large number of variables, it has been reported that embryos can be routinely obtained from as many as 5% of the microspores in

culture (Keller et al., 1987b), and this frequency can be improved by careful bud selection and/or fractionation of the microspores on Percoll gradients before culture (Fan et al., 1987; Fan et al., 1988).

1.I.4. Regulation of Plant Gene Expression

Plants respond to many environmental, developmental and hormonal cues by altering their patterns of gene expression. Excellent reviews have been written which discuss changes in gene expression in response to light (Gilmartin et al., 1990; Cuozzo et al., 1988; Dunsmuir et al., 1989; Harpster and Apel, 1985; Tobin and Silverthorne, 1985; Quail et al., 1986). Other environmental stimuli include heat shock (Mascarenhas and Altschuler, 1985; Schöffl et al., 1988), flooding, heavy metals, chilling, water stress and high salt conditions (Matters and Scandalios, 1986; Bailey-Serres et al., 1988; Tomsett and Thurman, 1988; Sachs and Ho, 1986; Kuhlemeier et al., 1987). Pathogens induce a number of responses in plants, such as the production of various proteins and secondary metabolites (Collinge and Slusarenko, 1987; Keen, 1986; Darvill and Albersheim, 1984; Pryor, 1987). Endogenous and exogenous plant hormones have also been shown to affect the synthesis of a wide range of

proteins (Szweykowska, 1987; Key et al., 1986; Guilfoyle et al., 1989).

Gene regulation plays a key role in plant development, both sporophytic and gametophytic. It has been estimated that 28% (approximately 8000 genes) of leaf mRNA sequences are shared by all other sporophytic organ systems. Each organ system also contains a set of organ-specific mRNAs that represent 23 to 40% of the structural genes expressed in that particular organ. These genes are believed to contribute to the development and maintenance of sporophyte differentiation (Kamalay and Goldberg, 1980; Okamuro and Goldberg, 1989). Extensive overlap has been detected in genes expressed in gametophytes and sporophytes (reviewed in Mascarenhas, 1989). Despite the large number of expressed genes shared by various plant organs and the two generations, there are also clear differences. This observation prompts one to ask how the expression of these genes is controlled.

Expression of a gene can be influenced by cis-acting elements, usually found in the 5' flanking region of the gene. The ability to transfer foreign DNA into plant cells has allowed for the elucidation of the location and function of some of these elements. Promoter and/or flanking sequences can be deleted or mutated, then expression of the gene can be analysed in transgenic cells, tissues or plants. Trans-acting factors are believed to interact with the cis

elements to regulate transcription of the gene. Differential control (ie. spatial, temporal, quantitative regulation) can be achieved by various combinations of cis- and trans-acting components.

Transcriptional control is a very important factor in gene expression but post-transcriptional mechanisms also influence the production of a functional protein. These mechanisms include processing of the initial RNA transcript, transport of the processed transcripts to the cytoplasm, translation and/or degradation of the mRNA, and post-translational modification of the protein (Alberts et al., 1983).

1.I.5. Gene Regulation in the Developing Seed

Some genes are expressed exclusively in the developing seed, for example, the seed storage protein genes. Much research has been devoted to the study of storage protein synthesis and deposition. In B. napus, mRNA for the 1.7S storage protein (napin) can be detected at 18 dpa, and levels peak at 21 dpa. Cruciferin (12S) mRNA is not seen until 21 dpa, with peaks detected at 40 dpa. Both mRNAs are undetectable in dry seeds (Crouch et al., 1984). The results of in vitro transcription studies in isolated nuclei indicate that napin is transcribed at a higher rate than

cruciferin, although cruciferin mRNA accumulates to a higher level than napin mRNA. DeLisle and Crouch (1989) suggest that this may be due to higher stability of the cruciferin message. Using in situ hybridization, Fernandez et al., (1991) determined the spatial and temporal distribution of napin and cruciferin mRNAs. Napin mRNAs start to accumulate during the heart stage in the cortex of the axis followed by deposition in the outer faces of the cotyledons during the torpedo stage, then the inner faces of the cotyledons during the cotyledon stage. Cruciferin mRNAs accumulate approximately 5 days later in a similar pattern. Late in embryo development, napin messages can be found in the shoot apical meristem, leaf primordia and vascular tissue. Cruciferin mRNAs accumulate only in the periphery of the meristem and flanks of the leaf primordia. Neither type of message can be detected in the root apical meristem at any time. In A. thaliana, the four 2S albumin genes are differentially expressed in the embryo, as evidenced by in situ hybridization studies (Guerche et al., 1990). These results indicate that seed mRNA and protein accumulation is highly regulated. It has been suggested that some regulatory mechanisms can be linked to events as early as the establishment of apical meristems (Fernandez et al., 1991).

Several conserved sequences in upstream regions of seed storage protein genes have been implicated in gene regulation. Bäumlein et al. (1986) compared legumin gene

sequences from soybean, pea, and field bean and discovered a 28 bp highly conserved sequence approximately 100 bp upstream of the cap site in each gene. They termed this element the "legumin box". When sequences containing this box were deleted from legA gene constructs, no expression was detected in transgenic tobacco plants (Shirsat et al., 1989a). However, destruction of the legumin box from a field bean legumin gene had no effect on the expression of a nptII gene driven by the deleted legumin gene 5' flanking sequence (Bäumlein et al., 1991). The role of the legumin box in seed-specific expression remains to be clarified. Another conserved region has been identified in legume seed genes. The "vicilin box" consists of 42 bases approximately 120 to 130 bases upstream of the cap site in various 7S globulin genes (Gatehouse et al., 1986; Newbigin et al., 1990). Conserved sequences have also been identified in the 5' flanking regions of hordein, gliadin and zein genes (34 bp prolamin "-300 element") (Forde et al., 1985). A separate zein gene sequence exists near the -300 position (maize 15 bp sequence) (Brown et al., 1986). In many cases, the functional significance of the conserved sequence has yet to be confirmed.

Presumptive regulatory elements from a gene encoding the α' subunit of the soybean β -conglycinin seed storage protein were functionally analysed by Chen et al. (1986, 1988). A 170 bp upstream DNA fragment containing five

repeats of the sequence AA/GCCCA enhanced the expression of the CAT gene with a truncated CaMV 35S promoter by 25 to 40 times. Enhancement was tissue-specific and temporally regulated during embryo development. The fragment could exert its effect in either orientation relative to the 35S promoter. A protein (SEF 3) from a nuclear extract of developing soybean seeds was shown to bind to sites on the α' subunit gene that included the AACCCA sequence. SEF 3 binding activity was found to increase during the time of β -conglycinin synthesis (Allen *et al.*, 1989). Nuclear proteins have also been shown to bind to 5' flanking regions (specifically A/T-rich regions) of a French bean phaseolin gene, and a sunflower helianthinin gene (Bustos *et al.*, 1989; Jordano *et al.*, 1989).

The study of a maize mutant has lead to the identification of a presumptive regulatory factor for zein synthesis. The opaque-2 mutation results in opaque seeds with soft powdery endosperms. This condition is correlated with a reduction in zein production, particularly zeins of the 22-kD class. The opaque-2 gene (*o2*) encodes a protein containing a leucine-zipper domain which binds to a genomic zein clone (Schmidt *et al.*, 1990). The *o2* protein is present in nuclei of maize endosperm tissues (Varagona *et al.*, 1991). This protein is thought to reduce zein transcription by direct interaction with zein promoter elements. A similar situation is believed to exist in the Risø mutant of barley.

The expression of hordein genes is affected by the trans-acting lys 3a gene product (Kreis et al., 1987).

The nutritional status of a plant can also have an effect on the accumulation of storage proteins. For example, sulfur stress has been shown to result in reduced levels of sulfur-rich proteins, particularly albumins, legumins and some hordeins. In peas, reduced mRNA levels were attributed to decreased mRNA stability rather than decreased transcription (for review, see Kreis et al., 1987; Spencer et al., 1990; Rerie et al., 1991).

There are many examples of post-transcriptional regulation in seeds. Genes with very similar transcription rates in isolated nuclei can have different levels (Sorensen et al., 1989; Thompson et al., 1989; Walling et al., 1986) or times (Harada et al., 1989a) of corresponding mRNA accumulation. This may be a result of differential mRNA turnover. During oat seed development, the levels of avenin mRNA can be approximately equal to, or greater than, the levels of globulin mRNA, but the oat seed contains much more globulin than avenin storage protein (Chestnut et al., 1989; Shotwell et al., 1990). Several regulatory possibilities for this case have been suggested. The globulin sequence at the initiation site for translation is closer to the plant consensus sequence and therefore, perhaps more efficient. A potential hairpin loop has been detected in the avenin coding region which may decrease the rate of translation.

Due to a difference in codon usage, the globulin mRNA may utilize the pool of tRNAs more effectively (Shotwell *et al.*, 1990). When the z4 zein coding region was placed under the control of the CaMV 35S promoter and transferred into tobacco, zein protein could be detected in roots, leaves and endosperm but not in embryos. Since the 35S promoter is known to be active in embryos, this suggests that the zein proteins may not be stable in tobacco embryos (Schernthaner *et al.*, 1988).

Abscisic acid (ABA) has been linked to the control of seed maturation, dessication and the onset of dormancy. When immature Brassica napus zygotic embryos are cultured in the presence of ABA, precocious germination is inhibited allowing embryogeny to continue (Crouch and Sussex, 1981). ABA will induce and maintain high storage protein mRNA levels (Crouch *et al.*, 1984). Embryos are most responsive to ABA during the phase of storage protein accumulation but both endogenous ABA and ABA sensitivity decline during the maturation of cultured embryos. In situ, ABA levels peak just before the embryos begin to dessicate. Due to the relative insensitivity of maturing seeds to ABA, it appears that other factors are responsible for the prevention of germination at this stage (Finkelstein *et al.*, 1985).

Genes other than those encoding storage proteins will respond to ABA. The late embryogenesis abundant (lea) genes encode proteins that may have a protective role during

dessication, perhaps by stabilizing membranes (Baker *et al.*, 1988; Harada *et al.*, 1989b; Dure *et al.*, 1989). Some of the lea genes can be induced, or mRNA levels increased, in excised young embryos by the application of ABA (Galau *et al.*, 1986; Harada *et al.*, 1989b).

Higher levels of wheat-germ agglutinin (WGA) mRNA are attained in wheat embryos cultured in the presence of ABA. An embryonic pattern of WGA expression can be maintained if exogenous ABA is applied to germinating seeds from the time of imbibition (Mansfield and Raikhel, 1990).

ABA-inducibility appears to be correlated with water stress (reviewed in Skriver and Mundy, 1990). Genes that respond to ABA are induced not only in times of seed dessication but also in response to dehydration, wounding and NaCl treatment, all conditions which lead to water stress (Gómez *et al.*, 1988; Mundy and Chua, 1988).

Responsiveness to ABA has been linked to two conserved sequences found in a wheat Em gene. A 50 bp oligonucleotide corresponding to sequence -152 to -103, relative to the cap site, was shown to confer ABA responsiveness on a truncated CaMV 35S promoter. The sequence was effective in either orientation relative to the 35S promoter. The oligonucleotide contained both consensus sequences. The Em1 box (ACGTGG/CG/CG/CC) and the Em2 box (CGAGCAG/C) (Marcotte *et al.*, 1989) are also found in the rice rab (Responsive to ABA) 21 gene. This gene is induced when plants are subjected

to water stress (Mundy and Chua, 1988). Homologous conserved motifs in the rice rab-16A gene were shown to bind nuclear proteins, although ABA treatment did not appear to promote binding. The nuclear factors may bind constitutively but may not activate transcription until after interaction with other factors (Mundy et al., 1990). The exact mechanism of gene inducibility by ABA has yet to be determined.

As discussed above, ABA has the ability to induce or extend a developmental pattern of gene expression, but as embryos mature, the ability to respond to ABA diminishes. Finkelstein et al. (1985) suggested that low water content rather than ABA prevents germination during the later stages of development. The switch to germination appears to be linked to seed dessication and subsequent rehydration (Bewley et al., 1989). Drying seems to permanently suppress the synthesis of developmental proteins. A germinative pattern of gene expression can be detected after rehydration. Comai and Harada (1990) showed that transcripts from genes active during late embryogeny could also be elongated in nuclei isolated from dry seeds. These results indicate that the switch to a germination-specific pattern of gene expression is due to an event that occurs after seed dessication.

It is obvious that a complex set of regulatory mechanisms exists to ensure correct seed development and maturation.

CHAPTER 2

MATERIALS AND METHODS

2.I. SCREENING OF THE ARABIDOPSIS THALIANA GENOMIC LIBRARY

2.I.1. Manipulation of the Lambda Phage Library

Protocols were based on those of Maniatis et al., 1982.

2.I.1.a. Source of library

The Arabidopsis thaliana genomic library was generously provided by Dr. Elliot Meyerowitz. It was prepared from DNA of the Arabidopsis thaliana strain Landsberg erecta and the vector Lambda EMBL 4 (Chang and Meyerowitz, 1986).

2.I.1.b. Plating

Bacteria for plating were prepared by centrifuging an overnight culture (grown in LB [10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5] plus 0.2% [w/v] maltose) of Escherichia coli LE392, and resuspending the pellet in 10 mM MgSO₄ to an O.D.₆₀₀ of 2. Twenty µl of phage (diluted in SM [10 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.01% (w/v) gelatin,

8 mM MgSO_4] to give the desired plaque density) plus 0.13 ml of bacteria were incubated for 30 min at 37°C then 4 ml of melted top agarose (LB plus 0.2% [w/v] maltose, 1.2 g/L MgSO_4 , 0.9% [w/v] agarose [Seakem ME, Mandel Scientific Co. Ltd., Guelph, Ontario]) were added and the mixture poured onto petri plates (100 mm X 100 mm X 15 mm) containing hardened medium (LB plus 1.2% [w/v] agar, [Difco Laboratories, Detroit]). The plates were incubated 10 to 12 hr at 37°C then chilled for 1 hr at 4°C prior to lifting the plaques.

2.I.1.c. Plaque purification

An agar plug containing the desired plaque was placed in 1 ml SM plus a drop of chloroform. To allow the phage to diffuse out of the agar, the tube was left at room temperature for 1 to 2 hr then stored at 4°C . The phage were replated as in 2.I.1.b. at a low density (100 to 200 pfu/plate), and positive plaques were identified by hybridization.

2.I.1.d. Large preparations of phage and phage DNA

Approximately 5×10^9 E. coli LE392 cells and 1×10^9 to 2×10^9 phage were added to 500 ml LB to give a final multiplicity of infection of 0.2 to 0.5. Flasks were placed on a shaker (250 rpm) at 37°C for 4 to 6 hr until the cells looked completely lysed. Five ml chloroform and 1 mg DNase I

(Boehringer Mannheim, Montreal, Quebec) were added and the flasks returned to the shaker for 15 min. The cultures were centrifuged at 8000 rpm for 15 min at 4°C then the supernatant was strained through cheesecloth into a cold graduated cylinder. NaCl was added to 0.5 M and polyethylene glycol (PEG) 8000 (BDH Inc., Toronto, Ontario) to 10% (w/v) then the mixture was stirred slowly at 4°C until the PEG dissolved. After centrifugation at 8000 rpm for 10 min at 4°C, the supernatant was decanted and 5 ml cold SM were added to the pellet. The centrifuge bottle was strapped to an aliquot mixer, pellet side down, and rocked for 20 min at 4°C to dissolve the pellet. This step was repeated with an additional 5 ml SM. CsCl was added to give 0.75 g/ml and the tube centrifuged at 25,000 rpm at 4°C for 18 hr. The blue phage band was extracted and dialysed overnight against three changes of SM at 4°C.

Phage DNA was extracted as follows: ethylene diamine tetraacetic acid (EDTA) to 10 mM, sodium dodecyl sulfate (SDS) to 0.3% (w/v) and Proteinase K (Boehringer Mannheim, Montreal, Quebec) to 100 µg/ml were added, and the phage mixture was incubated at 37°C for 80 min. An equal volume of water-saturated phenol was added and the tubes were rocked for 15 min at room temperature. The tubes were centrifuged at 3000 rpm for 10 min and the aqueous phase was reextracted with phenol 2 more times. The final aqueous phase was dialysed against cold TES (prepared

by adding 4 ml 100 mM EDTA pH 8.0 to 40 mM Tris-HCl, 50 mM NaCl, 0.1 mM EDTA, pH 7.6 in a final volume of 4 L) overnight.

2.I.1.e. Quantitation of DNA

The DNA was quantitated on a fluorometer (Model TKO 100) using Hoechst's 33258 dye specific for DNA (Hoefer Scientific Instruments, San Francisco, California).

2.I.2. Plaque Lifts

Bacteriophage plaques were lifted off the surface of agar plates using Hybond N (Amersham Canada Ltd., Oakville, Ontario) nylon membranes according to the method outlined in Amersham International plc Manual (1985). The membrane was placed on the surface of the agar, and the orientation was marked by puncturing the membrane and agar with a needle dipped in India ink. When multiple filters were prepared from a single plate, the first filter was left on the plate for 3 min, the second for 5 min and the third for 7 min. The membranes were transferred, plaque side up, onto 2MM paper (Whatman International Ltd., Maidstone, England) soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH). After 7 min, the membranes were moved to fresh 2MM paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) for 3 min. The filters were then washed in 2X SSC

(prepared from a 20X SSC stock consisting of 3 M NaCl, 0.3 M sodium citrate, pH 7.0) before air drying on Whatman 2MM paper. In order to crosslink the DNA to the membrane, the filters were wrapped in Saran Wrap (DowBrands Canada, Inc., Paris, Ontario) and placed plaque side down on a UV transilluminator for 5 min.

2.I.3. Probe Preparation

The A. thaliana genomic library was screened with a cDNA coding for a napin storage protein from Brassica napus. The cDNA was obtained by digesting pN2 (generously supplied by Dr. Martha Crouch [Crouch et al., 1983]) with Pst I (Bethesda Research Laboratories [BRL], Burlington, Ontario) (see 2.II.1.) to liberate pUC 8 and the 738 bp cDNA insert. The insert was isolated from a 1% (w/v) mini agarose gel electrophoresed for 1 hr at 80 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Diethylaminoethyl (DEAE) cellulose paper (Schleicher and Schuell, Inc., Keene, New Hampshire), (Dretzen et al., 1981; Lizardi et al., 1984) was inserted in the gel in front of the desired DNA band then the DNA was electrophoresed onto the paper. (Prior to use, the paper was pretreated with 2.5 M NaCl for several hours, washed 3 times in DDH₂O, and stored at 4°C in 1 mM EDTA pH 8.0). The membrane was cut into small pieces and placed in a 1.5 ml Eppendorf tube. The DNA was eluted in 200 µl 10 mM Tris-HCl

pH 7.5, 1.5 M NaCl at 65°C for 15 min. The supernatant was extracted twice with 400 μ l n-butanol saturated with 10 mM Tris-HCl pH 7.5, to remove the ethidium bromide. The DNA was ethanol precipitated (1/10 volume 3 M sodium acetate, 2.5 volumes ethanol) and resuspended in DDH₂O.

Radioactive ($[\alpha\text{-}^{32}\text{P}]\text{-dCTP}$; New England Nuclear [NEN] Research Products, Boston, Massachusetts) probes were prepared with the BRL Nick Translation Reagent Kit (BRL, Burlington, Ontario) according to the manufacturer's instructions. After incubation at 15°C for 2 hr, the reaction was stopped with an equal volume of 2% (w/v) SDS, 50 mM EDTA, 0.5% (w/v) bromophenol blue (BPB), and 0.5% (w/v) dextran blue. The labelled DNA was separated from the nucleotides by passage through a Nick Column (Sephadex G-50, DNA grade, Pharmacia Canada Inc., Montreal, Canada) equilibrated with 25 mM EDTA, 1% (w/v) SDS. The dextran blue fraction (approximately 300 μ l) was collected. The specific activity ranged from 10^7 to 10^8 cpm/ μ g DNA.

2.I.4. Hybridization and Washing Conditions

Filters were hybridized and washed according to the method of Bernatzky and Tanksley (1986). The nylon filters were washed for 30 sec in 0.4 N NaOH then neutralized briefly in 0.2 M Tris-HCl pH 7.5, 2X SSC. The prehybridization solution consisted of 5X SSC, 50 mM NaPO₄

pH 7.2, 0.4% (w/v) SDS, 5X Denhardt's (0.1% [w/v] Ficoll, 0.1% [w/v] polyvinylpyrrolidone [PVP], 0.1% [w/v] bovine serum albumin [BSA] Fraction V), 2.5 mM EDTA and 100 µg/ml low molecular weight denatured salmon sperm DNA (Sigma Chemical Co., St. Louis, Missouri). Approximately 25 ml of solution were added to 4 to 6 filters which were placed in Rubbermaid trays (Rubbermaid Canada Inc., Mississauga, Ontario) and covered with an acetate sheet to prevent evaporation. The filters were prehybridized for 6 to 18 hr at 68°C without shaking, then the radioactive probe (10^8 cpm) was added to the prehybridization solution and the filters were incubated at 68°C for an additional 18 hr.

Washings were all performed at 68°C: twice in 2X SSC, 0.1% (w/v) SDS for 20 min, twice in 1X SSC, 0.1% (w/v) SDS for 10 min; then once in 0.5X SSC for 10 min. Wire meshes were used to separate the filters during washes.

2.1.5. Autoradiography

The filters were sealed, while still wet, into heat sealable pouches (Kapak Corp., Minneapolis, Minnesota) then placed with Kodak XAR 5 film (Eastman Kodak Co., Rochester, New York) into Kodak X-Omatic film cassettes with 2 intensifying screens. The films were developed after 24 to 48 hr exposure at -80°C.

2.I.6. Selection of Lambda Clones

Plaques corresponding to a radioactive signal significantly above background were selected and taken through two rounds of plaque purification as described in 2.I.1.c.

2.II. SUBCLONING OF LAMBDA CLONES

2.II.1. Restriction Digests

Two to 5 μ g of Lambda DNA were digested with 5 Units restriction enzyme per μ g DNA. Digestions were carried out in a total volume of 20 to 50 μ l, containing 1/10 volume of 10X reaction buffer provided by the supplier. When DNA was digested with two enzymes requiring two different reaction buffers, the first reaction was carried out in the buffer of lower salt concentration, then the first enzyme was heat-inactivated and the buffer adjusted for the second enzyme. Reaction temperatures matched those suggested by the enzyme supplier.

2.II.2. Southern Blotting

2.II.2.a. Preparation of radioactive molecular weight markers.

A mixture containing 2.5 μ l React 2 buffer (BRL, Burlington, Ontario), 12.5 μ l of a dATP/dGTP/dTTP (Pharmacia Canada Inc., Montreal, Quebec) solution (0.1 mM each), 4 μ l (40 μ Ci) [α - 32 P]-dCTP (NEN, Boston, Massachusetts), 1 μ g of Lambda DNA previously cut with Hind III (BRL, Burlington, Ontario), (see 2.II.1.), and 1 Unit Klenow (BRL, Burlington, Ontario) was prepared in a total volume of 25 μ l. The reaction was incubated for 10 min at 25°C then terminated by the addition of 1 μ l 0.5 mM EDTA. Unincorporated nucleotides were removed by passage on a Nick Column (see 2.I.3.). The radioactivity of end-labelled Lambda DNA was measured by counting 1 μ l. Approximately 5000 Cerenkov cpm were loaded per lane.

2.II.2.b. Gel electrophoresis

DNA was separated on a 0.8% (w/v) agarose gel in TAE buffer and run overnight at 40 V. The 5X loading buffer consisted of 15% (w/v) Ficoll, 0.25% (w/v) BPB, 0.2 M EDTA. Molecular weight markers were obtained from Boehringer Mannheim, Montreal, Canada.

The DNA was stained by either adding 1 μ g/ml ethidium bromide directly to the gel before pouring or by immersing

the gel after electrophoresis in 1 $\mu\text{g/ml}$ ethidium bromide in water for 15 min. The gel was destained 15 min in water before photography.

2.II.2.c. DNA transfer to nylon membranes

Prior to transfer, the gel was treated with 2 to 3 volumes of denaturation solution (1.5 M NaCl, 0.5 M NaOH) twice for 15 min each. The gel was then soaked in neutralization solution (1.5 M NaCl, 1 M Tris-HCl pH 7.4) 2 times for 15 min each. The DNA was transferred to Nytran nylon membrane according to the manufacturer's (Schleicher and Schuell, Inc., Keene, New Hampshire) instructions using 10X SSC as the blotting buffer. After blotting, the membrane was soaked in 5X SSC then treated for 5 min on a UV transilluminator to crosslink the DNA to the membrane.

2.II.2.d. Probe preparation

Radioactive probes were prepared with BRL Nick Translation Reagent Kit, as described in 2.I.3., using pN2, a napin storage protein gene cDNA clone.

2.II.2.e. Hybridization and washing conditions

Membranes were prehybridized in 6X SSC, 10X Denhardt's, 1% (w/v) SDS, and 50 $\mu\text{g/ml}$ low molecular weight denatured salmon sperm DNA for 3 to 18 hours at 65°C. The probe was boiled for 10 min then chilled on ice before addition to the

prehybridization solution. Hybridization was carried out overnight at 65°C.

Washing conditions were as follows: 6X SSC, 0.5% (w/v) SDS, 2 times 15 min each at room temperature; 1X SSC, 0.8% (w/v) SDS, 2 times for 15 min each at 37°C; 0.1X SSC, 1% (w/v) SDS for 1 hr at 65°C. Wire meshes were used to separate the filters during washes.

2.II.2.f. Autoradiography

See 2.I.5.

2.II.3. Subcloning into M13mp18 and M13mp19

2.II.3.a. Manipulation of M13 phage

Methods used for M13 phage manipulations were adapted from Messing (1983).

2.II.3.a.i. Plating

One hundred μ l of the appropriate phage dilution were mixed with 10 μ l 100 mM isopropyl- β -D-thiogalactopyranoside (IPTG), (United States Biochemical Corp. [USB], Cleveland, Ohio), 50 μ l 2% (w/v) 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal), (Boehringer Mannheim, Montreal, Quebec) in dimethylformamide, 200 μ l log phase E. coli DH5 α F' cells and 3 ml soft agarose (10 g/L tryptone, 8 g/L NaCl, 8 g/L

agarose) and spread over the surface of the LB agar plates. The plates were incubated overnight at 37°C.

2.II.3.a.ii. Plaque purification

A single plaque was picked from an agar plate with a sterile Pasteur pipette and placed in 1.5 ml TB (12 g/L tryptone, 24 g/L yeast extract, 4 ml/L glycerol; 17 mM KH_2PO_4 , 72 mM K_2HPO_4 to achieve a pH of approximately 7.5) containing 50 μl of stationary phase E. coli DH5 α F'. The phage were grown overnight at 37°C, 300 rpm. The culture was centrifuged for 5 min at 5000 rpm at 4°C. The supernatant was stored at 4°C for use as phage stock.

2.II.3.b. Preparation of single-stranded M13 phage DNA

DNA was extracted from 10 ml of phage culture supernatant. Culture conditions were as stated in 2.II.3.a.i. using 50 μl phage stock as inoculum. The phage were precipitated overnight at 4°C with 20% (w/v) PEG, 3 M sodium acetate. After centrifugation at 4°C for 15 min at 5000 rpm, the pellet was resuspended in 650 μl STE (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA) then extracted twice with phenol/chloroform and once with chloroform alone. The DNA was ethanol precipitated then dissolved in DDH₂O.

2.II.3.c. Preparation of double-stranded M13 phage DNA

The double-stranded replicative form of M13 was extracted from the pellet obtained after centrifugation of a 25 ml phage culture (see 2.II.3.a.ii.). One hundred μ l of phage stock were used as the culture inoculum. The cell pellet was resuspended in 2 ml Buffer 1 (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) then 300 μ l Lysozyme (Boehringer Mannheim, Montreal, Quebec) (40 mg/ml in Buffer 1) were added. The mixture was incubated for 5 min at room temperature. The addition of 2 ml of fresh 0.2 N NaOH, 2% (w/v) SDS was followed by a 5 min incubation on ice. Three ml of 3 M sodium acetate, pH 5.5 were added and the tube was left on ice for 10 min then centrifuged at 4°C for 10 min at 10,000 rpm. RNase A (Boehringer Mannheim, Montreal, Quebec) was added to the supernatant to a final concentration of 10 μ g/ml followed by a 30 min incubation at 37°C. The double-stranded DNA was extracted with phenol, then phenol/chloroform, then chloroform alone. The DNA was precipitated with 2 volumes cold ethanol then resuspended in DDH₂O.

2.II.3.d. Quantitation of DNA

See 2.I.1.e.

2.II.3.e. Subcloning of the Lambda fragment

M13mp18 and M13mp19 were digested with Xba I (BRL, Burlington, Ontario) and Eco RI (BRL, Burlington, Ontario) (see 2.II.1.) in preparation for subcloning the 5.2 kb Xba I - Eco RI fragment from Lambda clone λ AtN8. The fragments were isolated with DEAE paper, as described in 2.I.3. Ligation mixtures consisted of 50 ng each of insert and vector DNA and 1 Unit Ligase (BRL, Burlington, Ontario) in ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 2 mM β -mercaptoethanol, 1 mM ATP [Pharmacia Canada Inc, Montreal, Quebec], 5% PEG 8000). Reactions were carried out overnight at 12°C.

2.II.3.f. Transformation of E. coli competent cells

Competent cells were prepared using a method based on the protocols of Hanahan (1985). Twenty-four ml of 2YT (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) plus 0.58 ml 1 M MgSO₄ were inoculated with 0.24 ml of a stationary phase culture of E. coli DH5 α and incubated at 37°C, 250 rpm to an O.D.₅₅₀ of 0.4 to 0.5. The cells were chilled on ice for 40 min, centrifuged for 8 min at 0°C, 5000 rpm then resuspended in 12 ml of a modified Simple Transformation Buffer (SB) (100 mM CaCl₂.2H₂O, 70 mM MnCl₂.4H₂O, 40 mM sodium acetate pH 5.5). The cells were chilled for 40 min and centrifuged again then resuspended in 2.4 ml SB. Various amounts (10 to 100 ng) of ligated DNA were added to 300 μ l of competent

cells then the mixtures were left on ice for 30 min. The cells were heat-shocked for 2 min at 42°C then returned to ice briefly. Ten μ l IPTG, 50 μ l X-gal, 200 μ l log phase E. coli DH5 α F' cells and 3 ml soft agarose (2.II.3.a.i.) were added to each tube then the contents were spread over the surface of LB agar plates. The plates were incubated overnight at 37°C.

2.II.3.g. Selection of M13 clones

Five white plaques (M13 phage containing an insert) from each (M13mp18 or M13mp19) subcloning experiment were picked. The size of the DNA from the clones was checked on a 0.7% (w/v) agarose gel in TBE buffer (89 mM boric acid, 2 mM EDTA, 89 mM Tris-borate) after rapid lysis of the phage. Five μ l of 250 mM EDTA pH 8.0, 1% (w/v) SDS, 0.1% (w/v) BPB, 15% (w/v) Ficoll were added to 25 μ l of phage culture supernatant, then the tubes were heated to 65°C for 10 min before loading onto the gel. Wild type M13 was used as a standard. One clone from each of the subcloning experiments was picked for further study.

A C-test was performed to prove that the two clones contained insert DNA that was complementary to each other (Miller, 1987). Ten μ l of both phage culture supernatants were mixed with 10 μ l 1% (w/v) SDS, 10 μ l 1X TBE, 10 μ l 20% (w/v) Ficoll, and incubated for 30 min at 55°C then 30 min at 50°C. The sample was electrophoresed in a 0.7% (w/v)

agarose TBE gel after the addition of 2.5 μ l 1% (w/v) BPB. Each supernatant was run separately as a control. The hybridization complex, consisting of the two complementary inserts, migrated more slowly in the gel compared to either supernatant alone.

2.III. DELETION BANK PREPARATION

2.III.1. Preparation of a Sequential Series of Overlapping DNA Molecules, using T4 DNA Polymerase

The deleted recombinant phage DNA molecules were prepared using a method based on the work of Dale et al. (1985). The single-stranded phage DNA was linearized after the hybridization of an oligomer which allowed cleavage by a restriction enzyme. The following were combined: 4 μ g phage DNA, 80 ng oligomer RD 22 (International Biotechnologies Inc. [IBI], New Haven, Connecticut) (for M13mp19) or 160 ng oligomer RD 29 (IBI, New Haven, Connecticut) (for M13mp18), 1 μ l 100 mM dithiothreitol (DTT), 40 Units Eco RI (mp19) or Hind III (mp18), in 20 μ l 1X appropriate enzyme reaction buffer. The Eco RI reactions were incubated at 42°C for 2 hr then the enzyme was inactivated by heating at 65°C for 10 min. The Hind III reactions were incubated at 50°C overnight before heat inactivation at 85°C for 10 min.

The linearized DNA was digested from the 3' end with the 3' to 5' exonuclease activity of T4 DNA Polymerase [IBI, New Haven, Connecticut]. To the above reaction mixture, 2.5 μ l 100 mM DTT, 1 μ l 10 mg/ml BSA and 5 Units T4 Polymerase were added. The reactions were incubated at 37°C and aliquots were removed at 10, 30, 50, 70, and 90 min. The reaction was terminated at each time point by heating to 65°C for 10 min. The size of the DNA from each time point was checked on a 0.7% (w/v) agarose TBE gel.

All the digests were pooled and the products either A-tailed (mp18) or G-tailed (mp19). Three μ l of 50 μ M dGTP or dATP were added to the above reaction mixture along with 15 Units Terminyl Deoxyribonucleotidyl Transferase [IBI, New Haven, Connecticut] and the mixture was incubated at 37°C for 10 min then at 65°C for an additional 10 min. The DNA was circularized by the addition of more oligomer which hybridizes to the homopolymer tail. Twenty ng of RD 22 or RD 29 were added and the tubes were incubated at 65°C for 10 min then cooled to 40°C before the addition of 3 μ l 10 mM ATP and 1 Unit T4 DNA Ligase. The ligation reaction was left for 2 hr at room temperature.

2.III.2. Transformation of E. coli Competent Cells

E. coli DH5 α cells were transformed as described in 2.II.3.f. Two to 200 ng of DNA were added to each aliquot of competent cells.

2.III.3. Estimation of the Length of the Cloned DNA

Individual plaques were picked and purified as in 2.II.3.a.ii. The sizes of the DNA from the deletion clones were estimated by a rapid phage lysis method as described in 2.II.3.g. Wild type M13 phage and the original full-length recombinant M13 phage were used as standards.

2.III.4. Selection of Clones for Sequencing

Eighteen deletion clones whose sizes spanned the range between the full-length original recombinant M13mp19 phage and wild type M13 were electrophoresed on a 0.7% (w/v) agarose TBE gel after rapid lysis of the phage (2.II.3.g.). The DNA was "Southern blotted" and probed with the napin cDNA pN2, as described in 2.II.2.b-f. The two M13 deletion clones that marked the boundary between the presence and absence of hybridization with the probe were chosen for sequencing. The orientation and approximate location of the gene within the 5.2 kb insert were determined by comparing

this initial sequence to the published sequence of a B. napus napin gene. Subsequent clones were selected for sequencing based on that information.

2.IV. SEQUENCING

2.IV.1. Single-stranded DNA Sequencing

2.IV.1.a. Preparation of template

Template DNA was prepared from 10 ml cultures (10 ml TB, 200 μ l lag phase E. coli DH5 α F', 50 μ l stock phage) according to 2.II.3.b.

2.IV.1.b. Chain termination sequencing reactions

Single-stranded DNA sequencing reactions were done according to the Sequenase (USB, Cleveland, Ohio) protocol, using either the Sequenase Kit M13 primer at M13 position -20 or the primer at M13 position -40 (Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta).

2.IV.1.c. Sequencing gel preparation

The acrylamide gel was poured so as to form a salt gradient to compress the DNA bands at the bottom of the gel. The gradient was created by the mixture of two acrylamide solutions. Solution 1 contained 6% (w/v) acrylamide (5.7%

acrylamide ["Ultrapure", BRL, Burlington, Ontario], 0.3% N,N'-methylene-bisacrylamide [bisacrylamide] ["Electran", BDH, Inc., Toronto]), 0.5X TBE, 7.8 M urea ["Ultrapure", BRL, Burlington, Ontario]. Solution 2 contained 6% acrylamide, 5X TBE, 7.8 M urea and 50 $\mu\text{g/ml}$ BPB.

To polymerize the acrylamide, 15 μl N,N,N',N'-tetramethylethylenediamine (TEMED) (Eastman Kodak Co., Rochester, New York) and 7.5 μl 25% (w/v) ammonium persulphate ("Ultrapure", BRL, Burlington, Ontario) were added per 10 ml acrylamide solution. The gradient was prepared by taking up 10 ml Solution 1 into a pipette then 10 ml Solution 2 and allowing several bubbles to rise through the pipette to partially mix the phases. The pipette was emptied between the glass plates then the remainder of the gel (55 ml) was poured using Solution 1.

2.IV.1.d. Electrophoresis

The gels were run overnight on a BRL Model S1 sequencing apparatus (BRL, Burlington, Ontario) using a gel slab of 82 cm X 17.8 cm X 0.4 mm for a total of 24,000 to 28,000 Vhr (until the first or second dye front ran off the gel). The wells were made with a 24 slot shark's tooth comb (BRL, Burlington, Ontario).

2.IV.1.e. Drying the gel

The acrylamide gel was dried in two pieces on Whatman 3MM paper for 2 hr on a gel dryer. Heat (80°C) was applied for the first hr.

2.IV.1.f. Autoradiography

The dried gel was placed directly against Kodak film in a Kodak X-Omatic film cassette, and left for 24 to 72 hr at room temperature before developing.

2.IV.1.g. Use of oligonucleotide primers

Three oligonucleotide primers were used to complete the sequence obtained using the deletion bank templates. The primers were synthesized in the laboratory of Dr. R. Watson, Plant Research Centre, Agriculture Canada, Ottawa. The sequences of the three primers were as follows:

1. ATTCTAAATATCCTAACT (position -559, Figure 3.2)
2. GAAGTAGACGCGGAAACT (position -209, Figure 3.2)
3. AAGGAGTTTCAGAAAGAA (position +142, Figure 3.2).

2.IV.1.h. Modifications for sequencing close to the primer

The "Poker Face" Sequencer SE 1500 (Hoefer Scientific Instruments, San Francisco) sequencing apparatus was used to obtain sequence information close to the hybridization site of the M13 universal primer because of the higher acrylamide concentration, hotter running temperature, and larger number

of sample slots available with this Sequencer. The gel was prepared according to the manufacturer's suggestions. The non-gradient, 8% (w/v) gel contained a final concentration of 7.7% acrylamide, 0.3% bisacrylamide, 4.7 M urea and 3.2X TBE. The 36 cm X 40 cm X 0.35 mm gel was pre-electrophoresed for 2 hr at 130 watts (1300 volts) then run at 130 watts for 2 hr. A conventional comb was used to make the 32 wells.

The reactions were carried out according to the Sequenase protocol. The labelling mix was diluted 1 in 10 rather than 1 in 5.

2.IV.2. Double-stranded DNA Sequencing

2.IV.2.a. Preparation of template

Template DNA was prepared from 250 ml cultures (TB plus 50 μ g/ml Kanamycin monosulfate [Km] [Sigma Chemical Co., St. Louis, Missouri]). The plasmids were isolated using alkaline lysis of the cells and purification on CsCl gradients (Maniatis et al., 1982).

2.IV.2.b. Modifications of Sequenase single-stranded DNA sequencing protocols

Plasmid DNA was sequenced as described in 2.IV.1.b-f. with some modifications. The double-stranded DNA was first denatured by an alkaline treatment. Three μ g DNA were treated with 0.2 M NaOH and 0.2 mM EDTA in 20 μ l total

volume at room temperature for 5 min. The DNA was neutralized with 2 μ l 2 M ammonium acetate, pH 4.6 then precipitated with 60 μ l ethanol at -20°C . The DNA was washed with 80% ethanol, vacuum dessicated, then used immediately in the Sequenase reactions.

The β -glucuronidase (GUS) primer (Clontech Laboratories, Inc., Palo Alto, California) was used in the sequencing of the constructed vectors (2.V.). Several changes (Zhang *et al.*, 1988) were made to the Sequenase protocol itself. Five ng (about 1 pmol) of primer and 3 μ g of template DNA were heated at 65°C for 5 min then allowed to cool to room temperature. The labelling mix was diluted 1 in 10. One μ l of [^{35}S]-dATP was added to the labelling step.

2.V. TRANSFORMATION VECTORS

2.V.1. Vectors Obtained from Other Sources

pBI 121.1 (Clontech Laboratories, Inc., Palo Alto, California) contains the Cauliflower Mosaic Virus (CaMV) 35S promoter within 800 bp, inserted in front of the GUS coding region in pBIN 19. Similarly, pBI 131.1 (generously provided by Dr. R. Jefferson; Jefferson *et al.*, 1987) includes the promoter from the small subunit of ribulose biphosphate carboxylase gene (*rbcS*) of tobacco. The *nos*-GUS vector, containing the nopaline synthase promoter, was constructed

by Jiro Hattori, Plant Research Centre, Agriculture Canada, Ottawa. The 1.48 kb Hind III - Bam HI fragment of pNCN (Pharmacia Canada Inc., Montreal, Quebec) was cloned into the Hind III - Bam HI sites of pBI 101.1 (Clontech Laboratories, Inc., Palo Alto, California).

2.V.2. Construction of the atar1-GUS Fusions

The atar1-GUS fusions were constructed using the pBI 101 cassettes (Clontech Laboratories, Inc., Palo Alto, California). atar1 fragments were obtained from deletion clones generated during the sequencing of the gene. The 3' ends of clones terminating in the desired area were sequenced (2.IV.1.h.). Two M13 clones were chosen: clone MP19-110 ending at nucleotide -44, and clone MP19-449 ending at nucleotide +70 of the arabin gene (Figure 3.2). Due to the protocol used to construct the deletion bank, clones also contained a short G-tail before the terminal 3' Eco RI site.

Translational fusions (pTLF1, pTLF2, pTLF3, pTLF1R) (Figure 3.5) contained an atar1 gene fragment from base -437 to +70. For pTLF1, pTLF2, pTLF3, M13 clone MP19-449 was cut with Eco RI and repaired with Klenow to obtain a blunt end, then cut again with Hind III. pBI 101.1, pBI 101.2, and pBI 101.3 were prepared by cutting with Sal I (BRL, Burlington, Ontario), repairing with Klenow then cutting

with Hind III and treating with Alkaline Phosphatase (Boehringer Mannheim, Montreal, Quebec). pTLF1, pTLF2 and pTLF3 were constructed by ligating the atar1 fragment to the Hind III site and the blunted Sal I site of the polylinker of the three pBI 101 cassettes to give the three reading frames relative to the atar1 ATG (+1 of fragment). The digestions were carried out as detailed in 2.II.1. The Sal I sticky ends of DNA were blunt-ended using 1 Unit Klenow in BRL Ligase Buffer containing mixed dNTP (Pharmacia Canada Inc., Montreal, Quebec) (0.1 mM each). The tubes were incubated at room temperature for 25 min, then at 70°C for 10 min to inactivate the enzyme. Proteins were precipitated by centrifugation at 12,000 rpm for 5 min after the addition of 1/2 volume of 7.5 M ammonium acetate. One unit of Alkaline Phosphatase was added to the completed restriction enzyme digests and the reactions were incubated at 37°C for 20 to 30 min then the phosphatase was inactivated by heating to 85°C for 10 min. Vector pTLF1R was constructed by cutting M13 clone MP19-449 with Eco RI and Hind III then filling in both ends using Klenow. The fragment was ligated into the repaired Sal I site of pBI 101.1.

Transcriptional fusions (pTCL, pTCLR, pTCS, pTCSR) (Figure 3.6) contained either the atar1 gene segment -764 to -44 bp (cut from M13 clone MP19-110 with Kpn I [Boehringer Mannheim, Montreal, Quebec] and Eco RI) or -437 to -44 bp (cut from clone MP19-110 with Hind III and Eco RI). pTCL,

pTCLR, and pTCSR fragments were repaired to yield blunt ends and inserted in pBI 101.1 prepared as for vector pTLF1R. For pTCLR, the atar1 fragment was blunt-ended by removal of the 3' overhang rather than by filling in the ends. To make pTCS, M13 clone MP19-110 was cut with Eco RI, the end was filled in by Klenow, then the clone was cut again with Hind III. Similarly, pBI 101.1 was cut with Sal I, treated with Klenow and cut with Hind III.

In all cases, linearized pBI 101 cassettes were treated with Alkaline Phosphatase. All fragments for vector construction were isolated on 0.8% (w/v) TAE agarose gels, using DEAE paper (see 2.I.3.) prior to ligation.

Ligation reactions contained about 25 to 35 ng insert DNA, 75 ng pBI 101 DNA and 1 Unit Ligase in ligation buffer (see 2.II.3.e.). The reactions were carried out overnight at 12°C.

2.V.3. Transformation of E. coli Competent Cells

A modified Hanahan (1985) protocol was used to prepare competent E. coli DH5 α cells. The cells were grown in 26 mls S.O.C. (2% [w/v] tryptone, 0.5% [w/v] yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) until reaching an O.D.₅₅₀ of 0.4. The cells were chilled for 40 min on ice then centrifuged at 5000 rpm for 8 min at 0°C. The pellet was resuspended in 13 ml TRB (100 mM KCl, 45 mM

MnCl₄·4H₂O, 10 mM CaCl₂·2H₂O, 3 mM HAcOCl₃, 10 mM K-[2(N-morpholino) ethane sulphonate acid]) and chilled for 40 min. The tubes were centrifuged for 8 min at 5000 rpm, 0°C, then the pellet was resuspended in 2.6 ml TRB.

The ligated DNA was mixed with 300 µl competent cells then chilled on ice for 30 min. The cells were heat-shocked at 42°C for 90 sec then returned to ice for 2 min. Two ml of S.O.C. were added and the tubes were incubated at 37°C for 40 min. The cells were spun down at 3000 rpm for 5 min then resuspended in 100 µl S.O.C. Aliquots of the cells were plated on LB plates containing 50 µg/ml Km.

2.V.4. Verification of Clones

Plasmid DNA was extracted from 10 ml cultures (TB plus 50 µg/ml Km), using an alkaline lysis method (Maniatis et al., 1982). The orientation of the inserts was determined by restriction mapping. Double-stranded sequencing (2.IV.2.) verified the vector fusion sites.

2.V.5. Transfer of Vectors to Agrobacterium

The vectors were transferred from the E. coli strain DH5α transformants (Kanamycin-resistant, Km^R) into A. tumefaciens strain GV3101 (pGV3850, Carbenicillin-resistant, Cb^R; Rifampicin-resistant, Rif^R), (Cb: "Pyogen", Ayerst

Laboratories, Montreal, Quebec; Rif: Sigma Chemical Co., St. Louis, Missouri) for N. tabacum transformation (Zambryski et al., 1983), or strain GV3101 (pMP90, Gentamycin-resistant, Gent^R; Rif^R), (Gent: Sigma Chemical Co., St. Louis, Missouri) for B. napus transformation (Koncz and Schell, 1986), via triparental mating (Van Haute et al., 1983). The transfer functions were provided by E. coli DH5 α (pBRK4013, Cb^R) (Hamel, 1987). The three strains of bacteria were mixed in equal proportions and collected on a 0.22 μ m sterile filter. The filter was placed on a LB plate overnight at 37°C then the bacteria were collected in 10 ml 0.85% (w/v) NaCl. Dilutions (1 in 10⁵ to 10⁸) were plated on LB plus appropriate antibiotics.

2.V.6. Growth and Storage of Clones

The bacteria were grown 24 to 48 hr in LB plus appropriate antibiotics then pelleted and resuspended in 5 mM MgSO₄, 50% (v/v) glycerol. Stock cultures were stored at -20°C, and at -80°C for long-term storage.

2.VI. PLANT TRANSFORMATION

2.VI.1. N. tabacum Transformation

N. tabacum transformation was based on the method of Horsch et al. (1985). Leaf pieces (1 cm X 2 cm) were excised from sterile in vitro grown N. tabacum cv. Petit Havana SR1 plantlets. The plantlets were maintained by meristem transfer on MSB5 (Charest, 1988; MS salts [Murashige and Skoog, 1962], plus B5 vitamins [Gamborg et al., 1968]), 3% (w/v) glucose, pH 5.7 plus 3 g/L agarose. A nurse culture (2 ml of N. tabacum cell suspension grown in MSB5, 2% [w/v] sucrose, 2 µg/ml 2,4-dichlorophenoxyacetic acid [2,4-D], [Sigma Chemical Co., St. Louis, Missouri], pH 5.7) was spread over the surface of TLD plates (MSB5, 3% [w/v] sucrose, 1 µg/ml naphthaleneacetic acid [NAA] [Sigma Chemical Co., St. Louis, Missouri], 1 µg/ml 6-benzylaminopurine [BA] [Sigma Chemical Co., St. Louis, Missouri], pH 5.7, plus 6 g/L agarose) and covered by 2 pieces Whatman #2 filter paper. The leaf pieces were placed on the filter paper and preconditioned for 2 days. The pieces were dipped in a 48 hr Agrobacterium culture, diluted 1 in 2 with LB, for 30 to 60 sec then blotted on sterile filter paper and returned to the nurse culture. After a cocultivation period of 2 days, the leaf pieces were transferred to TLD medium containing 500 µg/ml cefotaxime (Cef) ("Claforan", Roussel Canada Inc., Montreal, Quebec) and 200 µg/ml Km.

Km^R shoots (10 to 15 mm) were excised and transferred to MSB5, 3% (w/v) sucrose, pH 5.7 plus 6 g/L agarose, 500

$\mu\text{g/ml}$ Cef and 200 $\mu\text{g/ml}$ Km. Shoots (about 10 cm in height), with well-developed roots were transferred to the greenhouse. They were covered with plastic bags for a week after potting in soil, to allow adaptation to the lower humidity levels.

2.VI.2. B. napus Transformation

Transformation was performed according to Charest et al. (1988). Internodal stem sections were cut from B. napus cv. Westar greenhouse-grown plants after the first few flowers had opened. The pieces were sterilized in Javex containing a few drops of 10% Javex 20 (Fisher Scientific, Ottawa, Ontario) for 50 min with stirring, then transferred to sterile DH_2O for two 1 to 2 hr rinses. Cross-sections (2 to 4 mm thick) were cut and placed on 1/10 EM (1/10 MSB5, 1 $\mu\text{g/ml}$ BAA, 3% [w/v] sucrose, pH 5.8) plus 6 g/L agarose, 1 $\mu\text{g/ml}$ AgNO_3 (Biddington et al., 1988; De Block et al., 1989), and pre-conditioned for 4 days. The explants were inoculated as for N. tabacum then transferred to new 1/10 EM plates covered with 2 ml N. tabacum nurse culture and 1 piece Whatman #2 filter paper. After 2 to 3 days, the explants were placed on EM (MSB5, 1 $\mu\text{g/ml}$ BAA, 3% [w/v] sucrose) containing 500 $\mu\text{g/ml}$ Cb, 40 $\mu\text{g/ml}$ Km, 1 $\mu\text{g/ml}$ AgNO_3 and 6 g/L agarose.

Km^R shoots were transferred to rooting medium (MSB5, 1% [w/v] sucrose, 0.4 µg/ml NAA, pH 5.8, 6 g/L agarose) containing 500 µg/ml Cb and 40 µg/ml Km. After developing a well-formed root system, the plantlets were transferred to the greenhouse and bagged for 1 wk.

2.VII. PLANT GROWTH CONDITIONS

2.VII.1. Greenhouse

The greenhouse temperature varied from approximately 28°C day to 18°C night. Sunlight was supplemented by high pressure sodium lamps set for a 16 hr photoperiod with 600 µE/m²/sec intensity.

The soil mixture consisted of soil : peat moss : sand : perlite (1:2:1:1) with traces of lime and superphosphate. A slow release fertilizer, Nutricote (N:P:K = 14:14:14) was added to the soil. The plants were also fertilized with a Hoagland's solution and a solution of N:P:K (20:20:20) once a week. Plants were watered twice daily.

2.VII.2. Growth Room

Plant tissues and cell suspensions were grown at 25°C under constant illumination provided by fluorescent and

incandescent lights at $400 \mu\text{E}/\text{m}^2/\text{sec}$ intensity. Suspensions were continuously rotated at 140 rpm on a rotary shaker.

2.VIII. PLANT ANALYSIS

2.VIII.1. Sample Collection and Storage

Somatic samples (leaf, stem, roots, flower) were collected and fast-frozen in liquid nitrogen.

Flowers were self-pollinated and tagged on the day of anthesis. On appropriate days post-anthesis (dpa), N. tabacum seeds were scraped off the seed capsules and frozen in liquid nitrogen. Each sample consisted of seeds pooled from 2 to 3 capsules (approximately 1500 seeds/capsule). B. napus seeds from several siliques were picked with forceps then pooled and frozen (ranged from approximately 10 to 28 seeds/silique).

All tissues were stored at -80°C .

2.VIII.2. β -glucuronidase (GUS) Assays

All methods for GUS activity analysis were based on the work of Jefferson (1987a,b).

2.VIII.2.a. Fluorogenic assays

2.VIII.2.a.i. Lysis conditions

Tissues were homogenized in 3 ml ice-cold extraction buffer (50 mM NaPO₄ pH 7.0, 10 mM EDTA, 0.1% [w/v] sarcosyl, 0.1% [v/v] Triton X-100, 10 mM β -mercaptoethanol [added just prior to use]) per gram fresh weight tissue. An OMNI Model 10001 homogenizer (OMNI International, Waterbury, Connecticut) was used unless only a small amount of tissue (less than 30 mg) was available, in which case the frozen material was homogenized in a 1.5 ml Eppendorf tube with a ground glass tapered rod and a pinch of washed sea sand. The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C. Supernatants were frozen in liquid nitrogen and stored at -80°C.

2.VIII.2.a.ii. Microtiter plate assay

A rapid microtiter plate assay was used to initially screen Km^R plants for GUS activity. Five μ l of plant extract were added to 50 μ l assay buffer (1 mM 4-methyl umbelliferyl β -D-glucuronide [MUG] [Sigma Chemical Co., St. Louis, Missouri] in extraction buffer), or to 50 μ l extraction buffer alone and incubated in a microtiter plate well at 37°C in the dark. The reactions were stopped by the addition of 200 μ l stop buffer (0.2 M Na₂CO₃). The microtiter plate

was placed on a UV transilluminator and a Polaroid picture was taken. Fluorescence was scored visually.

2.VIII.2.a.iii. Fluorometer assay

Aliquots of 500 μ l assay buffer were warmed to 37°C before the addition of 20 to 40 μ l tissue extract. The tubes were returned to 37°C and 100 μ l were removed at 10, 30, 50 and 70 min, and added to 1.9 ml stop buffer. GUS activity was shown by Jefferson (1987a) to be linear under these conditions. Samples were done in duplicate. Controls consisted of assay buffer alone, extraction buffer and extract, or extraction buffer alone.

The amount of protein in the extracts was determined with the Bio-rad Protein Assay (Bio-Rad Laboratories, Richmond, Ontario) using BSA for the standard curve.

Fluorescence was measured on a Model TKO 100 DNA Fluorometer (Hoefer Scientific Instruments, San Francisco, California). The fluorometer was calibrated with known methylumbelliferone (MU) standards. The machine was set at 400 for a concentration of 100 nM MU then readings were taken for 10 nM, 50 nM, 250 nM and 500 nM MU. Linearity was maintained except at the highest concentration. A standard curve was prepared from the readings.

GUS fluorogenic activity was expressed as nmol MU produced per min per mg protein.

2.VIII.2.b. Histochemical assay

The histochemical substrate used for the GUS enzyme was 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) (Clontech Laboratories Inc., Palo Alto, California) dissolved at a concentration of 1 mg/ml in 0.1 M NaPO₄, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆].3H₂O and 10 mM EDTA (Jefferson, 1987b). Fresh N. tabacum seeds were rinsed with 0.1 M NaPO₄ pH 7.0 then dissected. Embryos, endosperms and seed coats were incubated separately in X-gluc at 37°C overnight. After staining, the tissues were fixed in FAA (42.5% (v/v) ethanol, 5% (v/v) glacial acetic acid, 10% (v/v) formalin) overnight then stored in 70% ethanol (Hinchee et al., 1988).

2.VIII.3. Southern Blots

2.VIII.3.a. Isolation of plant DNA

Genomic DNA was isolated from approximately 2.5 g of freeze-dried leaf tissue using a modification of the Lichtenstein and Draper method (1985). Leaves were collected and frozen in liquid nitrogen before drying. The tissue was ground to a fine powder in a mortar and pestle with 0.2 g aluminium oxide (BDH, Inc., Toronto) per gram original fresh weight. Ten ml extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 50 mM NaCl, 1% [w/v] sarcosyl, 400 μ g/ml ethidium bromide [added just prior to use]) was added and

the material was ground for another 5 min then centrifuged at 5000 rpm for 5 min. CsCl was added to the supernatant at 1 g/ml then the solutions were centrifuged overnight in Quick-seal tubes (Beckman Instruments Inc., Toronto) at 60,000 rpm at 20°C. The DNA band was collected under UV illumination and extracted several times with an equal volume of n-butanol saturated with 10 mM Tris-HCl, pH 7.5. Two volumes DDH₂O and 2 volumes ethanol were added and the DNA was collected several hr later with a bent Pasteur pipette. The DNA was dipped in 70% ethanol and dissolved in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

2.VIII.3.b. DNA quantitation

See 2.I.1.e.

2.VIII.3.c. Restriction digests

Restriction enzyme digestions of DNA were carried out as described in 2.II.1. with the addition of 2.5 mM spermidine (Sigma Chemical Co., St. Louis, Missouri) to the reactions.

2.VIII.3.d. Gel electrophoresis

Genomic DNA was separated on a 0.7% (w/v) agarose TAE gel (see 2.II.2.b.). The gel was stained in ethidium bromide after electrophoresis.

2.VIII.3.e. DNA transfer to nylon membranes

DNA was transferred according to the method of Rutledge et al. (1991). The gel was soaked in 2 to 3 volumes of 0.5 M NaOH, 1.5 M NaCl for 45 min then transferred to 1.5 M NaCl, 1 M Tris-HCl pH 7.0 for two rinses of 25 min. The Nytran membrane was soaked in DDH₂O then 20X SSC before use. The blot consisted of 4 layers of 3MM paper (Whatman) wet with 20X SSC, the gel, the nylon membrane, 1 layer wet (20X SSC) 3MM, 1 layer dry 3MM, a stack of paper towels and a glass plate held down with a beaker containing about 250 ml water. Saran wrap was placed around the gel to prevent the buffer from reaching the dry layers directly. After overnight transfer, the membrane was dried, treated for 2 min (DNA side down) on a UV transilluminator, rinsed for 5 min in 5X SSC, dried, and UV treated for an additional 30 sec.

2.VIII.3.f. Probe preparation

A fragment was cut from the GUS gene coding region with Sma I and Sst I. The fragment was isolated from a 1% agarose TAE gel with GeneClean (BIO/CAN Scientific Inc., Mississauga, Ontario).

A radioactive probe was prepared with the fragment and the Stratagene (La Jolla, California) Prime-It Random Primer Kit according to the manufacturer's instructions. Incorporation of the [α -³²P]-dCTP resulted in a specific activity of approximately 10⁹ cpm/ μ g DNA.

2.VIII.3.g. Hybridization and washing conditions

Filters were hybridized and washed using the conditions of Rutledge *et al.* (1991). The filters were prehybridized for 1 to 2 hrs at 65°C in 1 M NaCl, 1% (w/v) sarcosyl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 µg/ml yeast tRNA (Boehringer Mannheim, Montreal, Quebec). The filters (1 to 3) were placed in a Rubbermaid tray and covered with approximately 20 to 25 ml of solution and an acetate sheet. Hybridization was carried out overnight at 65°C in the same buffer. The probe (approximately 10 to 12 X 10⁶ cpm) was boiled for 10 min then left on ice for 5 min before addition to the original prehybridization solution.

The filters were washed two times, 15 min each at room temperature in 1X SSC, 0.1 M NaPO₄ pH 7.0, 0.1% (w/v) sarcosyl. This was followed by a second set of washes (2 times, 15 min each, room temperature) in 0.2 M NaCl, 0.5% (w/v) sarcosyl, and TE pH 8.0. The most stringent washes were carried out at 60°C in 0.5% (w/v) sarcosyl, TE pH 8.0 (two 1 hr washes).

2.VIII.3.h. Autoradiography

See 2.I.5.

2.VIII.3.i. Elution of the probe

Some filters were probed more than once. The probe was stripped off the filter by boiling it for 20 min in 0.01X

SSC, 0.1 mM NaPO₄ pH 7.0, 0.01 mM EDTA, 0.5% (w/v) SDS then the filter was rinsed in 2X SSC before drying on 3MM paper. The thoroughness of the elution procedure was checked by exposing the stripped filters to Kodak XAR 5 film (see 2.I.5.) and confirming the absence of autoradiography.

2.VIII.4. Segregation Analysis of N. tabacum F₁ Generation

2.VIII.4.a. GUS histochemical staining of embryos

N. tabacum seeds were dissected and stained as described in 2.VIII.2.b. Embryos showing any blue staining were considered GUS-positive.

2.VIII.4.b. Kanamycin resistance of germinating seeds

N. tabacum seeds were enveloped in Whatman 3MM paper and sterilized in 70% ethanol (for 1 min) then undiluted Javex (Bristol-Myers, Toronto) containing a few drops of Tween 20 (for 25 min). The seeds were rinsed three times with sterile DDH₂O and dried overnight in a laminar flow hood.

Seeds were germinated on MSB5, 1% (w/v) sucrose, 6 g/L agarose with 100 µg/ml Km. The numbers of Km^R (green) and Km^S (bleached) seedlings were counted 5 to 6 wk after plating. Seeds that did not germinate were not included in the analysis.

2.IX. BRASSICA NAPUS MICROSPORE CULTURE

2.IX.1. Growth Conditions of B. napus Plants

Brassica napus cv. Topas or Westar plants were grown in a growth cabinet at 20°C/15°C (day/night) with a 16 hr photoperiod (400 $\mu\text{E}/\text{m}^2/\text{s}$) provided by VHO (very high output) cool white fluorescent lamps. Four wk after germination, the plants were transferred to embryogenic (10°C/5°C) or non-embryogenic (25°C/20°C) (day/night) conditions.

Three populations of buds were collected. The most embryogenic microspores were obtained from buds of 3.0 to 5.0 mm in size (Fan et al., 1988). The population consisted of mid-uninucleate to early binucleate stage microspores. Small buds (1.5 to 3.0 mm) contained tetrads and early uninucleate microspores. In large buds (5.0 to 8.0 mm), late binucleate microspores to mature pollen were found.

2.IX.2. In vitro Culture Conditions

Culture conditions have been described by Keller et al. (1987b). Buds were surface sterilized in 7% (w/v) calcium hypochlorite for 15 min then rinsed 3 times, 5 min each, in sterile DDH₂O. To release the microspores, the anthers were mashed with a flat-bottomed ground glass rod in a beaker with B5 medium (Gamborg et al., 1968) and 13% (w/v) sucrose,

no hormones (B5-13). The macerate was filtered through a 44 μm pore size nylon mesh and the microspores were washed 3 times by centrifugation in B5-13 (1000 rpm, 3 min). The microspores were resuspended in 1/2X salts NLN (1/2 NLN) plus 13% (w/v) sucrose (Keller *et al.*, 1987b) at a concentration of 5×10^4 to $1 \times 10^5/\text{ml}$. Ten ml of microspore suspension were added to 100 X 15 mm petri dishes. The plates were sealed with Parafilm (American Can Co., Greenwich, Connecticut) and incubated in the dark at 32.5°C for 4 days then transferred to 25°C. When globular embryos were first detected (approximately day 10 of culture), the plates were placed on a rotary shaker at 50 rpm (Swanson *et al.*, 1987). Uninduced cultures were incubated exclusively at 25°C.

Under these culture conditions, the embryos reached the cotyledon stage but did not undergo the final maturation as seen in zygotic embryos. Therefore, cotyledon-stage embryos were purified and replated in a second medium consisting of 1/2 NLN plus 1% (w/v) sucrose, 5 μM abscisic acid (ABA) (Sigma Chemical Co., St. Louis, Missouri), 0.35 M mannitol.

2.IX.3. Embryo Collection

Large populations of microspore-derived embryos were purified for RNA extraction by filtration through various size nylon meshes (Fyntex, Switzerland) as described in

Giuliano et al. (1983). At 9 to 10 days of culture, early and late globular forms were collected on 62 μm and 88 μm meshes, respectively. Early heart-shaped embryos were retained on 125 μm membranes. Cultures of 11 to 12 days contained heart-shaped embryos (200 μm mesh). Torpedo stage embryos were collected at 14 to 15 days of culture on a 250 μm mesh. A 500 μm membrane was used to collect cotyledon stage embryos at 21 days. Embryos transferred to the second medium for maturation were collected at 4, 5, 6 and 7 wk after the original cultures were initiated. Embryos were removed by rinsing the meshes in B5 then the embryos were pelleted and frozen in liquid nitrogen. Small numbers of embryos (for GUS assays) were collected with a Pasteur pipette or small paintbrush.

2.IX.4. Northern Blot Analysis

2.IX.4.a. Isolation of microspore and embryo RNA

All solutions and glassware were treated with diethylpyrocarbonate (DEPC) to inhibit RNase activity (Maniatis et al., 1982).

RNA was isolated using a CsCl procedure adapted from Chirgwin et al. (1979) and Glisin et al. (1974). Frozen material was ground with a mortar and pestle and a small piece of dry ice. Ice-cold extraction buffer (10 ml Guanidine isothiocyanate [GuSCN] stock solution [4.0 M

2

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GuSCn, 25 mM sodium citrate, 0.5 M EDTA] plus 150 ml Antifoam A 30% Emulsion [Sigma Chemical Co., St. Louis, Missouri] plus 100 μ l β -mercaptoethanol) was mixed with the powder (10 to 16 ml per g tissue). The plant material was homogenized on ice with a Polytron (Brinkman Instruments, Rexdale, Ontario) at speed 6 for 1 min. Sarcosyl was added to a final concentration of 2% and the sample was again homogenized for an additional min. The homogenate was centrifuged at 7000 rpm for 15 min at 4°C several times until the supernatant was clear. The supernatant was loaded onto 2 ml of a CsCl stock (5.7 M CsCl in 0.1 M EDTA, pH 7.0) in 14 X 95 mm polyallomer tubes (Beckman Instruments Inc., Toronto, Ontario). The tubes were spun at 30,000 rpm for 16 to 20 hr at 18°C in a SW40.Ti rotor (Beckman Instruments Inc., Toronto, Ontario). After centrifugation, the supernatant was removed to reveal a clear pellet which was suspended in 600 μ l of GHC (7.5 M guanidine HCl, 25 mM sodium citrate pH 7.0, 5 mM DTT). The RNA was precipitated at -20°C with 15 μ l 1 M acetic acid and 300 μ l ethanol. The precipitate was rinsed with 150 μ l 70% ethanol and spun for 5 min at 12,000 rpm, 4°C. The partially dried pellet was resuspended in 200 μ l DDH₂O by alternately vortexing and heating (60°C, 1 min). The suspension was spun for 5 min at room temperature and the supernatant removed to a new tube. This procedure was repeated until all the RNA in the original pellet was dissolved (repeated 2 or 3 times to give

a final volume of 450 μ l). The RNA was precipitated overnight at -20°C with 150 μ l ammonium acetate and 900 μ l ethanol. The precipitation cycle was performed 3 times. The final RNA pellet was resuspended in 200 μ l DDH_2O .

2.IX.4.b. Quantitation of RNA

The RNA was quantitated on a UV spectrophotometer (1 O.D.₂₆₀ Unit = 40 $\mu\text{g/ml}$ RNA).

2.IX.4.c. Gel electrophoresis

RNA was denatured in glyoxal/dimethylsulfoxide (DMSO) prior to electrophoresis (Thomas, 1983). Seven μ l of glyoxal/DMSO buffer (125 μ l DMSO, 42 μ l deionized 40% glyoxal, 3 μ l 1 M NaPO_4 pH 7.0) were added to a 3 μ l RNA sample. The mixture was heated at 50°C for 1 hr, then 1 μ l marker dye (5 mg xylene-cyanol, 5 mg bromophenol blue, 10 μ l 1 M NaPO_4 , 990 μ l DDH_2O) was added and the sample was loaded onto a 1% agarose gel in 10 mM NaPO_4 pH 7.0. The gel was run overnight at 30 V with buffer circulation. The gel was stained in 0.1 $\mu\text{g/ml}$ ethidium bromide (see 2.II.2.b.) then photographed before transfer.

2.IX.4.d. RNA transfer to nylon membranes

RNA was transferred overnight to BIOTRANS nylon membranes (ICN Biomedicals, Inc., St. Laurent, Quebec) according to the manufacturer's instructions.

2.IX.4.e. Probe preparation

Probes were prepared using a random primer kit (see 2.VIII.3.f).

2.IX.4.f. Hybridization and washing conditions

The filters were prehybridized 1 to 2 hr at 42°C in 5X Denhardt's, 5X SSC, 50 mM sodium phosphate pH 6.5, 0.1% (w/v) SDS, 250 µg/ml denatured salmon sperm DNA and 50% (v/v) formamide (BDH, Inc., Toronto, Ontario; Analar grade). The filters (1 to 3) were placed in a Rubbermaid tray covered with approximately 20 ml of prehybridization solution and an acetate sheet. The random primer labelled probe (approximately 5 to 10 X 10⁶ cpm) was added to fresh hybridization solution then boiled for 10 min before being cooled on ice. The blots were hybridized at 42°C.

After hybridization, the blots were washed in 3 changes of 2X SSC, 0.1% (w/v) SDS for 15 min each then 2 changes of 0.1X SSC, 0.1% (w/v) SDS for 30 min each at 50°C.

2.IX.4.g. Autoradiography

See 2.I.5.

2.IX.5. GUS Assays

See 2.VIII.2.

CHAPTER 3

ISOLATION AND SEQUENCING OF AN ARABIDOPSIS THALIANA 2S STORAGE PROTEIN (ARABIN) GENE, AND VECTOR CONSTRUCTION

3.I. INTRODUCTION

The arabin gene encodes a 2S albumin seed-specific storage protein in Arabidopsis thaliana. The mature 13kD protein consists of a large and small subunit linked by disulfide bridges. The subunits are produced together on a large precursor polypeptide which is proteolytically cleaved to release a signal peptide and three other fragments besides the two subunits (Krebbers et al., 1988). Napin, the corresponding 2S albumin in B. napus, was shown to be localized in B. napus embryo protein bodies using immunogold labelling (Ericson et al., 1989). Arabin is deposited in the protein bodies of both endosperm and embryo in A. thaliana and transgenic N. tabacum seeds (De Clercq et al., 1990). The signal peptide is believed to be involved in targeting the protein to the protein bodies (Crouch et al., 1983).

Napin mRNA can first be detected at 18 dpa in B. napus embryos, with a peak at 27 dpa (Crouch et al., 1984). Napin protein is first detectable at 25 dpa and accumulates to day 42 then napin levels in the maturing embryo drop slightly

then level off (Crouch and Sussex, 1981). As well as being influenced by the developmental stage of the embryo, the synthesis of napin protein and napin mRNA is stimulated by the presence of abscisic acid (ABA) in B. napus embryo cultures (Crouch et al., 1984; DeLisle and Crouch, 1989). Differential expression of various members of the 2S albumin gene family has been reported in A. thaliana (Guerche et al., 1990). The four arabin genes (at2S1, at2S2, at2S3, at2S4; Table 1.1) exhibit similar temporal patterns of mRNA expression during seed development, but expression differs spatially and quantitatively. It is evident from these studies that developmental, hormonal, spatial, and quantitative factors can influence gene expression for this class of storage proteins.

Transcriptional responses to regulatory cues occur via the interaction of trans-acting transcription factors with cis elements usually found in the gene 5' to the translation initiation site. Therefore, the first stage of this project was to isolate and sequence an arabin gene (designated atar1) and search its 5' regulatory region for possible cis-acting transcriptional control elements. These steps, as well as the construction of atar1-GUS transformation vectors, are described in this chapter.

3.II. RESULTS

3.II.1. Isolation of Genomic Arabin Clones

An Arabidopsis thaliana genomic library was screened with a Brassica napus napin cDNA probe. Three Lambda clones hybridizing to the probe were isolated. DNA from each of the three clones (λ AtN6, λ AtN8, λ AtN10) was digested with a variety of restriction enzymes. Restriction patterns indicated that two clones (λ AtN6 and λ AtN8) were identical. Consequently, Southern blots of clones λ AtN8 and λ AtN10, only, were hybridized with the napin probe in order to identify a fragment suitable for subcloning into M13mp18 and M13mp19 for sequencing. No single hybridizing band could be detected for λ AtN10 after digestion with eight restriction enzymes or pairwise combinations of those enzymes (results not shown). Digestion of Lambda clone λ AtN8 DNA yielded several candidates for subcloning (Plate 3.1). A single fragment of approximately 5.2 kb which hybridized to the napin probe was released by Xba I/Eco RI digestion (Plate 3.1). This 5.2 kb fragment was small enough to be easily manipulated yet large enough to allow for the possibility of containing not only the hybridizing transcribed region but also the 5' and 3' sections of the gene. This band was chosen over three other bands of similar size (ie. Xho I/Eco RI, Eco RI, Xba I/Xho I lanes) since the probe appeared to

Plate 3.1 Southern Blot Analysis of λ AtN8

A. Agarose gel. Each lane contains 4 ug λ AtN8 DNA digested with the indicated restriction enzyme(s): Hd, Hind III; Xb, Xba I; Hf, Hinf I; Xh, Xho I; E, Eco RI. M, Molecular weight markers. The sizes of the bands are given in kilobase pairs to the right of the gel.

B. Autoradiograph. The Southern blot was probed with a B. napus napin cDNA. M. Radioactive molecular weight markers. The sizes of the bands are given in kilobase pairs to the right of the autoradiograph. Arrow indicates band subcloned into the M13 vectors.

hybridize to this band more intensely than to the others. A second hybridizing band (approximately 6.2 kb) in the Xba I/Eco RI lane was later proven to be the result of incomplete digestion of the DNA (results not shown). The 5.2 kb Xba I - Eco RI fragment was subcloned into M13mp18 and M13mp19 for sequencing.

3.II.2. Sequencing of the Arabin Gene

3.II.2.a. Sequencing strategy

The Xba I - Eco RI fragment of λ AtN8 was subcloned into the multiple cloning site of the replicative form of both M13mp18 and M13mp19, in order to sequence both strands of the gene. A T4 DNA polymerase deletion clone bank was prepared for each strand. A diagram of the sequencing strategy is presented in Figure 3.1. The deletion bank prepared from M13 subclone JDM18 did not evenly span the determined location of the arabin gene within the subclone. Therefore, three oligonucleotide primers were used to fill the gap in sequence information for subclone JDM18.

3.II.2.b. Analysis of the Arabin Gene (atar1) sequence

The gene sequence of atar1 is shown in Figure 3.2. The gene contains no introns. A TATA box is found at -66. No CAAT or AGGA boxes can be detected at the locations upstream

Figure 3.1 Sequencing Strategy for the Arabin Gene

The scale shows the location of the arabin gene (coding region: ATG to TAA) within the sequenced portion of λ AtN8. JDM18 and JDM19 are M13 subclones (in M13mp18 and M13mp19, respectively) containing complementary strands of the 5.2 kb fragment of λ AtN8. Arrows represent sequenced areas of the overlapping deletion bank clones. Numbered bold lines represent the three oligonucleotide primers used to complete the sequence information for JDM18. The sequences of the primers are as follows:

1. ATTCTAAATATCCTAACT (found at position -559, Fig. 3.2)
2. GAAGTAGACGCGGAAACT (found at position -209, Fig. 3.2)
3. AAGGAGTTTCAGAAAGAA (found at position +142, Fig. 3.2)

The gene outlined in this figure was designated atar1.

JDM18

JDM19

Figure 3.2 General Features of the Arabin Gene (atar1)
Sequence

Nucleotides are numbered from the first base of the coding region (A). Corresponding amino acids appear below the nucleotide codons of the coding region. The stop codon is marked by an asterisk. A dotted line indicates the position of the TATA box. Possible polyadenylation signals are double underlined. The location of two restriction sites, Kpn I and Hind III, are indicated. These sites were used in the construction of vectors for transformation (3.II.3.).

The sequence of atar1 differs from at2S1 (Krebbers et al., 1988) by one nucleotide. at2S1 contains an additional T at position -211.

-940 -930 -920 -910 -900 -890 -880 -870
GATATTTATTTATTTTCCATGGATAGAGAGCTAGGCATTCCGGTTATTTGGAGATTTTGGAAATTTCAATTTTGC

-860 -850 -840 -830 -820 -810 -800 -790
GGTTTGGTATTTATTTATTTATCAATTTGAACGAAACAGAGCTTTGTTTGGTTACGATGCGGTGGATTTTGGTTC

Kpn I

-780 -770 -760 -750 -740 -730 -720 -710
GGTTTAGAGTGATATATTTGGTACCAAAATTAACCAAGATTCGTTTTCGGTAAAAACAAATTTGATTTTAAAGCAT

-700 -690 -680 -670 -660 -650 -640 -630
TTTGGAAAAATAGTGTATATATATGAGATTTCTTAATCAAAATCTCACTTTTATCCGATTTAGTGGTAGTTCATAA

-620 -610 -600 -590 -580 -570 -560 -550
AGTGGTTTCATGTATATGATACCTGAATAACCAACATATGTATTTAAGAGACACTTGAATAATAATCTAAATATCC

-540 -530 -520 -510 -500 -490 -480 -470
TAACTACTCGTGTCCGTATGTTTGTACGGTGAAACGTGAGAGGACTAGTTTTGTACCCGTCATAACATTCCTTAG

Hind III

-460 -450 -440 -430 -420 -410 -400 -390
ACATACATTACTTTGGGAGTGAAAAACATTAAGCTTATCTTTATCCATATATTGTCTTACCATCAATAGACAATATCCA

-380 -370 -360 -350 -340 -330 -320 -310
ATGGACCGGTGACCTGCGTGTATAAGTAATTTTCAAGATGCTAAACTTTTATGTATTTTCAAGATTAACCTCCAAAAA

-300 -290 -280 -270 -260 -250 -240
CATTTATTGACACACTACTCTTTCCGTATTGACTCTCACTAGTCATTTCAAAATAATTGACATGTCAGAACATGA

T
v
230 -220 -210 -200 -190 -180 -170 -160
GTTACACATGGTTGCATATTGCAAGTAGACGCGGAACTTGTCACTTCCTTTACATTTCAAGTTTCCAACCTAATCAC

-150 -140 -130 -120 -110 -100 -90 -80
GACAACAATCATATAGCTCTCGCATACAAACAAACATATGCATGTATTCTTACACGTGAACCTCCATGCAAGTCTCTTTT

-70 -60 -50 -40 -30 -20 -10 1
CTCACCTATAAATACCAACCACACCTTCACCACATTCTTCACTCGAACCAAAACATACACATAGCAAAAA ATG
M

10 20 30 40 50 60
GCA AAC AAG TTG TTC CTC GTC TGC GCA GCT CTC GCT CTC TGC TTC CTC ACC AAC GCT
A N K L F L V C A A L A L C F L L T N A

70 80 90 100 110 120
TCC ATC TAC CGC ACC GTC GTT GAG TTC GAA GAA GAT GAC GCC ACT AAC CCC ATA GGC CCA
S I Y R T V V E F E E D D A T N P I G P

130 140 150 160 170 180
AAA ATG AGG AAA TGC CGC AAG GAG TTT CAG AAA GAA CAA CAC CTA AGA GCT TGC CAG CAA
K M R K C R K E F Q K E Q H L R A C Q Q

190 200 210 220 230 240
TTG ATG CTC CAG CAA GCA AGG CAA GGC CGT AGC GAT GAG TTT GAT TTC GAA GAC GAC ATG
L M L Q Q A R Q G R S D E F D F E D D M

250 260 270 280 290 300
GAG AAC CCA CAG GGA CAA CAG CAG GAA CAA CAG CTA TTC CAG CAG TGC TGC AAC GAG CTT
E N P Q G Q Q Q E Q Q L F Q Q C C N E L

310 320 330 340 350 360
CGC CAG GAA GAG CCA GAT TGT GTT TGC CCC ACC TTG AAA CAA GCT GCC AAG GCC GTT AGA
R Q E E P D C V C P T L K Q A A K A V R

370 380 390 400 410 420
 CTC CAG GGA CAG CAC CAA CCA ATG CAA GTC AGG AAA ATT TAC CAG ACA GCC AAG CAC TTG
 L Q G Q H Q P M Q V R K I Y Q T A K H L

430 440 450 460 470 480
 CCC AAC GTT TGC GAC ATC CCG CAA GTT GAT GTT TGT CCC TTC AAC ATC CCT TCA TTC CCT
 P N V C D I P Q V D V C P F N I P S F P

490 500 510 520 530 540 550
 TCT TTC TAC TAA ATCTCAACAAACCCTCAAAGCGTATGAGAGTGTGGTTGTTGATATACATGTTGACACTTG
 S F Y *

560 570 580 590 600 610 620 630
 ACACATACCACACCTCATCGTGTGTTTTATGATAAATGTAAGCTTTTGGATGTTTGAGGGTAATGTAAGCACTACTTAA

640 650 660 670 680 690 700 710
TAAATAAGAGTTTTGTTTATATTTTTTTGTTGTACATTGCTGCTCTCTAAAAAGTCATGACGCTTACATAAAAAATA

720 730 740 750 760 770 780 790
 ACTTTACTCCAGTGAAGTGGAAAAATTATAGAGTACCGCTCCACCTCCACGGAAGATCATAGACTAGTACTACCCAGA

800 810 820 830 840 850 860 870
 GATATAAATCGTAATTAAGACTTCAAATGCGTAGAGACAAATGGAATTATGACTTGGCAATTTGATCTTTCTTGATATT

880 890 900 910 920 930 940 950
 TTTAACAAATGGTTTACGTATGTCTTTATCAAACAAAAAAGTAAATAATGTGTTTAATTATTGAG*TGTTGGCTAATA

960 970 980 990 1000 1010 1020 1030
 GCACAATTTAAAAAAGTATGTCCAATTTTTCTTTTAAAAAAGCTATAACGAAGCATGTAAAAATGAACACTTAAA

1040 1050 1060 1070 1080 1090 1100 1110
AAATAAAGTTTCATTTACGAATCATGTACAATTGTGGATTTGAGATTAAGAATCCATCTGAGAATAGTATACCATTTG

1120 1130 1140 1150 1160
 TGATTTGTTTATGAATGGTATGACACCATAAACGACTATACTACAGTTTGAGA

of the TATA box reported for other plant genes (Heidecker and Messing, 1986; Elliston and Messing, 1989; Elliston and Messing, 1988). Three possible polyadenylation signals are found at positions 636, 919 and 1034. The gene codes for a 13 kD processed protein consisting of a large and small subunit (De Clercq, *et al.*, 1990).

The region of the gene upstream of the translation initiation site contains multiple direct and inverted repeats (Figure 3.3). Sequences A, F, and K are perfect direct repeats, and I and L are perfect inverted repeats. Imperfect repeats are shown by B, D, E and H (direct) and C, G, J and M (inverted). A palindrome exists at position -248. There are several stretches of alternating purine and pyrimidine residues within the 5' region of the *atar1* gene. Some of these regions overlap Boxes I and II. Sequences identical or similar to these Boxes have been reported for the napin genes of *B. napus* (*napA*, Josefsson *et al.*, 1987; *gNa*, Scofield and Crouch, 1987; *napB*, Ericson *et al.*, 1991; *Bng NAP1*, Baszczyński and Fallis, 1990; see Table 1.1). Sequences outlined by Boxes III and IV are similar to those shared by genes known to be regulated by ABA (Ericson *et al.*, 1991; Mundy and Chua, 1988; Gómez *et al.*, 1988).

Figure 3.3 Analysis of atar1 Promoter and Upstream Sequences

Sequences upstream of the translation initiation codon of the atar1 gene are presented. The TATA box is marked by a dotted line. The locations of the Hind III and Kpn I restriction sites are marked above the site. A palindrome at position -248 is double underlined. Arrows mark the locations of direct repeats (solid arrows) and inverted repeats (dotted arrows). Boxes I to IV are described in the text.

-940 -930 -920 -910 -900 -890 -880 -870
 GATATTTATTTTATTTTCCATGGATAGAGAGCTAGGCATTCCGGTTATTTGGAGATTTTGAATTTCAATTTTGC
 A

-860 -850 -840 -830 -820 -810 -800 -790
 GGTTTGGTATTTTATTTTATTTATCAATTTGAACGAAACAGAGCTTTGTTTGGTTACGATGCGGTGGATTTTGGTTC
 A

Kpn I

-780 -770 -760 -750 -740 -730 -720 -710
 GGTTTAGAGTGATATATTTGGTACCAAATTAAACCAAGATTTCGTTTTCGGTAAAAACAAAATTTGATTTTAAAGCAT
 B

-700 -690 -680 -670 -660 -650 -640 -630
 TTTTGGAAAAATTAGTGTATATATATGAGATTTCTTAATCAAATCTCACTTTTATCCGATTTAGTGGTAGTTCATAA
 C D

-620 -610 -600 -590 -580 -570 -560 -550
 AGTGGTTCATGTATATGATACCTGAATAACCAACATATGTA1TTAAGAGACACTTGAATAATAATTTCTAAATATCC
 D E F G

-540 -530 -520 -510 -500 -490 -480 -470
 TAACTACTCGTGTCCGTATGTTTTGTCACGGTGAAACGTGAGAGGACTAGTTTGTGACCCCTCCATAACATTCTTAG
 H I J

Hind III

-460 -450 -440 -430 -420 -410 -400 -390
 ACATACATTACTTTGGGAGTGAACAAACATTAAAGCTTATCTTTATCCATATATTGCTTACCATCAATAGACAATATCCA
 K L

-380 -370 -360 -350 -340 -330 -320 -310
 ATGGACCGGTGACCTGCGTGATAAGTAATTTTCAAGATGCTAAACCTTTATGTATTTTCAAGATTAACCTCCAAAAA
 M C F G K

-300 -290 -280 -270 -260 -250 -240
 CATTIATTGACACACTACTCTTTCCGTATGACTCTCAACTAGTCATTTCAAAATAATTGACATGTCAGAACATGA
 J

-230 -220 -210 -200 -190 -180 -170 -160
 GTTACACATGGTTGCATATGCAAGTACACGCGGAACTTGCACITCCATTACATTGAGTTTCCAACACCTAATCAC
 M

-150 -140 -130 -120 -110 -100 -90 -80
 GACAACAATCATATAGCTCTCGCATACAAACAAACATATGCATGTATCTTACACGTGAACCTCCATGCATGTCTCTTT
 B E

-70 -60 -50 -40 -30 -20 -10
 CTCACCTATAAATACCAACCACACCTTCACCACATTCTTCACTCGAACCACAAACATACACACATAGCAAAAA ATG
 I

3.II.3. Vector Construction

Vectors were constructed using the pBI 101 cassettes available from Clontech (Palo Alto, California). Figure 3.4 outlines the general scheme for vector construction. Different promoters were cloned upstream of the coding region of the *E. coli* β -glucuronidase (GUS) reporter gene. The pBI 101 cassettes also contain a bacterial nptII gene which confers kanamycin resistance upon transformed plant cells. The nos-GUS vector, pBI 131 and pBI 121 were obtained from other sources (see 2.V.1.). The atar1-GUS vectors are described in more detail in Figures 3.5 and 3.6.

Translational fusions give rise to proteins encoded by DNA from two or more sources. The translational fusions described in Figure 3.5 contain the first 23 amino acids of the arabin protein. The signal peptide of arabin consists of the first 21 amino acids (Krebbers et al., 1988). The arabin fragment was obtained from an M13 deletion bank clone (MP19-449) by digestion of the replicative form of the clone with Hind III (see Figure 3.2) and Eco RI. Due to the protocol used to construct the deletion bank, a G-tail and Eco RI site are attached to the 3' end of the atar1 sequence at position 70. An additional 15 amino acids are contained in the fusion protein between the 23 amino acids coded by the arabin sequence, and the GUS protein. These amino acids are contributed by the G-tail, Eco RI site and the nucleotides

Figure 3.4 General Vector Scheme *

BOTTOM. T-DNA region of the pBI 101-based vectors. The polylinker cloning sites are upstream of the GUS coding region and the nopaline synthase polyadenylation site (NOS-term). Kanamycin resistance is conferred by a bacterial nptII gene flanked by a nos promoter and NOS-term. RB, right border; LB, left border.

TOP. Various promoter regions were cloned in the polylinker sites. pBI 131 (tobacco rbcS promoter) was provided by Dr. R. Jefferson, and pBI 121 (CaMV 35S promoter) was obtained commercially. A 1480 bp fragment containing the nos promoter was cloned into the Hind III, Bam HI sites of pBI 101. Different arabin gene fragments from atar1 were spliced into the polylinker region. For more detail, see Figures 3.5 and 3.6.

*Figure was adapted from Jefferson et al., 1987.

Figure 3.5 Vector Construction: Translational Fusions

A 506 bp arabin gene (atar1) fragment (-436 bp to 70 bp, relative to the ATG) was inserted into the polylinker region of pBI 101, upstream of the GUS coding region. The coding region was placed in three different reading frames relative to the arabin ATG, by the use of the three GUS cassettes (pBI 101.1, pBI 101.2, pBI 101.3). pTLF1 (frame 1) contains the GUS coding region ATG correctly aligned with the arabin gene ATG (in order to give rise to a fusion protein composed of the first 23 amino acids of the arabin protein plus 15 amino acids from the G-tail, Eco RI site and polylinker, and the entire GUS protein). The arabin gene fragment in pTLF1R was inserted in the reverse orientation.

pTLF1: translational fusion, frame 1

pTLF2: translational fusion, frame 2

pTLF3: translational fusion, frame 3

pTLF1R: translational fusion, frame 1, reversed

TRANSLATIONAL FUSIONS

Figure 3.6 Vector Construction: Transcriptional Fusions

Two arabin gene (atar1) fragments with a common 3' end were used to make the transcriptional fusions. A 717 bp segment was cloned in both orientations (pTCL, pTCLR) into the polylinker region upstream of the GUS coding region of pBI 101.1. A smaller fragment (392 bp) was also cloned in both orientations (pTCS, pTCSR). Where necessary, the original cleaved ends (Hind III, Kpn I or Eco RI) were blunt-end repaired to allow ligation in a repaired Sal I site.

pTCL: transcriptional fusion, long arabin fragment

pTCLR: transcriptional fusion, long arabin fragment,
reversed

pTCS: transcriptional fusion, short arabin fragment

pTCSR: transcriptional fusion, short arabin fragment,
reversed

TRANSCRIPTIONAL FUSIONS

in the pBI 101 cassette polylinker. None of the nucleotide triplets in this region encode a translation termination signal.

The arabin gene fragment was cloned in the three different reading frames relative to the GUS gene ATG by using the three pBI 101 cassettes. pTLF1 contains the arabin ATG and the GUS ATG aligned in the same reading frame. Vector pTLF1R contains the arabin gene fragment in the reverse orientation relative to the GUS coding region. The arabin fragments were cloned into the Hind III and Sal I sites, or the Sal I site only (pTLF1R) of the pBI 101 polylinker.

The fusion sites of the vectors were checked by sequencing (Figure 3.7).

Transcriptional fusions give rise to proteins encoded by DNA from only one source. The fusion site is upstream of the coding region. The transcriptional fusions described in Figure 3.6 contain either a 717 bp or 392 bp arabin gene fragment from atar1. The fragments were obtained from an M13 deletion bank clone (MP19-110) by digestion of the replicative form of the clone with Eco RI and either Kpn I or Hind III (see Figure 3.2). As with clone MP19-449, clone MP19-110 also contained a G-tail and an Eco RI site attached to the arabin sequence at position -44. In vectors pTCLR and pTCSR, the arabin fragments were inserted in a reverse orientation relative to the GUS coding region.

The arabin fragments were cloned into the Hind III and Sal I sites, (pTCS) or the Sal I site only, of the pBI 101.1 polylinker. The Sal I site was chosen for several reasons. The Pst I and Sph I restriction sites in the polylinker are not unique on the plasmid. Sma I is already blunt-ended, but it is on the periphery of the polylinker which could make additional modifications of the vector, if necessary, more difficult. The main reason for choosing Sal I was to increase the distance between the TATA box on the arabin gene fragment (only 18 bp upstream of the 3' end of the arabin fragment) and the GUS ATG. By using the Sal I site, an extra 14 nucleotides were contributed by the polylinker, placing the TATA box 62 bases upstream of the GUS ATG. This spacing reflects the distance between the TATA box and the ATG in the original atar1 gene.

The fusion sites were checked by sequencing (Figure 3.7).

Figure 3.7 Sequences at the Vector Fusion Sites

In each case, the 3' end of the arabin fragment was blunt-ended and cloned into the blunt-ended Sal I site of the pBI cassette.

A. Translational Fusions

The arabin gene fragment was cloned into the three pBI 101 cassettes to place the atar1 translation initiation site in the three different reading frames relative to the GUS gene ATG.

pTLF1R contains the arabin fragment in the reverse orientation.

B. Transcriptional Fusions

The two atar1 gene fragments were cloned into the pBI 101.1 cassette in the forward (pTCL, pTCS) or reverse (pTCLR, pTCSR) orientations.

fusion site
↓

pTLF1

•70 Eco RI / Sal I

TCC ATC T GGGGGAATT / TCGACTCTAGAGGATCCCCGGGTGGTCAGTCCCTTATG

G-tail

pBI 101.1 polylinker

pTLF2

•70 Eco RI / Sal I

TCC ATC T GGGGGAATT / TCGACTCTAGAGGATCCCCGGGTAGTTCAGTCCCTTATG

G-tail

pBI 101.1 polylinker

pTLF3

•70 Eco RI / Sal I

TCC ATC T GGGGGAATT / TCGACTCTAGAGGATCCCCGGGTACGGTCAGTCCCTTATG

G-tail

pBI 101.3 polylinker

pTLF1R

Hind III / Sal I

TATGGTATAAAGATAAGCT / TCGACTCTAGAGGATCCCCGGGTTTCAGTCCCTTATG

pBI 101.1 polylinker

pTCS
and
pTCL

-44 Eco RI

CACACCTTCAGGGGAATT / TCGACTCTAGAGGATCCCCGGGTTCAGTCCCTTATG

G-tail

pBI 101.1 polylinker

pTCSR

Hind III Sal I

ATATGGATAAAGATAAGCT / TCGACTCTAGAGGATCCCCGGGTTCAGTCCCTTATG

pBI 101.1 polylinker

pTCLR

Kpn I Sal I

TGGGTTTAATTGGTAC / TCGACTCTAGAGGATCCCCGGGTTCAGTCCCTTATG

pBI 101.1 polylinker

3.III. DISCUSSION

3.III.1. atar1 Gene Analysis

The sequence of atar1 from A. thaliana Landsberg erecta was found to be essentially the same as that reported by Krebbers et al. (1988) for at2S1, a 2S albumin gene from A. thaliana seeds collection number C24. Upon comparison of the two sequences (for 1637 bp), only one difference could be detected. at2S1 contains an additional thymine at position -211. Surprisingly, sequence divergence between at2S1 and each of the other three A. thaliana 2S albumin genes in C24 reported by Krebbers et al. (1988) (Table 1.1) is substantially greater than that between at2S1 and atar1.

The gene contains no introns, as is the case for other 2S albumin genes (Krebbers et al., 1988; Josefsson et al., 1987; Ericson et al., 1991). A perfect TATA box is found at -66 (Figure 3.2). The TATA box is necessary for efficient and accurate initiation of transcription (Breathnach and Chambon, 1981). A CAAT box consensus sequence is absent in atar1. However, at position -84, CAAGT can be found. This corresponds to CAAT at the equivalent position in at2S2, and CAAAT in at2S3. No AGGA box such as that described by Messing et al. (1983) for zein genes is present. It is not unusual to find little or no homology to CAAT or AGGA boxes in plant genes (Elliston and Messing, 1988).

The translation start site consensus sequence for plants is TAAACAATGGCT (Lütcke *et al.*, 1987; Joshi, 1987). The corresponding sequence in *atar1* is CAAAAAATGGCA. At the -2 position, A is the second most prevalent base (39% versus 47% for C) in a survey of 79 plant genes (Joshi, 1987). Based on sequence alone, one can predict that translation at the *atar1* initiation codon is efficient, but functional analysis would be required to prove this.

The deduced consensus for the transcription initiation site in plants is CTCATCA. In 85% of the genes surveyed, A was found at the transcription initiation site (Joshi, 1987). The initiation site was not determined for *atar1*, but it is known for several napin genes (*napA*, Josefsson *et al.*, 1987; *gNa*, Scofield and Crouch, 1987). Based on comparison with the napin genes, it is likely that the transcription initiation site occurs at an adenine residue at -27 or -32, although the sequence surrounding an A at -44 (TTCACCA) is closer to the consensus sequence mentioned above. Depending on which of the three possible initiation sites one chooses, the leader sequence can be calculated to be 64 to 70% A+T-rich. Leader sequences which are rich in A and T residues may be more easily "melted" by scanning ribosomal subunits (Heidecker and Messing, 1986; Joshi, 1987; Elliston and Messing, 1988).

Three possible polyadenylation signals appear in *atar1*. Two napin cDNA clones, pN1 and pN2 (Crouch *et al.*, 1983)

have a polyadenylation signal (AATAAA) approximately 130 bases downstream of the termination codon, and 25 bases upstream of the poly-A addition site. It is likely that the first polyadenylation signal in atar1 (at position 636) is the functional one. It appears approximately 140 bp downstream of the termination codon. The other two possible polyadenylation signals occur after the point in the atar1 sequence which corresponds to the site where the poly-A tail is added in pN1 and pN2. Further study will be required to elucidate the function, if any, of the three presumptive polyadenylation signals.

The promoter and upstream regions of atar1 contain a number of direct and inverted repeats. Repetition of a particular DNA motif suggests that it may play a role in the functioning of a gene. Regulation of transcription involves the interaction of protein factors with specific DNA sequence elements in a gene. Regulatory regions of a gene are believed to contain distinct functional modules consisting of the recognition sites for one or more of these transcriptional activator proteins (Dyner, 1989). These modules can either cooperate or work independently. A repeated element may act as two independent recognition sites for two copies of a transcription factor, or two related factors. Many factors act as either homodimers, or heterodimers with other members of the same family.

Different combinations may have different regulatory properties (reviewed in Jones, 1990).

Transcription factors themselves are modular in structure and contain DNA-binding domains and/or transcriptional activation domains (Frankel and Kim, 1991). There are two classes of activators. Universal activators contain both a DNA-binding region and an activation region which interacts with the target protein. Non-universal activators contain only one of the regions and therefore must cooperate with another factor which provides the missing function (Ptashne and Gann, 1990). Factors that are originally bound to the DNA at separated locations may require that the gap be closed before they can interact. For example, two molecules of a factor recognizing sequence F (Figure 3.3) would be bound approximately 250 bp apart. In order to bring potentially interactive factors together, DNA looping may occur (Schöler *et al.*, 1988). This would not be an option for factors separated by short segments. In this case, interaction may require cis-acting elements to be found on the same side of the DNA helix. The helical twist of DNA in the nucleosome is approximately 10 bp per turn (Morse and Simpson, 1988). For repeats A, D, H and L, the centre of the elements in each pair are separated by approximate multiples of 10 bp (80, 69, 30 and 19, respectively) which would place both elements of the repeat on the same side of the helix.

Repeat L is an inverted repeat potentially capable of forming a hairpin loop. Formation of the loop would require local unwinding of the DNA helix. The loop could act as a recognition signal for some component of the transcriptional machinery, or perhaps its formation could result in a conformational change at another point in the DNA thereby increasing accessibility of the DNA by transcription factors.

Inverted repeat G contains a sequence (AGAAT) proposed to act as a general enhancer in Arabidopsis thaliana genes (Ott and Chua, 1990). The inverse of the enhancer sequence (ATTCT[T]) can be found at positions -39, -106, and -475 (Figure 3.3) in addition to the one found within repeat G (-559).

The majority of the repeated sequences in atar1 are A+T-rich. Ten out of 13 different repeats consist of 75% or more A+T residues. The average A+T content of A. thaliana DNA is 60% (Leutwiler et al., 1984). A+T-rich cis-acting elements have been identified in other genes. A conserved motif in a class I patatin promoter was found to bind a nuclear protein present in leaf and tuber extracts (Liu et al., 1990). An upstream A+T-rich sequence from a French bean phaseolin gene which bound a nuclear factor from immature bean cotyledons was shown to activate transcription from a minimal 35S promoter (Bustos et al., 1989). Similar results were reported for an A+T-rich region from a sunflower

helianthinin gene (Jordano et al., 1989). A nuclear High Mobility Group (HMG) protein was shown to bind to A+T-rich stretches of the P2 promoter of a gene encoding a 19kD zein storage protein, although not all A+T-rich regions bound the nuclear protein, pointing to the involvement of other factors (Grasser et al., 1990). A pea ferredoxin I gene and a soybean nodule gene were also reported to bind HMG proteins at A+T-rich sequences (Pedersen et al., 1991; Jacobsen et al., 1990). A general role for HMG proteins in transcriptional activation has been proposed. The proteins may bind to A+T-rich tracts near promoters or enhancers in order to keep the chromatin accessible to transcription factors. The HMG proteins, which are acidic, may interact with basic histones or with transcription factors themselves (Pedersen et al., 1991; Grasser et al., 1990).

A 10 bp palindrome which is found at position -248 may act as a cis element. A 16 bp palindrome in the octopine synthase gene was shown to bind two identical proteins. Occupation of both sites was necessary for transcriptional enhancement (Tokuhisa et al., 1990). The smaller size of the atar1 palindrome (10 bp) may rule out the possibility of double binding but it may still act as a recognition site for a single factor. A degenerate form of this sequence exists in the napin genes (napA, Josefsson et al., 1987; gNa, Scofield and Crouch, 1987; napB, Ericson et al., 1991; Bng NAP1, Baszczynski and Fallis, 1990) but the sequence is not

a true palindrome in any of these cases. As with all the other potential cis elements in atar1, the palindrome would require functional testing before a role in transcription could be assigned.

A number of alternating purine/pyrimidine stretches overlap Boxes I and II in the arabin gene. These areas of alternating purine and pyrimidine residues have the potential to form Z-DNA. It has been suggested that Z-DNA may influence gene expression, perhaps by maintaining an active configuration of the regulatory region by enhancing interactions between trans-acting factors and cis-acting sequences through the binding of Z-DNA-specific proteins, or by altering chromatin structure or the level of negative supercoiling (An, 1987; reviewed in Rich et al., 1984). Deletion analysis of the regulatory regions of a wheat gliadin gene (Aryan et al., 1991), an A. thaliana chlorophyll a/b binding protein gene (Ha and An, 1988) and an Agrobacterium tumefaciens nopaline synthase gene (An et al., 1990; Mitra and An, 1989) supported the possibility of a role for Z-DNA-forming elements in the regulation of these genes.

Variations of Box I (TACACAT) are found four times in the 5' upstream regions of atar1 (Figure 3.3) and also within the four napin and other three arabin genes reported (Josefsson et al., 1987; Scofield and Crouch, 1987; Ericson et al., 1991, Baszczynski and Fallis, 1990; Krebbers et al.,

1988; Table 1.1). A nuclear factor in an extract from immature seeds binds to the Box I motif (Ericson *et al.*, 1991). The sequence TG/TCAT/AGTAT/G occurs three times in atar1 (Box II). This motif encompasses the palindrome TGCATGCA described by Ericson *et al.* (1991) which is located in the napin and arabin genes referred to above. This is similar to the sequence CATGCATG found in various legume seed protein genes (Dickinson *et al.*, 1988) and composing part of the "legumin box" of legume 11S globulin seed storage genes (Bäumlein *et al.*, 1986). Since this sequence is distributed among different seed genes, it is possible that this element plays a role in seed-specific gene regulation.

The consensus sequence GTCTCCCT comprises Box III. Three different forms of the sequence appear in the atar1 upstream region. It matches 1 of 4 G+C-rich repeats in the rice rab 21 gene described by Mundy and Chua (1988). The rab (Responsive to ABA) gene is induced by water stress and abscisic acid (ABA). Another motif detected in genes regulated by ABA is shown in Box IV (TC/GACTT/CC/TC). The sequence is found three times in atar1, once in the reverse orientation (Box IVR). Various forms of the motif occur in the rice rab 21 gene (Mundy and Chua, 1988), an ABA-inducible maize embryo gene (Gómez *et al.*, 1988), and napin and arabin genes (Ericson *et al.*, 1991; Baszczynski and Fallis, 1990; Josefsson *et al.*, 1987; Scofield and Crouch,

1987; Krebbers et al., 1988). An ABA response element (CGAGCAG) for the Em gene of wheat was proposed by Marcotte et al., (1989). An inverted version of this sequence (CTACTCG) is present at position -543. ABA response elements are not unexpected in atar1. In the presence of ABA, storage protein synthesis in cultured B. napus embryos is maintained at levels close to those found in seeds (Crouch et al., 1984). Levels of cruciferin and napin mRNAs as well as transcription rates are elevated in embryos cultured with ABA (DeLisle and Crouch, 1989). Another B. napus seed protein gene, encoding a late embryogenesis-abundant protein, has been shown to respond to ABA (Harada et al., 1989b). The mechanism(s) linking ABA response elements to ABA-induced physiological changes in seeds remains to be elucidated.

3.III.2. Vector Construction

In order to study the expression of the arabin gene in transgenic plants, transformation vectors were constructed that contained promoter and upstream sequences of atar1 cloned upstream of a promoterless GUS gene. One set of vectors (transcriptional fusions) contained only regulatory sequences from atar1. Two convenient restriction sites were utilized, Hind III and Kpn I (Figure 3.2). At the time that these vectors were constructed, only two napin genomic

sequences were published, gNa (Scofield and Crouch, 1987) and napA (Josefsson *et al.*, 1987). By comparing the sequence of atar1 with that of these two genes, it could be seen that significant homology was only detectable within the first 150 to 200 bp upstream of the translation initiation codon. Bearing this in mind, the Hind III site at position -436 was chosen for vector pTCS and the control vector, pTCSR. This same 5' site was used for the translational fusions, but an additional 114 bp downstream were included. Part of this region codes for the signal peptide of arabin plus two additional amino acids. These vectors were included to examine the possibility that 5' leader and/or signal peptide coding region sequences might contribute significantly to arabin gene expression. It was difficult to unambiguously determine the number of G residues in the tail generated during deletion bank preparation on the clone that was used as a source of arabin sequences. The number of G residues would determine the reading frame of the translational fusions. Therefore, all three possible reading frames were constructed using the three pBI 101 GUS cassettes. Sequencing of the fusion sites identified the correct reading frame for the fused protein (pTLF1). The last pair of vectors pTCL and pTCLR contained atar1 sequences upstream as far as the Kpn I site (-761). These vectors were included to address the possibility that significant cis elements exist in atar1 upstream of the Hind III site.

N. tabacum and B. napus explants were transformed with the aforementioned vectors. Analyses of GUS expression in transformed plants are detailed in Chapters 4 and 6.

CHAPTER 4

NICOTIANA TABACUM TRANSFORMATION AND GENE EXPRESSION ANALYSIS

4.1. INTRODUCTION

Transcription of a gene is regulated by a set of cis-acting control elements that interact with sequence-specific trans-acting factors (Dyner, 1989). Response to a particular stimulus is dependent upon the presence of a certain cis sequence or sequences in the gene and the availability of the corresponding trans-acting factors. Cis elements associated with different stimuli have been identified. Perhaps the best characterized plant gene cis elements are those involved in light regulation of gene expression (Cuozzo et al., 1988; Gilmartin et al., 1990). Another example of a cis response sequence is the plant heat shock element (Schöffl et al., 1988). Specific sequence elements have not been identified in every case. Rather, inducibility or enhancement of a gene has been associated with a larger region of the gene sequence.

Analyses of the regulatory region of a gene often utilize constructs containing various fragments of the 5' regions of a gene fused to a reporter molecule such as GUS. In some cases such constructs have been analysed in a transient assay system (Van der Meer et al., 1990; Lawton et al., 1991; Staiger et al., 1990). Transient assays allow many constructs to be analysed in a relatively short time but the transferred genes often do not become stably integrated (Fromm and Walbot, 1987). Intracellular conditions within protoplasts may not completely mimic those in vivo and therefore, results obtained in transient systems may not faithfully reflect gene expression in the plant.

It is possible to stably integrate foreign DNA into the genome of a plant cell. In dicotyledonous plants this is usually accomplished using Agrobacterium tumefaciens-mediated transformation. There are numerous accounts in the literature of the use of transgenic plants to study gene expression. Frequently, tobacco is the plant of choice. It is easy to transform and regenerate, and yields a large number of seeds. The expression of genes encoding seed storage proteins of dicotyledonous plants has been studied in transgenic tobacco (Rerie et al., 1991; Newbigin et al., 1990; Chen et al., 1988; Bogue et al., 1990; Bustos et al., 1989). The presence of an endosperm as well as an embryo in tobacco seeds permits the regulation of storage protein genes from monocotyledonous plants to be investigated as

well (Goldberg et al., 1989) since the expression of these genes is often endosperm-specific (Takaiwa et al., 1991; Schernthaner et al., 1988; Marris et al., 1988; Robert et al., 1989).

In this study, the expression of an Arabidopsis thaliana 2S albumin storage protein gene (atar1) was investigated. Vectors containing atar1 5' sequence and the GUS coding region were transferred to N. tabacum (tobacco) using A. tumefaciens-mediated transformation. The spatial, temporal and quantitative aspects of GUS expression were analysed in somatic tissues and developing seeds of transgenic N. tabacum plants. A comparison of fluorogenic GUS activity in N. tabacum transformants containing different constructs indicated that there is an enhancer-like element in the atar1 gene 5' sequence. Gene expression was limited to the seeds, from the late globular/early heart stage to the end of embryo development. Histochemical localization of the GUS activity showed that the atar1-GUS fusion (construct pTCS) was not induced uniformly throughout the embryo. Activity was first detected in the upper axis of the embryo and radiated out through the embryo as it developed and matured. The expression of the arabin gene is discussed as compared to that of napin and other seed storage protein genes.

4.II. RESULTS

4.II.1. Nicotiana tabacum Transformation

N. tabacum cv. Petit Havana SR1 was transformed with 11 different vectors (pBI 131, pBI 121, nos-GUS, 8 atar1-GUS vectors; see Figures 3.4, 3.5, 3.6) according to the scheme in Figure 4.1. Transformants were selected on kanamycin (100 $\mu\text{g/ml}$). Cefotaxime was used to clear the tissues of A. tumefaciens after cocultivation. Approximately 10 Km^R shoots per vector were selected and most of those were transferred to the greenhouse upon rooting. On the day of anthesis, flowers were self-pollinated and tagged. Seed samples, collected at weekly intervals, as well as somatic tissue samples were tested for GUS enzyme activity. A rapid microtiter plate assay was used to initially screen the samples for GUS activity.

4.II.2. Results of Initial Screening of N. tabacum Plants

4.II 2.a. Microtiter plate assay

Extracts from the various tissues were prepared on a fresh weight basis. The extracts were tested for fluorogenic GUS activity by addition to the substrate MUG. Endogenous fluorescence was monitored by adding the extracts to extraction buffer only (-MUG). Plates were incubated

Figure 4.1 Nicotiana tabacum Transformation and Sample
Collection Scheme

Km^R, kanamycin-resistant.

Precondition 2-3 days. 25^oC

Inoculate with 48 hr culture of
Agrobacterium tumefaciens
containing vector

Return to plates for 48 hr

Transfer to TLD selection plates
containing Kanamycin (Kn) and
Cefotaxime (Cef)

Maintain Kn^R shoots on selection
(MSB5, 3% sucrose, Kn, Cef)
until develop roots

Transfer to greenhouse

Tag flowers on day of pollination (anthesis)

Samples collected for initial screening
seeds 1,2,3,4 wks: dry
leaf stem, flower, roots

overnight at 37°C, reactions were terminated by the addition of stop buffer, and the fluorescence was graded visually after placing the plates on a UV transilluminator. Examples of the grades of GUS activity are given in Plate 4.1. The levels of endogenous fluorescence (-MUG wells) in the extracts were taken into account during grading. When assessing activity in seed samples, both the intensity of fluorescence and the time of onset of fluorescence during seed development were considered.

Plants transformed with GUS vectors containing previously characterized promoters were tested first in the plate assay. The patterns of GUS activity obtained with the known promoters (Plate 4.2) were compared to published reports of gene expression for these promoters (Nagy *et al.*, 1985; An *et al.*, 1988; Coruzzi *et al.*, 1984). Plants transformed with pBI 131 (containing the tobacco ribulose-1,5-bisphosphate carboxylase small subunit [*rbcS*] promoter) had the highest GUS activity in leaf and stem samples. Plants from the pBI 121 (containing the Cauliflower Mosaic Virus [CaMV] 35S promoter) group possessed GUS activity in all samples tested, although at varying levels. Plants transformed with *nos*-GUS constructs (containing the nopaline synthase promoter) also had GUS activity in most tissues but the levels were lower than those for pBI 121. These results did not differ greatly from expected activity patterns (see 4.III.1.). Therefore, it was decided that the microtiter

Plate 4.1 Microtiter Plate Assay: Relative Levels of
GUS Activity

Fluorescence was scored according to an arbitrary scale from high, to no detectable activity. All extracts were tested with (+) and without (-) the substrate (MUG, 4-methylumbelliferyl glucuronide). The last column contains fluorescence standards (MU, 4-methylumbelliferone).

Plate 4.2 Microtiter Plate Assay: Characterized Promoters

A plant from each of three vector groups, and an untransformed N. tabacum plant were used for the assay presented in this figure. (The nos-GUS representative shown here produced the highest level of fluorescence observed in that group.) Each plant extract was tested with (+) and without (-) the substrate (MUG, 4-methylumbelliferyl glucuronide). The last column contains fluorescence standards (MU, 4-methylumbelliferone).

plate assay could be used as a reliable method by which to initially screen the 68 remaining Km^R plants for GUS activity. The assay was used to estimate the relative activity levels in selected tissues from GUS-positive plants. The fluorometer assay was used for more quantitative analyses.

4.II.2.b. Summary of initial screening of N. tabacum plants

Initial results are summarized in Table 4.1. There was a great deal of variation in the GUS activity profile in plants transformed with pBI 131, pBI 121 and nos-GUS. The plants containing atar1-GUS vectors showed more uniformity of activity, mainly because no activity was detectable in somatic tissues (except in one plant transformed with pTLF1). Most of the Km^R plants (94.4%) were also positive for fluorogenic GUS activity. Seed samples from plants identified as GUS-positive were subsequently analysed with the more sensitive fluorometer method. A few plants were discarded before the fluorometer analysis due to the physical state of the plants.

4.II.3. Comparison of Fluorogenic GUS Activity in Seeds from N. tabacum Plants Transformed with Various Vectors

Seeds from plants identified as GUS-positive in
4.II.2.b. were tested quantitatively for fluorogenic GUS

Table 4.1 Summary of the Initial Screening of the Km^R
N. tabacum Plants

Various tissues of kanamycin-resistant (Km^R) transformed plants were tested for GUS activity using the microtiter plate assay.

(See Plates 4.1, 4.2 for examples.)

ND, not detectable; med, medium.

VECTOR	Total no. Kn ^R plants	Total no. GUS ⁺ plants	BREAKDOWN OF RELATIVE GUS ACTIVITY LEVELS IN VARIOUS TISSUES OF GUS-POSITIVE PLANTS					
			GUS ACTIVITY PROFILE					no. plants with profile
			seeds	leaf	stem	roots	flower	
pBI 131 (<u>rbcS</u> - GUS)	8	8	low	high	high	ND	med	2
			ND	high	high	ND	low	3
			ND	high	high	ND	ND	1
			ND	high	med	ND	ND	1
			ND	high	low	ND	low	1
pBI 121 (<u>CoMV 35S</u> -GUS)	5	5	high	high	med	med	med	1
			high	high	high	low	med	1
			high	high	low	med	low	1
			high	med	high	high	low	1
			low	high	high	high	med	1
<u>nos</u> -GUS	8	8	med	low	med	med	ND	1
			low	ND	ND	ND	ND	4
			low	low	ND	ND	ND	1
			ND	ND	low	ND	ND	1
			ND	med	ND	ND	ND	1
pTCS	8	8	high	ND	ND	ND	ND	7
			med	ND	ND	ND	ND	1
pTCL	10	10	high	ND	ND	ND	ND	5
			med	ND	ND	ND	ND	4
			low	ND	ND	ND	ND	1
pTLF1R	8	6	high	ND	ND	ND	ND	2
			med	ND	ND	ND	ND	3
			low	ND	ND	ND	ND	1
pTCSR	10	9	high	ND	ND	ND	ND	2
			med	ND	ND	ND	ND	4
			low	ND	ND	ND	ND	3
pTLF1	8	7	high	ND	ND	ND	ND	2
			med	ND	ND	ND	ND	1
			low	ND	ND	ND	ND	3
			med	med	med	med	low	1
pTLF3	8	8	med	ND	ND	ND	ND	3
			low	ND	ND	ND	ND	5
pTLF2	10	9	med	ND	ND	ND	ND	3
			low	ND	ND	ND	ND	6
pTCLR	6	6	med	ND	ND	ND	ND	2
			low	ND	ND	ND	ND	4

Table 4.2 GUS Activity in Transgenic N. tabacum
Seeds (25 dpa)

A. Two seed samples (2 to 4 seed capsules/sample) were collected from each transformed plant at 25 dpa. Each sample was assayed in duplicate for GUS activity using the fluorometer method. Results were grouped according to the vector used for transformation.

Dispersion in the data was related to the mean. Therefore, a log transformation of the data prior to an Analysis of Variance was deemed appropriate.
SR1: untransformed N. tabacum; MU: 4-methylumbelliferone.

B. The data were examined with an Analysis of Variance with a Hierarchical Model. Terms in the model were: vectors; plants within vectors; seed samples within plants; duplicates within seed samples. Differences among vectors were tested using plant variation as the error term. All calculations were done using PROC GLM in SAS (SAS Institute Inc., Cary, North Carolina) on the VAX/VMS computer at Agriculture Canada, Ottawa, with the assistance of Dr. Mark Wolynetz.

*: denotes a significant difference at $\alpha=0.05$ in GUS activity between groups of plants transformed with different vectors

		nmol MU produced per min per mg protein					
		ORIGINAL DATA			LOG TRANSFORMATION OF DATA		
VECTOR	No. PLANTS	MEAN	STANDARD ERROR	MIN. - MAX. VALUES	MEAN	STANDARD ERROR	MIN. - MAX. VALUES
pTCS	8	23.6	5.6	2.6-80.3	2.6	0.3	1.0-4.5
pTCL	10	25.9	9.3	1.3-95.5	2.5	0.3	0.3-5.2
pTLF1R	6	24.5	6.6	1.3-64.5	2.5	0.4	0.3-4.2
pTCSR	9	4.9	0.9	0.6-16.8	1.4	0.2	-0.5-2.9
pTLF1	6	2.4	0.4	0.9-4.4	0.7	0.2	-0.1-1.5
pTLF3	7	3.1	0.4	0.6-6.6	1.0	0.2	-0.5-1.9
pTLF2	8	1.4	0.4	0.3-5.9	0.02	0.2	-1.2-1.8
pTCLR	6	1.5	0.4	0.5-6.1	0.1	0.2	-0.7-1.8
pBI 121	5	16.6	6.6	0.4-70.3	2.1	0.5	-1.0-4.3
SR1	7	0.4	0.1	0.08-1.2	-1.3	0.2	-2.6-0.2

[illegible]

activity using the fluorometer assay (Table 4.2). The analysis was restricted to plants transformed with the atar1-GUS fusions and pBI 121 since the initial results indicated that pBI 131 and nos-GUS directed little GUS activity in seeds of transgenic plants (Plate 4.2). Seeds were harvested at 25 dpa. By this stage, protein accumulation in the seed has tapered off (Sano and Kawashima, 1983) and dessication has begun. Small differences in the rate of development of individual seeds would have less effect on GUS enzyme accumulation at this stage than at an earlier stage when storage protein is being rapidly synthesized. Values generally reflected the ranking obtained in the initial screening although a great deal of plant-to-plant variation occurred within each vector group.

The highest level of GUS activity was detected in plants carrying the vectors pTCS, pTCL and pTLF1R. pTCS and pTCL are the two transcriptional fusions containing arabin 5' sequences in the correct orientation relative to the GUS coding region (Figure 3.6). pTCL contains an additional 325 bp of upstream sequence compared to pTCS. There was no significant difference in mean GUS activity between the groups of plants transformed with these two vectors (Table 4.2). Therefore, sequences responsible for high levels of developmentally-regulated and seed-specific GUS activity appeared to reside within bases -436 to -44 in the atar1 gene. The translational fusion, pTLF1R, containing the

arabin gene fragment in the reverse orientation relative to the GUS coding region (Figure 3.5), also directed high levels of GUS activity in transgenic N. tabacum. There was no statistical difference between the levels obtained with these three vectors and the GUS activity found in plants transformed with pBI 121, which contains the CaMV 35S promoter (Table 4.2).

All vectors resulted in GUS activity above that detected in untransformed plants. This was true even for vectors which contained the arabin fragment out-of-frame (pTLF2, pTLF3) (Figure 3.5) or in the reverse orientation (pTCLR, pTCSR) (Figure 3.6) relative to the GUS coding region. Vector pTLF1 is a translational fusion containing 436 bp of the regulatory region and sequence encoding the first 21 amino acids of atar1, spliced in the correct reading frame to the GUS coding region (Figure 3.5). Unexpectedly, GUS activity due to the integration of this vector in N. tabacum plants was not significantly different from that obtained with pTLF2 and pTLF3, where the fragment was spliced into the second and third reading frames.

The results of these experiments indicated that the cis elements responsible for seed-specific and developmental regulation of the atar1-GUS fusions resided within 392 bp of the atar1 5' sequence. Integral to this interpretation was the assumption that the GUS activity observed in transgenic N. tabacum was the result of expression of an intact

integrated atar1-GUS fusion. Southern blot analysis was performed to try to confirm this assumption and to determine the number of integrated genes in the transgenic plants.

4.II.4. Southern Blot Analysis of Transformed N. tabacum Plants

DNA was isolated from two transformed GUS-positive plants per vector group. The probe encompassed the GUS coding region.

Plant DNA was digested with Hind III (Plate 4.3). The enzyme cuts either within the polylinker region or the arabin insert, and also within the plant DNA, outside of the inserted T-DNA. The second Hind III cut, and therefore the size of the DNA fragment recognized by the probe, will vary depending on the site of integration of the T-DNA. This allows one to estimate the number of T-DNA insertions within a plant. As many as 8 hybridizing bands could be seen in individual lanes in the Southern blot (Plate 4.3). Some of the bands appeared to be more dense than others, suggesting the presence of more than 1 copy of the gene at that location in the gel. No copies of the gene were detected in untransformed N. tabacum (SR1).

Enzyme activity does not appear to be correlated with GUS gene copy number. For example, N. tabacum plants TCS-T1 and TCS-T5, which had similar copy numbers (Plate 4.3) had

Plate 4.3 Southern Blot Analysis of Transformed N. tabacum

Plants: Hind III Digestion, GUS Probe

A. This figure is a composite of two Southern blots. The molecular weight markers are placed to the side of each autoradiograph. Individual plants are listed across the top of the figure. The designation above each lane indicates the vector used for transformation and the particular N. tabacum transformant tested from that vector group (eg. TCSR-T7: vector pTCSR, transformant 7).

SR1: untransformed N. tabacum.

B. Diagram indicating the segment of the GUS gene used to make the radioactive probe for Southern blot analysis. The plant DNA was digested with Hind III which cuts either within the polylinker or the atar1 insert, and again at an unidentified site within the plant DNA external to the inserted T-DNA.

A

B

GUS activity levels of 17.84 and 66.87 nmol/min/mg protein, respectively (GUS activity summarized in Table 4.2; individual results not shown). Plants TCS-T3, TCS-T7 and TCS-T11 all contained 1 copy of the gene (results not shown) but GUS expression levels were 3.97, 10.81 and 19.42 nmol/min/mg protein, respectively.

In order to detect any rearrangements in the inserted GUS gene, an internal T-DNA fragment was cut out by digestion with Hind III and Eco RI (Plate 4.4). Fragments recognized by the probe were 2.5 to 2.6 kb (transcriptional fusions) or 2.7 kb (translational fusions). A few larger molecular weight bands were observed as well, perhaps due to a deletion or rearrangement that affected a restriction enzyme site. In all 10 plants studied, an additional band was detected at approximately 2.1 to 2.2 kb indicating rearrangement of one or more copies of the transferred DNA. In plant TCSR-T9, only this smaller band was seen. This fragment is shorter than that expected for an intact atar1-GUS fusion. However, this plant still expresses GUS in a seed-specific manner (pTCSR group summarized in Table 4.1) at 11.24 nmol/min/mg protein (group summarized in Table 4.2, individual results not shown).

Plate 4.4 Southern Blot Analysis of Transformed N. tabacum

Plants: Hind III/Eco RI Digestion, GUS Probe

A. Autoradiograph from Southern blot. Individual plants are listed across the top of the figure. The designations indicate the vector used for transformation and the N. tabacum plant tested. Molecular weight markers are placed to the right.

B. Diagram indicating the segment of the GUS gene used to make the radioactive probe for Southern blot analysis. The plant DNA was digested with Hind III which cuts within the polylinker or the atar1 insert. The DNA was cut a second time with Eco RI to yield a fragment of approximately 2.5 to 2.7 kb (depending on the vector) that would be recognized by the probe.

A

B

4.II.5. Choice of Plants For Further Study

Initial analyses of the transgenic N. tabacum plants indicated which vectors were associated with the highest level of GUS activity. The N. tabacum study was streamlined based on this information. Subsequent research was restricted to plants transformed with pTCS for the following reasons: 1. The pTCS vector resulted in high GUS activity in mature seeds and no detectable activity in somatic tissues, as would be expected for a storage protein promoter; 2. It was the simplest vector used since, a. there was no coding sequence for the arabin signal peptide, b. the vector contained the smallest amount of upstream sequence, and, c. the arabin promoter was cloned in the forward orientation relative to the GUS gene.

4.II.6. GUS Gene Expression in Transgenic N. tabacum Plants Transformed With pTCS.

4.II.6.a. GUS fluorogenic activity during N. tabacum seed development

Fluorogenic GUS activity was first detected at 12 dpa in plant TCS-T1 (Plate 4.5). This corresponds to the late globular or early heart stage of embryo development. The other 7 plants transformed with pTCS were also tested, and activity was first detected by 11 dpa (2 plants), 12 dpa (3

Plate 4.5 Time of Onset of GUS Fluorogenic Activity During
Seed Development

TOP. Fluorogenic activity of plant TCS-T1 was tested in the microtiter plate GUS assay. Extracts were prepared from seed on a fresh weight basis (3:1, μ l extraction buffer:mg plant material). All extracts were tested with (+) or without (-) the substrate (MUG, 4-methylumbelliferyl glucuronide). dpa, days post anthesis.

BOTTOM. Fluorescence standards. MU, 4-methylumbelliferone; MUG only, substrate without plant extract.

plants) or 13 dpa (2 plants) (results not shown). Maximum expression was seen in the 28 dpa and dry seed samples.

Gus activity in plant TCS-T1 was studied in more detail throughout seed development using the fluorometer assay (Figure 4.2). Activity was low at 10 dpa but rose almost 10-fold to peak at 21 dpa, then tapered off again as the seeds matured. The GUS enzyme activity in somatic samples (leaf, stem, flower, root) was near background (untransformed SR1) levels.

4.II.6.b. Spatial localization of GUS expression during N. tabacum seed development.

Spatial localization of GUS activity was determined by histochemical staining. Embryos of different stages of development were removed from the seed and stained overnight in X-gluc. Staining was first detected at 15 dpa (Plate 4.6, D) in the longitudinal centre of the embryo. As development proceeded, GUS activity spread towards each end until the entire embryo was stained by day 25 (Plate 4.6, E-H). Cross-sections revealed that staining began in the ground meristem of the embryo at day 15. GUS activity was detected shortly thereafter in the protoderm (Plate 4.6, L). Staining reached the cross-sectional centre (vascular cylinder) of the upper axis as the embryo progressed through the cotyledon stage (Plate 4.6, M). In the cotyledons, the GUS staining was detected along the inner faces before staining could be seen

**Figure 4.2 GUS Activity in Developing Transgenic
N. tabacum Seeds**

Seeds from transgenic N. tabacum plant TCS-T1 were harvested at various days post anthesis and pooled. A representative sample from each time point was tested in duplicate for GUS fluorogenic activity using the fluorometer method. Somatic tissues were also tested.

SR1: GUS activity in 18 dpa seeds from untransformed N. tabacum plants.

Plate 4.6 Spatial Localization of GUS Expression During
N. tabacum Seed Development

Seeds were dissected, and separated embryos, endosperms and seed coats were stained overnight in X-gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide). Whole embryos of plant TCS-T1: A, 12 dpa; B, 13 dpa; C, 14 dpa; D, 15 dpa; E, 16 dpa, (arrow refers to cross-section L); F, 18 dpa; G, 21 dpa, (arrow refers to cross-section M); H, 25 dpa. I, 25 dpa embryo from untransformed SR1; J, 25 dpa endosperm from TCS-T1; K, 25 dpa seed coat from TCS-T1; L, hand-cut cross-section from 16 dpa embryo from TCS-T1 (taken at position marked with arrow in E); M, hand-cut cross-section from 21 dpa embryo from TCS-T1 (taken at position marked with arrow in G); N, microtome-cut * cross-section through 25 dpa embryo cotyledons. Bar=100 μ m.

* Microtome sections were prepared by Dr. Mary Whiteley at the University of Ottawa using the JB-4 Embedding Kit (J.B. Em Services, Inc., Montreal) and a Sorval microtome. Whole embryos were stained in X-gluc overnight then embedded in glycol methacrylate (GMA) and cut into 10 μ m sections.

in the vascular cylinder of the axis. By approximately day 18, the wave of GUS activity had started to spread up both the inner and the outer faces of the cotyledons. However, the intensity of the stain was greater in the cells of the outer faces (Plate 4.6, N). GUS activity was detected in the endosperm as well (Plate 4.6, J). The seed coat did not stain (Plate 4.6, K) nor did untransformed N. tabacum embryos (Plate 4.6, I).

4.II.7. Segregation Analysis of Transgenic N. tabacum Seeds

Gene segregation in seeds from a self-pollinated transgenic N. tabacum plant (TCS-T1) was looked at in two ways. Sterile seeds were germinated on a medium containing 100 $\mu\text{g/ml}$ Km. The numbers of Km-sensitive and Km-resistant seedlings were counted. The cotyledons of untransformed seedlings, and some transformed seedlings, bleached after 2 to 3 wk on the medium (data not shown). Km-resistant transformed seedlings remained green and went on to form true leaves. Untransformed seeds produced green seedlings when plated in the absence of Km.

To look at segregation in another way, dry seeds from the same seed lot were imbibed then the embryos were removed and stained in X-gluc.

These two experiments track traits encoded by each half of the T-DNA. In both cases, the segregation ratio was approximately 3:1 (Table 4.3).

Table 4.3 Segregation Analysis of Seeds from N. tabacum
Plant TCS-T1

A. Sterile seeds from transgenic N. tabacum plant TCS-T1 were germinated on medium containing 100 $\mu\text{g/ml}$ kanamycin. Km^{R} , kanamycin-resistant; Km^{S} , kanamycin-sensitive.

B. Embryos were removed from imbibed mature seeds (plant TCS-T1) and stained in X-gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) overnight. GUS^+ , positive for GUS histochemical staining; GUS^- , negative for GUS histochemical staining.

A. Kanamycin resistance of germinating seeds

No. Km ^R	No. Km ^S	Km ^R : Km ^S
885	257	3.4 : 1

B. Histochemical GUS staining of mature embryos

No. GUS ⁺	No. GUS ⁻	GUS ⁺ : GUS ⁻
121	37	3.3 : 1

4.III. DISCUSSION

4.III.1. Screening of Km^R N. tabacum Plants for GUS Activity

A large number of transformed plants were generated for this study resulting in the need for a rapid screening procedure. Fluorogenic GUS activity was assessed in a microtiter plate assay. Samples from plants transformed with pBI 131, pBI 121 and nos-GUS yielded predicted results (Plate 4.2). pBI 131 contains the tobacco rbcS gene promoter fused to the GUS coding region. The rbc enzyme catalyzes the fixation of CO₂ during photosynthesis. The highest GUS activity for this vector was found in leaves and stems, which would be among the most photosynthetic tissues in the plant. Moderate fluorescence was detected in flowers but negligible activity was seen in roots and seeds. Coruzzi et al. (1984) reported mRNA levels for pea rbcS in roots, seeds, and petals to be only 0.7%, 8.0% and 8.0%, respectively, of that found in leaves. The flower samples used for the GUS assay contained all the flower components, including the green sepals, which may account for the moderate level of fluorescence seen.

The intact CaMV 35S promoter drives expression of a heterologous gene in all organs of a plant (Nagy et al., 1985). GUS activity was detected in all samples from plants

transformed with pBI 121, the 35S-GUS construct. The levels of activity varied among tissues within the same plant and also from plant to plant when comparing the same type of sample (summarized in Table 4.1). This may be due to position effects on the integrated gene(s). The nopaline synthase (nos) promoter, although developmentally regulated, is also active in a wide variety of plant tissues (An et al., 1988). In the microtiter plate assay, GUS activity of the nos-GUS group of plants seemed to be near the threshold of detection. Activity could not be detected in many samples (Table 4.1), although in one transgenic plant (Plate 4.2) the GUS enzyme was active in all samples except the flower. Activity in some samples may have been below the limit of detection. The 35S promoter has been shown to be 20 to 30 times stronger than the nos promoter (Harpster et al., 1988; Sanders et al., 1987).

Based on the results obtained with plants from the three vector groups discussed above, a decision was made to screen the remaining Km^R plants for GUS activity using the microtiter plate assay. Out of the 68 remaining Km^R plants, 63 were also GUS-positive. This high percentage was surprising since not all of the 8 atar1-GUS constructs were expected to direct the production of functional GUS molecules (3 contained the arabin fragment in the reverse orientation relative to the GUS coding region; 2 contained the arabin ATG out of frame with the GUS ATG; see 4.III.2.).

GUS activity levels in plants transformed with atar1-GUS constructs ranged from activities as low as those obtained with the nos-GUS vector to activities as high as those for pBI 131 and pBI 121 (summarized in Table 4.1). The vast majority of plants had GUS activity only in the seed samples. The 2S albumin storage proteins are known to be seed-specific (Crouch and Sussex, 1981; Heath et al., 1986). GUS expression was detected in the somatic tissues of one transgenic plant carrying an atar1-GUS fusion. Integration of the gene near a general enhancer could have accounted for this breakdown in seed-specificity. Such enhancers have been identified in A. thaliana by constructing a library in a bidirectional enhancer cloning vehicle and studying expression in transgenic N. tabacum (Ott and Chua, 1990).

4.III.2. T-DNA Integration in Transformed Plants

Southern blot analysis indicated that the transformed plants carried 1 to 8 or more copies of the GUS gene. Others report similar integration results for this set of pBI vectors in the presence of various Ti plasmids (Jefferson et al., 1987; Robert et al., 1990; Guerrero et al., 1991). As well as full-length T-DNA, truncated or rearranged copies of introduced genes can also be integrated after transfer to the plant cell (Deroles and Gardner, 1988; Jones et al., 1987; Van Lijsebettens et al., 1986; Spielmann and Simpson,

1986). Deletions, additions or rearrangements may occur as a result of the integration process. Short homologous sequences or partial homologies between T-DNA and plant target sequences have been postulated to promote initial physical contacts between the DNA molecules prior to integration (Gheysen et al., 1987; Matsumoto et al., 1990). Base-pairing in regions of partial homology could lead to deletions or duplications by endonuclease or exonuclease digestion or DNA repair synthesis (Mayerhofer et al., 1991; Bakkeren et al., 1989).

The Southern blot in Plate 4.4 shows an expected hybridizing band at 2.5 to 2.7 kb plus another major band at approximately 2.1 to 2.2 kb when the DNA is cut at internal sites in the T-DNA. In the unaltered pBI 101 cassette, a 2.2 kb fragment is released by a Hind III - Eco RI digestion. Perhaps the arabin insert in the cassette is unstable in N. tabacum and is excised from some copies of the T-DNA. In the case of plant TCSR-T9, the GUS probe only hybridized to a DNA band of 2.1 to 2.2 kb. Although this DNA fragment is smaller than that expected to be released by Hind III/Eco RI digestion of the intact atar1-GUS fusion, this plant still expresses GUS activity in a developmentally regulated and seed-specific manner. The GUS coding region in pBI 101 carries its own translation initiation site so translation from this ATG is possible (see 4.III.3. for more detail) but loss of the arabin promoter from the construct would make

regulated transcription unlikely. Perhaps a deletion occurred within the GUS coding region which did not affect the enzyme's activity. The enzyme can tolerate large amino-terminal additions without an effect on activity (Jefferson, 1987a).

Enzyme activity does not appear to be correlated to copy number. A lack of correlation between increased copy number and increased activity has been shown elsewhere (Shirsat et al., 1989b; Vyskot et al., 1989; Jones et al., 1987). In fact in some cases, a negative correlation has been reported (Jones et al., 1987; Linn et al., 1990; Matzke et al., 1989; Hobbs et al., 1990). Segregation of both GUS expression and kanamycin resistance was studied in seeds from the N. tabacum plant TCS-T1 (Table 4.3). In both cases, the segregation was 3:1 in favour of expression of the trait. These results indicated that a single dominant locus controlled expression of each of these two transferred genes in this plant. This finding was surprising in view of the fact that TCS-T1 appeared to contain at least 5 copies of the GUS gene integrated at 5 different sites in the genome (Plate 4.3). Plate 4.4 shows that at least some of those integrated T-DNAs were rearranged in TCS-T1. Rearrangement might interfere with the production of a functional protein. However, other factors such as methylation (Vyskot et al., 1989; Weber et al., 1990; Linn et al., 1990; Matzke et al., 1989; Hobbs et al., 1990) and position effects (Jones et

al., 1987) may also modulate the expression of an integrated gene. In some cases increased copy number of the introduced gene was correlated to both increased methylation and decreased expression of the gene (Linn et al., 1990; Matzke et al., 1989; Hobbs et al., 1990). The 3:1 segregation pattern might also be due to clustering of the integrated genes at a single locus in the genome.

4.III.3. Comparison of GUS Activity in Seeds from N. tabacum Plants Transformed with Various Vectors

The comparison of GUS activity levels among different vector groups of transgenic N. tabacum plants was complicated by the fact that individual transformants contained different numbers of the integrated T-DNA molecules, and some of those copies appeared to be rearranged (see 4.III.2.). The number of functional copies of the gene fusions, and the effect of the rearranged copies, if any, on GUS activity levels in these plants was unknown. However, for discussion purposes in the following section, only the original sequences of the atar1-GUS fusions will be considered when discussing the possible relationships between atar1 5' sequences and GUS activity levels observed in the different groups.

In general, the ranking of activity levels among the various vector groups based on the microtiter plate assay was confirmed by the fluorometer analysis of 25 dpa seeds.

There was no significant difference between the activity levels in vector groups pTCS and pTCL. The additional 325 bp in pTCL appeared to have no effect on GUS activity in transgenic *N. tabacum*, although the possibility of individual elements within the fragment exerting opposing influences has not been ruled out. It is also possible that activity is dependent upon interaction with other elements outside of the fragment.

When the arabin fragments were reversed in the transcriptional fusions (pTCSR, pTCLR), GUS activity above baseline levels was detected in transgenic plants. Similar results were reported for constructs containing either 1400 or 433 bp of upstream sequence from a High Molecular Weight (HMW) glutenin gene or 938 bp from a Low Molecular Weight (LMW) glutenin gene fused in the 3' to 5' orientation to the CAT gene (Colot *et al.*, 1987; Robert *et al.*, 1989). Seed-specific CAT activity was observed in these cases, although at a reduced level compared to constructs with the glutenin fragments fused 5' to 3'. Presumably, there is some sequence acting as a promoter in these constructs. In pTCSR, a TATA sequence (TATACA) is present approximately 100 bp upstream of the GUS ATG. This sequence can be found at -370 to -365

(TGTATA) in Figure 3.2. It is also possible that the atar1 gene promoter functions bidirectionally.

The presence of regulated GUS activity in plants transformed with vectors carrying the arabin gene fragment in the reverse orientation indicates that the fragment may include an enhancer element. Enhancers are cis elements that can increase the efficiency of transcription of a gene in a manner that is independent of the position and orientation of the enhancer relative to the gene. They also have the ability to work over long distances (reviewed in Khoury and Gruss, 1983; Schöler et al., 1988; Dynan, 1989). In some cases, enhancers have been associated with cell- and tissue-specificity of transcription (see reviews Schöler et al., 1988; Voss et al., 1986; Dynan, 1987; Okamura and Goldberg, 1989). Chen et al. (1988) reported an enhancer-like element from a soybean gene encoding the α' -subunit of β -conglycinin. The element could enhance gene expression in a tissue-specific and temporally-regulated manner during embryo development. A 404 bp upstream sequence from a sunflower helianthinin gene fused to a truncated 35S promoter was capable of enhancing GUS expression in transgenic tobacco embryos (Jordano et al., 1989). Similarly, an enhancer element from a wheat HMW glutenin gene was shown to be responsible for developmentally-regulated endosperm-specific expression (Thomas and Flavell, 1990).

The results obtained with the translational vectors were unexpected. The mean GUS activity observed in plants transformed with pTLF1 (atar1 ATG in-frame with GUS ATG) was not significantly different from that obtained with pTLF2 or pTLF3 (Table 4.2). These vectors contained the same atar1 fragment as pTLF1 but fused in the other two reading frames (not in frame with the GUS coding region).

Since the GUS gene in the pBI cassettes contains its own ATG, it is possible that in the translational fusions, translation could be initiated at this point rather than at the plant ATG. Kozak (1978) proposed a ribosome scanning model to explain the choice of a translation initiation site. Perhaps the GUS ATG is preferred by the N. tabacum translational machinery, although, as discussed in Chapter 3, the sequence surrounding the translation initiation site in atar1 is close to the plant consensus sequence, unlike the sequence surrounding the GUS ATG (TCCCTTATGTTA). Putterill and Gardner (1989) have presented evidence for the initiation of translation at one ATG followed by termination, then a reinitiation of translation at a different ATG. In the translational fusions used in this work, there are no termination codons between the plant and GUS ATG's in any frame. It is possible that this particular combination of arabin and GUS sequences leads to secondary structure which interferes with the use of the arabin ATG.

This possibility is consistent with the similar activity levels seen with pTLF1, pTLF2 and pTLF3.

The effects of post-transcriptional and post-translational mechanisms cannot be ruled out. In A. thaliana, at2S1 encodes the most abundant 2S albumin isoform (Krebbers et al., 1988) but at2S1 mRNA contributes only about 10% of the total mRNA encoding 2S albumins (Guerche et al., 1990). It has been suggested that the signal peptide is removed cotranslationally as the albumin storage protein crosses the membrane of the endoplasmic reticulum (ER) (Crouch et al., 1983; Ericson et al., 1989; Krebbers et al., 1988). The GUS protein can be glycosylated in the lumen of the ER in N. tabacum cells. The glycosylation appears to inhibit GUS enzyme activity (Iturriaga et al., 1989). Perhaps the major protein produced from pTLF1 is inactivated in the lumen. The full-length proteins produced from pTLF2 and pTLF3 are likely to be inactive even before processing since the plant and GUS ATG's are out-of-frame. The low levels of activity seen in these three vectors could be due to small amounts of native GUS protein produced from the GUS ATG.

Another possible alternative is that each plant transformed with a translational fusion contains all three proteins (all three frames) due to slippage of the RNA polymerase at the G-tail generated during the preparation of deletion bank clones. Slippage of the ribosome at the

corresponding stretch of C residues in the mRNA(s) could also lead to a mixed population of proteins. On a run of 4 or more identical bases, ribosomes can sometimes disengage from the mRNA then reengage at an adjacent base (reviewed in Atkins et al., 1990).

Differences in translational efficiency or mRNA stability may also contribute to varying GUS activity levels. The high level of activity in plants transformed with pTLF1R could be accounted for by a reversed RNA enhancer in the region of the arabin gene coding for the 5' untranslated region of the atar1 transcript. Since RNA enhancers are orientation dependent (reviewed in Sharp and Marciniak, 1989), the effect would only be seen when the arabin gene fragment was placed in the reverse orientation relative to the GUS coding region.

The number of possible explanations for the observed results could be narrowed by mapping the transcriptional start site for each vector. For example, slippage of the RNA polymerase on the G tract could be detected as could variations in the transcriptional start site due to the lack of a TATA box in the reverse orientation vectors.

In summary, the 392 bp arabin fragment appears to direct the developmentally-regulated, seed-specific expression of GUS in transgenic N. tabacum when placed in either orientation relative to the GUS gene. However, as noted at the beginning of this section, regulated GUS

activity cannot be conclusively attributed to sequences in this fragment since some rearrangements of the transferred T-DNA molecules were detected in the transgenic plants. Further studies will be required to locate the enhancing element within the arabin 5' sequence and to test whether the element is a true enhancer as defined above, and whether the enhancing activity, seed specificity and temporal regulation are associated with a single cis element or multiple elements.

4.III.4. GUS Gene Expression in N. tabacum Plants

Transformed with pTCS

GUS expression in pTCS transgenic N. tabacum plants was developmentally regulated and seed specific. Using the microtiter plate assay, fluorogenic activity could be detected in seeds as early as 11 dpa. The slight variation in onset of activity (11 to 13 dpa) could be accounted for by slight differences in the rate of seed development from plant to plant, and within individual capsules, although the plants were grown under similar greenhouse conditions. The highest GUS activity was seen in 28 dpa and dry seeds. Some of the fluorescence seen in these last two samples could be attributed to endogenous fluorescence (Plate 4.5, -MUG). Fluorescence in the absence of the MUG substrate increased as the seeds matured, even in untransformed N. tabacum

plants (Plate 4.2). Jefferson (1988) has suggested that endogenous fluorescence in plant samples may be due to intermediates in lignin biosynthesis or other phenylpropanoid pathway compounds. Fluorescence due to the activity of GUS or GUS-like enzymes has not been seen in untransformed N. tabacum (Hu et al., 1990; Jefferson, 1988).

The fluorometer assay provides a slightly different profile of GUS activity for TCS-T1 (Figure 4.2) than that seen with the microtiter plate assay (Plate 4.5). Activity peaks about 21 dpa then drops off as the seeds dessicate, unlike the result presented in Plate 4.5 which shows peak activity in the most mature seeds. Assay differences may account for this inconsistency. Samples for the microtiter plate were prepared on a fresh weight basis. In the early stages of development, water contributes a greater proportion of the fresh weight of the tissue than at later stages (Vickery et al., 1932), presumably leading to higher protein levels per unit fresh weight in the more mature seeds. GUS activities presented in Figure 4.2 were calculated per mg protein. The effect of endogenous fluorescence was also circumvented in the fluorometer assay since the rate of fluorogenic activity was determined. Endogenous fluorescence does not increase over the time of the assay (results not shown) and therefore does not contribute to the rate.

2S storage proteins accumulate specifically in maturing seeds (Heath et al., 1986; Crouch and Sussex, 1981). The pattern of fluorogenic GUS expression in N. tabacum plant TCS-T1 (Figure 4.2) was consistent with the presence of 5' regulatory sequences from the 2S storage protein gene atar1 in the pTCS vector. Little or no activity was seen in somatic tissues. The low level of activity seen in the root sample was also observed in untransformed N. tabacum (results not shown). Since plants were grown in the greenhouse, it is possible that this GUS activity could have been due to enzymes from rhizosphere bacteria.

Although GUS activity was maximal in cotyledon stage embryos, the initial expression of GUS was detected earlier than expected. Heath et al. (1986) reported that accumulation of storage proteins occurs in A. thaliana primarily during the late cotyledon stage of embryo development. GUS activity was detected in embryos of globular or early heart stage in transgenic N. tabacum (Figure 4.2). This discrepancy could be attributed to the sensitivity of the GUS assay. However, the early appearance of arabin mRNA was also observed in transgenic N. tabacum (De Clercq et al., 1990) as compared to its appearance in A. thaliana (Guerche et al., 1990).

The GUS reporter molecule allows one to study spatial regulation of gene expression through the use of a histochemical assay. Plate 4.6 shows the localization of GUS

expression in developing N. tabacum embryos. GUS activity was first detected at 15 dpa. Fluorogenic activity was detected at a slightly earlier stage of development, probably due to the greater sensitivity of the fluorogenic method (Jefferson, 1987a).

No activity was found in the seed coat (Plate 4.6, K). However, the blue compound released by cleavage of the X-gluc substrate accumulated in endosperm tissue (Plate 4.6, J). As in embryos, the blue colour was first observed at 15 dpa in the endosperm, but there was no obvious reproducible pattern to the staining. Patches of blue expanded, and often darkened as development proceeded. In early papers, 2S albumin storage protein accumulation was reported to be embryo-specific in B. napus (Crouch and Sussex, 1981). Later results in A. thaliana and transgenic N. tabacum (De Clercq *et al.*, 1990) indicated that 2S storage protein could also be found in the endosperm. The apparent discrepancy could be accounted for by an increased sensitivity in analytical methodology and the large proportion of endosperm found in mature N. tabacum seeds as compared to B. napus and A. thaliana.

The spatial distribution of GUS activity in the embryo changed as development proceeded. A typical profile is presented in Plate 4.6. Initial activity was detected in the ground meristem of the upper axis at 15 dpa (Plate 4.6, D). Staining appeared to be more intense in the outer layers of

the upper axis and outer faces of the bases of the cotyledons at day 16 (Plate 4.6, E,L). As development proceeded the GUS staining spread towards the apices of the embryo and also internally towards the vascular cylinder in the axis and towards the inner faces of the cotyledons. Fernandez et al. (1991) have recently reported similar, although not identical patterns of napin mRNA accumulation in developing B. napus embryos. Napin mRNA was detected by in situ hybridization in the late heart/early torpedo stage of embryogeny in the ground tissue (cortex) of the upper axis. Napin mRNA did not accumulate in epidermal tissue until the late torpedo stage. By this time, napin mRNAs were also detectable in cells of the outer faces of the B. napus cotyledons. The inner faces of the cotyledons accumulated mRNA as the embryo progressed through the cotyledon stage of development. A similar cotyledon accumulation pattern of GUS occurred in transgenic N. tabacum but at a slightly earlier stage of embryogeny. Perez-Grau and Goldberg (1989) also reported a wave-like pattern of accumulation of soybean seed protein mRNAs. Each seed protein mRNA accumulated progressively from the outer to the inner faces of the cotyledons but the timing and extent of each wave was specific for its particular mRNA. No Kunitz trypsin inhibitor mRNA was detected in the vascular tissue of soybean or transgenic tobacco embryos (Perez-Grau and Goldberg, 1989), nor was napin mRNA localized in the

vascular tissue in B. napus embryos (Fernandez et al., 1991), unlike the pattern of GUS expression in embryos of transgenic N. tabacum plant TCS-T1.

In the B. napus embryos, the last area to accumulate napin mRNA was identified as the epiphysis, a group of cells which contributes to the development of the shoot apical meristem (Fernandez et al., 1991). A corresponding lack of GUS activity in this area was not observed in immature N. tabacum embryos. The blue precipitate formed by the action of GUS on the X-gluc substrate is insoluble (Jefferson, 1987a) but leakage of the precipitate into adjacent tissues has not been ruled out. However, when whole mature seeds were stained, only the embryo and endosperm developed an intense blue colour (results not shown). The complete lack of stain in the seed coat argues against diffusion of the precipitate. Alternatively, the relatively small areas of the epiphysis and vascular tissue may have been masked by the intensity of the blue colour in the rest of the whole embryo. More detailed studies of these areas would be required to test these possibilities.

The similarities in expression of seed protein mRNA and GUS protein observed among these three systems indicate that transcriptional regulation of gene expression plays an important role in the accumulation of seed proteins. Differences in expression may be accounted for in part by post-transcriptional mechanisms, but the differences between

the work described here, and previously published reports may also be due to the use of a chimaeric gene (atar1-GUS) in a heterologous plant (N. tabacum). Any contributions made by atar1 sequences 3' to the coding region would be eliminated in this construct. Regulatory mechanisms for storage protein gene expression may not be identical in A. thaliana, B. napus and N. tabacum. In addition, members of a gene family can be differentially regulated. Guerche et al., (1990) showed by in situ hybridization that at2S1 (equivalent to atar1) is expressed only in the axis of A. thaliana embryos. The presence of GUS activity in the cotyledons of N. tabacum plant TCS-T1 suggests that the regulatory mechanisms controlling storage protein gene expression differ in N. tabacum and A. thaliana plants. Temporal expression differences were discussed earlier in this section. These variations are not surprising in view of the fact that N. tabacum lacks a major albumin seed storage protein (Sano and Kawashima, 1983).

Fernandez et al. (1991) have suggested that the pattern of storage protein gene expression in B. napus embryos could be achieved by differentially setting the internal clocks of groups of cells. They proposed that the clocks could be set via a spatial gradient of some factor centred at the apex. Cells exposed to lower levels of the factor (farther from the apex) would begin a maturation program earlier. The GUS staining pattern seen here in N. tabacum could be explained

by a simpler mechanism. A gradient with its source centred within the outer upper cortex cells of the axis could account for the pattern seen in N. tabacum. Cells closest to the source would be first to express GUS. Expression of the atar1-GUS construct would be induced in a progressive fashion as the factor diffused out from its source.

One can speculate as to the identity of the "factor". A trans-acting transcriptional factor would be a likely candidate but this solution raises another question. What activates the gene activator? Perhaps the "factor" is a hormone. The atar1 gene contains cis elements which are shared with other ABA-responsive genes (see Chapter 3). Napin expression has been shown to be maintained or enhanced in cultured B. napus embryos by ABA (Crouch et al., 1984; Delisle and Crouch, 1989). An A. thaliana double mutant that produces reduced levels of endogenous ABA and has a reduced responsiveness to ABA failed to accumulate arabin during seed development (Koorneef et al., 1989). These studies do not prove that ABA acts directly on the seed storage protein genes. It is possible that the effect is indirect, perhaps through the induction of a transcription factor, the lifting of a repression, or through post-transcriptional mechanisms. As yet, the nature and the transmission of the signal are still unknown.

In a previous report (De Clercq et al., 1990), seed-specific and developmentally-regulated expression in

transgenic N. tabacum and B. napus was conferred upon a reporter gene by 1.8 kbp of 5' flanking sequence from the arabin gene at2S1. The results presented in this report, suggest that the regulatory elements are found within a 392 bp (-436 to -44 bp) fragment of atar1 (equivalent to at2S1). Radke et al. (1988) showed that 300 bp 5' to the translation start site of a napin gene (gNa) was sufficient to direct expression of a tagged napin gene in embryos but not leaves. Gustavsson et al. (1991) studied the ability of specific promoter sequences (prepared as oligonucleotides) from the napin gene napA to bind seed specific nuclear factors. All four oligonucleotide probes formed an additional retarded band after they were mixed with seed nuclear extracts and analysed in a gel retardation assay. Some of the oligonucleotides used in the study encompass sequences discussed in Chapter 3, specifically Boxes I and IV in Figure 3.3. Interestingly, the oligonucleotide probes also formed retarded bands when the nuclear extracts were obtained from cells of leaves or non-napin-expressing seeds. The bands migrated with similar mobilities in all cases. No obvious seed-specific factor was detected but the possibility that the binding proteins for a particular oligonucleotide were different in the different extracts was not ruled out.

The information presented in Chapters 3 and 4 has set the stage for further studies of the cis and trans elements

involved in the regulation of atar1. Although not proven conclusively, temporal and spatial regulation appears to reside within 392 bp of the atar1 gene 5' sequence. The sites and sequences of the controlling elements within this fragment remain to be determined. Some candidates were outlined in Chapter 3. The fragment contains a palindrome at position -248, the inverted repeat L, and at least one copy of each of the Boxes I to IV. Deletion analysis of the fragment could be used to test the functional role of these potential regulatory elements.

CHAPTER 5

GENE EXPRESSION IN DEVELOPING BRASSICA NAPUS MICROSPORE-DERIVED EMBRYOS

5.1. INTRODUCTION

The study of biochemical and molecular events during embryo development can be hindered by the lack of sufficient embryonic material. The excision of B. napus zygotic embryos is almost impossible to achieve before 15 dpa (heart stage) (Crouch and Sussex, 1981). Consequently, researchers often turn to in vitro systems for an abundant supply of embryos, particularly when studying early embryonic stages.

Carrot suspension cultures can give rise to somatic embryos. There are at least two phases in the differentiation of embryos from carrot cells. Small spherical (type 1) cells were shown to divide and form embryogenic cell clusters when cultured in the presence of low levels (5×10^{-8} M) of auxin. Development of embryos from the embryogenic cell clusters was seen only after transfer of the clusters to an auxin-free medium (Komura and Komamine, 1985). Protein profiles from in vitro-translated mRNA from carrot somatic embryo populations and

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proembryogenic masses were reported to be very similar. One cDNA (Dc3) hybridized to RNA from embryos and proembryogenic masses but not RNA from hypocotyls, leaves or single cells. The possibility of using Dc3 as a marker for the acquisition of embryogenic potential was raised (Wilde *et al.*, 1988).

In newly initiated hypocotyl-derived suspension cultures (starting cultures), somatic embryogenesis could be stimulated by the addition of medium conditioned by a 7 day embryogenic suspension culture (de Vries *et al.*, 1988a). Further studies indicated that several extracellular glycoproteins are essential for carrot somatic embryogenesis to occur in these cultures (de Vries *et al.*, 1988b). One glycoprotein has been identified as a peroxidase with a suggested role in restricting cell expansion during early embryogenesis (Van Engelen and de Vries, 1992).

Embryos can be derived from gametophytic cells. Haploid embryos from anthers or isolated microspores have been reported in over two hundred plant species (Dunwell, 1986; Keller *et al.*, 1987a). Haploid embryos are particularly useful in the establishment of homozygous lines for plant breeding or for the selection of mutants or as potential recipients for gene transfer (Keller *et al.*, 1987a).

The production of male gametophytes (pollen) (described in Mascarenhas, 1989; Scott *et al.*, 1991) occurs in the anther, where pollen mother cells (microsporocytes) develop

from the sporogenous tissue. These cells undergo meiosis to produce tetrads of haploid microspores. The tetrads and the individual microspores are surrounded by a callose wall. The wall is digested by a β -(1-3)-glucanase secreted by the tapetum, a nutritive layer surrounding the microsporocytes. The released uninucleate microspores increase in volume, round up, storage reserves are deposited, and the pollen coat is completed. Two mitotic divisions also occur during gametophyte maturation. The first division is asymmetrical. The resulting binucleate microspore contains a large vegetative cell and a small generative cell which lies within the cytoplasm of the vegetative cell. The generative cell undergoes a second mitotic division to form two sperm cells. The mature gametophyte is trinucleate, consisting of the two sperm cells within the vegetative cell cytoplasm.

Microspores can be diverted from the pollen pathway of development to form embryos. Microspores are isolated from plants grown at 10°C day/5°C night then incubated for 4 days at 32.5°C before transfer to 25°C. In B. napus, large preparations of isolated microspores can give rise to embryos at frequencies up to 5% (Keller et al., 1987b). B. napus microspores in the late uninucleate stage have been shown to have the greatest potential for embryo formation (Fan et al., 1988; Kott et al., 1988; Pechan and Keller, 1988). Within the first 24 hr of culture, uninucleate microspores swell and become spherical. The nucleus moves to

the centre and a symmetrical mitosis takes place to give rise to two equal-sized nuclei. Usually only one of the nuclei continues to divide and form the embryo (Fan et al., 1988; Zaki and Dickinson, 1990). Microspores in the early binucleate stage can also give rise to embryos. In this case, only the vegetative nucleus contributes to embryo formation (Fan et al., 1988; Pechan and Keller, 1988). The majority of binucleate microspores, however, do not go on to form embryos. It has been suggested that they may already be committed to the second pollen mitosis. In culture, these microspores swell enormously and take on a distinctly oval shape. They often burst after 3 or 4 days in culture (Fan et al., 1988; Kott et al., 1988).

The feasibility of using the microspore embryo system as a model for embryo development has been investigated. Fatty acid accumulation has been compared in developing zygotic and microspore-derived embryos of B. napus (Pomeroy et al., 1991; Taylor et al., 1990; Wiberg et al., 1991). Taylor et al. (1990) also compared levels of storage protein (cruciferin and napin) mRNA in torpedo stage microsporic embryos cultured in the presence or absence of ABA. In the study presented in this chapter, the expression of storage protein mRNAs was tested over a range of developmental stages in microsporic and zygotic embryos. Results indicated that the temporal pattern of storage protein gene expression is similar in the two types of embryos but there is a

quantitative difference. A unique finding was the detection of napin mRNA in microspores after the 4-day treatment at 32.5°C. The induction of napin expression and its correlation to the potential of a sample to form embryos is discussed.

5.II. RESULTS

The research presented in this chapter was carried out in collaboration with Dr. María-Jesús Ginés.

5.II.1. Generation and Collection of B. napus

Microspore-derived Embryos

Under certain conditions, microspores can be diverted from their normal pathway of pollen development to an embryogenic pathway. To obtain microspore-derived embryos, plants were preconditioned by growth at 10°C day/5°C night then the microspores were isolated and cultured for 4 days at 32.5°C before transfer to 25°C. B. napus cv. Topas plants were used for all the experiments presented in this chapter. All microspore cultures were prepared by John Grainger, Nancy Long and Dr. Wilf Keller at the Plant Research Centre.

Buds 3 to 5 mm in length contained microspores which gave rise to the highest percentage of proembryos (Plate 5.1, Mi). The first few cell divisions towards embryo development occur within 3 to 7 days in culture (Coventry et al., 1988). If the heat treatment was omitted, the microspores did not produce embryos but maintained pollen development, as indicated by microspores with a greatly swollen, oval appearance (Plate 5.1, Mu). Small buds (1.5 to 3 mm) contained a mixture of tetrads and early microspores

Plate 5.1 Populations of Tetrads, Microspores and Pollen

Plants were grown at 10°C day/5°C night. B. napus buds of varying sizes were selected and cultured under embryogenic conditions (4 days, 32.5°C) or non-embryogenic conditions (4 days, 25°C). **M**, microspores obtained from medium-sized buds (3-5 mm) at time of collection (time zero); **Mu**, uninduced microspores: **M** sample cultured for 4 days at 25°C; **Mi**, induced microspores: **M** sample cultured for 4 days at 32.5°C; **E**, mixture of tetrads and microspores obtained from small buds (1.5-3 mm); **Eu**, **E** sample cultured for 4 days, 25°C; **Ei**, **E** sample cultured for 4 days, 32.5°C; **P**, mixture of microspores and pollen obtained from large buds (5-9 mm); **Pu**, **P** sample cultured for 4 days, 25°C; **Pi**, **P** sample cultured for 4 days, 32.5°C. Bar=50 um.

(Figure provided by M.-J. Ginés, with permission.)

Mi

Ei

Bi

Ma

Eu

Pu

Ni

E

P

(Plate 5.1, E) which yielded a small number of embryos (approximately 20% of Mi levels) after heat treatment (Ei). Presumably this is due to the smaller number of microspores within the population that are responsive to embryogenic conditions. Fan et al. (1988) reported that late uninucleate and early binucleate microspores are the most embryogenic under these conditions. When Sample E was cultured at 25°C for 4 days, the percentage of microspores increased, the percentage of tetrads decreased and some pollen-like structures were observed (Plate 5.1, Eu). These results indicated that normal pollen development proceeded in the Eu cultures during the 4 days at 25°C. Large buds (5 to 9 mm) contained a mixture of microspores, mostly in the late binucleate or trinucleate stage. In culture, these late-stage microspores have been shown to swell and take on an oval shape (Kott et al., 1988) (Plate 5.1, P). Embryos were not induced at either incubation temperature (samples Pu and Pi). Perhaps at these later stages, the commitment to pollen formation cannot be altered by culture conditions.

When the induced cultures were transferred to 25°C, many of the proembryos continued dividing and gave rise to globular embryos. Embryo development in the B. napus microspore cultures was only partially synchronous. For this reason, embryos of the desired stages had to be separated before collection. The embryo cultures were poured through a stacked set of nylon meshes of various sizes (Plate 5.2).

Plate 5.2 Collection of B. napus Microspore-derived
Embryos

Embryos from microspore cultures were collected on nylon meshes of varying pore size. 9, 11, 14, 21: approximate day of microspore culture. G, globular embryos; H, heart stage embryos; T, torpedo stage embryos; C, cotyledon stage embryos. Bar=200 um.

(Photographs provided by M.-J. Ginés, with permission.)

MESH
PORE
SIZE

62.88 μm

125 μm

200 μm

250 μm

500 μm

Globular embryos were most abundant at 9 to 10 days after the initiation of the culture, and were collected at that time on a 62 μm mesh (early globular) and an 88 μm mesh (large globular). The populations were at least 95% pure. Early heart stage embryos were also isolated at this time with a mesh of 125 μm . The heart stage embryos at day 11 or 12 were retained on a 200 μm mesh. The pooled samples consisted of over 90% heart-shaped embryos. The torpedo stage embryos were collected at 14 or 15 days of culture using a 250 μm nylon mesh to give 75 to 85% purity. A 500 μm mesh trapped embryos at the cotyledon stage. This 21 day sample was 80 to 90% pure. The embryos were quickly frozen in liquid nitrogen and stored at -80°C until the RNA was extracted.

5.II.2. Storage Protein mRNA Expression in Zygotic and Microsporic Embryos.

The expression of napin and cruciferin genes was compared in zygotic and microspore-derived embryos of B. napus. RNA was extracted from embryos of various stages of development. These included globular, heart, torpedo and early cotyledon stages as well as maturing cotyledonary embryos. Early cotyledonary embryos were transferred at 21 days of culture to a maturation medium containing 5 μM ABA in an attempt to prevent precocious germination. Under these

conditions the cotyledons expanded but early germination was seen by 4 to 5 wk of total culture as revealed by elongation of the radicles. RNA was also extracted from seeds collected at weekly intervals. Other samples included microspores collected at the time of initial isolation, or after 4 days of culture at 25°C (embryogenic pathway not induced) or 32.5°C (embryogenesis induced). A cell suspension culture derived from microsporic embryos was also incubated at 25°C or 32.5°C for 4 days.

The expression of napin mRNA in developing embryos is presented in Plate 5.3. Northern analysis verified the specificity of the probe derived from a napin cDNA. One band of the expected size (850 bp) was seen (Plate 5.3, A). Napin mRNA was first detected in 21 dpa early cotyledon stage microsporic embryos (Plate 5.3, A,B). In seed samples, small amounts of napin mRNA were detectable by 14 dpa (Plate 5.3, C), with levels peaking at 28 dpa before dropping off to very low levels in dry seeds. The lower intensity of the hybridizing signal in the 6 wk samples in the napin and cruciferin Northern blots could be due to a proportionately smaller amount of mRNA in these lanes. Hybridization with a soybean 26S rRNA probe indicated that the 6 wk samples contained slightly less rRNA than the other samples (results not shown). These results confirmed those of Crouch *et al.* (1984) and DeLisle and Crouch (1989). Cruciferin expression was initiated at a slightly later stage of development than

Plate 5.3 Napin mRNA Expression in Zygotic and Microsporic Embryos

A. Northern blot analysis of B. napus microspores and microsporic embryos using a napin cDNA as a probe. The probe hybridized to a band of 850 bp. Each lane contained 5 μ g total RNA. Mu, uninduced microspores (cultured 4 days at 25°C); M, microspores at time of collection (time zero); Mi, induced microspores (cultured 4 days at 32.5°C); G, globular stage microsporic embryos; H, heart stage microsporic embryos; T, torpedo stage microsporic embryos; C, cotyledon stage microsporic embryos; 4*, cotyledon stage microsporic embryos at 4 weeks of culture; 5*, cotyledon stage microsporic embryos at 5 weeks of culture; 6*, cotyledon stage microsporic embryos at 6 weeks of culture; 7*, cotyledon stage microsporic embryos at 7 weeks of culture; S, zygotic embryos (28 dpa whole seeds).

* C samples were purified at 21 days and transferred to a second culture system to try to induce embryo maturation.

B. Slot blot analysis of B. napus microspores and microsporic embryos using a napin cDNA as a probe. Samples same as in A. Slots contained 6 μ g total RNA.

C. Slot blot analysis of B. napus zygotic embryos using a napin cDNA as a probe. Mu, uninduced microspores (cultured 4 days at 25°C). Time of seed collection (dpa): 7, early globular stage; 14, heart stage; 18, torpedo stage; 21, late torpedo/early cotyledon stage; 28, early cotyledon stage; 35, mid cotyledon stage; 45, late cotyledon stage; D, mature dry seeds; K, B. napus microspore-derived embryo cell culture (kindly supplied by Z. Fan) incubated at 25°C, 4 days; Ki, B. napus microspore-derived embryo cell culture incubated at 32.5°C, 4 days. Slots contained 6 µg total RNA. (Figure provided by M.-J. Ginés, with permission.)

A

B

C

napin expression in both seed samples and microsporic embryos but like napin, persisted until culture termination in microspore-derived embryos (Plate 5.4).

Unexpectedly, napin mRNA was detected in the 4-day induced microspore samples (Plate 5.3, A,B; sample Mi). The response appeared to be specific for napin. A faint band could be seen in the Mi lane of the cruciferin Northern blot (Plate 5.4, A,B). A case could be made that this band could become significant if the blot was exposed to the same degree as the napin blot. However, under no circumstances did the expression of cruciferin in the Mi sample ever reach the level seen in cotyledon stage microsporic embryos (additional results not shown). Uninduced microspores (Mu, incubated at 25°C) did not produce napin message. Occasionally napin mRNA was detected in RNA samples from microspores at the time of initial collection (M) but the response varied from blot to blot and microspore preparation to preparation. It is unknown if 4-day zygotic embryos express napin. The high levels of napin expression observed in induced microspores prompted further study.

5.II.3. Is the Induction of Napin Expression Linked to Embryogenic Potential?

Since napin expression was detected in induced but not uninduced microspores, the possible link between the

Plate 5.4 Cruciferin mRNA Expression in Zygotic and
Microsporic Embryos

A. Northern blot analysis of B. napus microspores and microsporic embryos using a cruciferin cDNA as a probe. The probe hybridized to a band of 1750 bp. Each lane contained 5 μ g total RNA. **Mu**, uninduced microspores (cultured 4 days at 25°C); **M**, microspores at time of collection (time zero); **Mi**, induced microspores (cultured 4 days at 32.5°C); **G**, globular stage microsporic embryos; **H**, heart stage microsporic embryos; **T**, torpedo stage microsporic embryos; **C**, cotyledon stage microsporic embryos; **4***, cotyledon stage microsporic embryos at 4 weeks of culture; **5***, cotyledon stage microsporic embryos at 5 weeks of culture; **6***, cotyledon stage microsporic embryos at 6 weeks of culture; **7***, cotyledon stage microsporic embryos at 7 weeks of culture; **S**, zygotic embryos (28 dpa whole seeds).

* C samples were purified at 21 days and transferred to a second culture system to try to induce embryo maturation.

B. Slot blot analysis of B. napus microspores and microsporic embryos using a cruciferin cDNA as a probe. Samples same as in A. Slots contained 6 μ g total RNA.

C. Slot blot analysis of B. napus zygotic embryos using a cruciferin cDNA as a probe. Mu, uninduced microspores (cultured 4 days at 25°C). Time of seed collection (dpa): 7, early globular stage; 14, heart stage; 18, torpedo stage; 21, late torpedo/early cotyledon stage; 28, early cotyledon stage; 35, mid cotyledon stage; 45, late cotyledon stage; D, mature dry seeds; K, B. napus microspore-derived embryo cell culture incubated at 25°C, 4 days; Ki, B. napus microspore-derived cell culture incubated at 32.5°C, 4 days. Slots contained 6 µg total RNA.

(Figure provided by M.-J. Ginés, with permission.)

A

B

C

induction of napin expression and the induction of embryogenesis was explored. Populations of microspores in various stages of development (described in 5.II.1.) were treated for 4 days at either 32.5°C or 25°C. Microspores from medium-sized buds were again shown to express napin message when heat treated (Plate 5.5, Mi). RNA from the Ei sample (32.5°C-treated mixture of tetrads and uninucleate microspores obtained from small buds) also contained high levels of napin message. The Mi and Ei microspore samples were the most embryogenic (Plate 5.1). When microspores from large buds were incubated at 32.5°C, negligible levels of napin mRNA were detected (Plate 5.5, Pi). This Pi sample did not yield embryos (Plate 5.1). The uninduced cultures (Mu, Eu, Pu) did not produce embryos (Plate 5.1), and very little or no napin message was observed in RNA from these samples (Plate 5.5). As noted above, samples collected on the day the buds were harvested sometimes contained low levels of napin mRNA (Samples M, E). The levels were inconsistent but always substantially lower than those in the induced sample. The time zero RNA sample from large buds (P) did not hybridize to the napin probe, nor did this late microspore sample give rise to embryos under either non-inducing (25°C) or inducing (32.5°C) conditions (Plate 5.1).

Microsporic embryos which do not normally express napin were shown to be susceptible to heat induction (Plate 5.5, T versus Ti). Embryos were transferred from 25°C to 32.5°C for

Plate 5.5 Effect of Induction Conditions on Napin mRNA
Expression in Microspore Populations and
Microspore Embryos

Slot blots were probed with a radioactively-labelled napin cDNA. Each slot contained 6 μ g total RNA. **M**, microspores obtained from medium-sized buds (3-5 mm) at time of collection (time zero); **Mu**, uninduced microspores: M sample cultured for 4 days at 25°C; **Mi**, induced microspores: M sample cultured for 4 days at 32.5°C; **E**, mixture of tetrads and microspores obtained from small buds (1.5-3 mm); **Eu**, E sample cultured for 4 days, 25°C; **Ei**, E sample cultured for 4 days, 32.5°C; **P**, mixture of microspores and pollen obtained from large buds (5-9 mm); **Pu**, P sample cultured for 4 days, 25°C; **Pi**, P sample cultured for 4 days, 32.5°C; **T**, torpedo stage microsporic embryos; **Ti**, torpedo stage microsporic embryos (treated for 2 days, 32.5°C before collection); **S**, zygotic embryos (28 dpa whole seeds).
(Figure provided by M.-J. Ginés, with permission.)

REPORTS
T T S

2 days prior to collection. Napin message was found in the heat-treated sample (Ti) but not in the untreated embryos (T). The same result was observed in heart-shaped embryos (results not shown). However, the effect of the heat treatment on napin expression in heart and torpedo stage microsporic embryos was not as pronounced as that in 4-day microspores.

The experimental results presented in Plate 5.5 point to a link between the production of napin mRNA and the ability of the corresponding population to form embryos. Embryogenesis in the B. napus microspore system is dependent upon two sets of conditions, before and after bud collection. Plants are preconditioned by growth at 10°C/5°C night. Isolated microspores are treated for 4 days at 32.5°C followed by incubation at 25°C. The next set of experiments was designed to separate the general effects of the heat treatment from the role heat plays in blocking pollen development/inducing embryogenesis.

Plants grown at preconditioning (10°C/5°C) or normal (25°C/20°C) temperatures were heat treated (32.5°C or 25°C) for 4 days before collection. Napin mRNA was not detected in any somatic tissue samples from these plants (Plate 5.6, A,B).

The severe reduction or abolishment of embryo formation that occurs when the preconditioning or heat treatment of the microspores is omitted was exploited in the experiment

Plate 5.6 Lack of Napin mRNA Induction in Somatic Tissues
and Non-embryogenic Control Cultures

6 μ g total RNA were applied per slot. Somatic tissues were collected 7 weeks after germination. A napin cDNA was used as a probe.

A. B. napus plants were grown at preconditioning temperatures (10°C day/5°C night). Some plants (i) were placed at 32.5°C for 4 days prior to sample collection. L, Li, leaves; F, Fi, stems; R, Ri, roots; S, zygotic embryos (28 dpa whole seeds).

B. B. napus plants were grown at normal temperatures (25°C day/20°C night). Some plants (i) were placed at 37°C for 4 days prior to sample collection. l, li, leaves; f, fi, stems; r, ri, roots; s, zygotic embryos (28 dpa whole seeds).

C. **m**, microspores collected from non-conditioned plants (grown at 25°C day/20°C night) (time zero); **mu**, **m** cultures after 4 days at 25°C; **mi**, **m** cultures after 4 days at 32.5°C; **M**, microspores collected from pre-conditioned plants (grown at 10°C day/5°C night) (time zero); **Mu**, **M** samples after 4 days at 25°C; **Mi**, **M** samples after 4 days at 32.5°C; **Mx**, **M** samples incubated at 25°C for 1 day then at 32.5°C for 3 days; **S**, zygotic embryos (28 dpa whole seeds).

(Figure provided by M.-J. Ginés, with permission.)

A

L L_i F F_i R R_i S

B

l l_i f f_i r r_i S

C

m m_u m_i M M_u M_i M_x S

presented in Plate 5.6,C. When plants were grown at normal temperatures ($25^{\circ}\text{C}/20^{\circ}\text{C}$) prior to bud harvesting, embryos were not produced in induced microspore cultures (results not shown). Napin mRNA was not detected in any of the samples derived from these plants (m, mu, mi). Microspores from preconditioned plants form embryos if treated for 4 days at 32.5°C (Mi). If the treatment includes an incubation at 25°C for 1 day before 3 days at 32.5°C , few, if any, embryos are formed (results not shown), and napin mRNA is not detected (Mx).

The results presented in this chapter clearly demonstrate that there is a correlation between the ability to form embryos and the expression of napin. The exact nature of the relationship is still unknown.

5.III. DISCUSSION

Embryos develop through a series of distinctive morphological stages. This initial phase of cell differentiation is followed by a maturation phase during which the major reserves are accumulated, then the embryo dessicates prior to germination. Much of the research on embryo development has centred on the seed-specific storage proteins. Early embryonic events are more difficult to study due to the small amount of tissue available. A tissue culture system that allows the formation of embryos from isolated B. napus haploid microspores has been developed by Keller et al. (1987b). Large numbers of the cultured embryos can be collected at desired stages. The similarity of microsporic and zygotic embryos was investigated by comparing storage protein gene expression during development.

The expression of storage protein genes in microsporic and zygotic embryos was temporally similar but mRNA levels were lower in the microspore-derived embryos. Low levels of 12S storage protein were reported in embryos derived from microspores in anther culture, but unlike embryos from isolated microspores, the 12S protein was detected as early as the globular stage (Crouch, 1982). Taylor et al. (1990) also found low levels of storage protein transcripts in B. napus torpedo stage microsporic embryos. The quantitative

differences in transcript levels between zygotic and microsporic embryos may be even greater than indicated by the blots in Plates 5.3 and 5.4 since the zygotic samples were prepared from whole seeds and therefore the embryo mRNA within the total RNA sample might be diluted by RNA from the other seed components.

Cruciferin gene expression lagged behind that of napin, as described in zygotic B. napus embryos by Crouch et al. (1984) and DeLisle and Crouch, 1989. In microsporic embryos, the levels of napin (Plate 5.3) as well as cruciferin (Plate 5.4) mRNA were maintained up to 7 wk of culture even though the embryos had begun to germinate. When mid cotyledon B. napus embryos were removed from the seed and cultured on hormone-free medium, they germinated but retained many embryonic characteristics, including the continued synthesis and accumulation of low levels of storage proteins (Finkelstein and Crouch, 1984). The addition of 5 μ M ABA to the culture medium delayed germination and increased the levels of storage protein mRNA by 2 to 3 fold (DeLisle and Crouch, 1989). The response of microsporic embryos to maturation culture conditions appears to be similar to that exhibited when maturation phase zygotic embryos are placed in culture. The levels of storage protein gene transcripts were not determined in the maturing microsporic embryos in the absence of ABA but others have shown that both transcript and protein levels in 21-day torpedo stage

microspore-derived embryos could be enhanced by ABA treatment (Taylor et al., 1990).

Although the patterns of gene expression are not identical, the similarities indicate that molecular events in B. napus microsporic embryos reflect those that occur during normal zygotic embryo development. The similarity has been confirmed on a biochemical level. Fatty acid and triacylglycerol profiles have been shown to be qualitatively and often quantitatively similar (Pomeroy et al., 1991; Taylor et al., 1990)

The presence of high levels of napin transcript in the 4-day induced samples (data provided by M.-J. Ginés) suggested that there is a link between the induction of napin expression and the induction of the embryogenic pathway in microspores. Subsequent experiments supported this idea. Napin mRNA could be induced in heart and torpedo stage microsporic embryos after 2 days incubation at 32.5°C. The napin message could not be induced in non-embryogenic samples:

1. microspores from large buds, which presumably had developed beyond the "embryogenic window" (late uninucleate to early binucleate; Pechan and Keller, 1988)
2. microspores from non-preconditioned plants
3. microspores incubated at 25°C for 24 hr before the 32.5°C heat treatment

4. somatic tissues from preconditioned or non-preconditioned plants.

One of the limits of the microspore cell culture system is the small proportion (approximately 5%) of microspores actually induced to undergo embryogenesis. The small size difference between the dividing proembryos and the remaining microspores precludes enrichment by filtration. It is possible that the non-dividing microspores are responsible for the napin expression observed. This seems unlikely since the Mx (25°C for 24 hr, then 32.5°C), Mu (uninduced), and M (zero time) samples also contain total 4-day sample populations but do not express napin message.

The embryogenic response was dependent upon two key factors. Preconditioning of the plants by growth at 10°C/5°C was essential but not sufficient for embryogenesis. A second signal (heat treatment) was also required. The molecular effect of preconditioning is unknown but may serve to prepare the genes for the second signal, perhaps by opening up the chromatin to allow transcription factors triggered by the heat to access the DNA. Alternatively, rather than actively inducing embryogenesis, these treatments may work by blocking pollen formation. Perhaps preconditioning reversibly blocks an essential step in pollen development. The heat treatment may be required for irreversible blockage.

The switch from the pollen to the embryogenic pathway of development is accompanied by changes in the protein profiles in microspores (L. Holbrook and B.L. Miki, unpublished results). Alterations in translatable mRNA have been detected in microspores incubated at 32.5°C as early as 4 hr after culture initiation. The role of the induced proteins has not been determined but it is possible that Heat Shock Proteins (HSPs) are among those that are newly synthesized (Pechan et al., 1991). HSPs have been previously characterized in Brassica species (Fabijanski et al., 1987; Baszczynski, 1988). It is unknown if the HSPs are directly involved in the embryogenic response, but functions have been proposed for these proteins in normal embryonic development, differentiation and cell proliferation (Domoney et al., 1991; Gyorgyey et al., 1991; reviewed in Bond and Schlesinger, 1987; Lindquist, 1986; Pechan, 1991).

Embryogenesis can also be induced in microspores by γ -irradiation and ethanol treatment (Pechan and Keller, 1989). This indicates that heat shock per se does not trigger embryogenesis, but that the embryonic pathway is induced by stress in general. HSPs can also be induced by a number of stress stimuli, including water stress, ABA, wounding, arsenite, ethanol and heavy metals (Lin et al., 1984; Heikkila et al., 1984; reviewed in Lindquist and Craig, 1988). It is not known if napin expression is induced by the alternative triggers of embryogenesis, but this experiment

could provide an opportunity to study the link between napin expression and embryogenic potential in the absence of the heat treatment.

The pattern of napin gene expression in microsporic embryos is not consistent with a typical heat shock response. HSPs are induced rapidly, usually within the first hour or two after being exposed to elevated temperatures. No samples of microsporic embryos were taken before 4 days after the initiation of the heat treatment so the timing of the increase in napin mRNA is unknown. In Brassica oleracea, maximal synthesis of HSPs occurs 60 to 120 min after the shift from 20°C to 37°C. The rate of synthesis falls dramatically for most of the proteins after 120 min but the synthesis of a few of the HSPs is maintained during the third and fourth hour of heat shock (Fabijanski et al., 1987). During prolonged exposure to high temperatures in maize, tobacco and soybeans, HSPs disappear within 4 to 10 hr after the initiation of heat treatment (Cooper and Ho, 1983; Barnett et al., 1980; Altschuler and Mascarenhas, 1982). The synthesis of HSPs in cultured maize cells maintained at 40°C is prolonged compared to that in maize seedlings but begins to decline by 10 hr (Cooper et al., 1984). In the microsporic embryos, high levels of napin mRNA were detected 4 days after transfer to 32.5°C. This indicates that napin does not respond like a HSP. As well, the napin gene promoter does not contain a sequence

homologous to the heat shock regulatory element determined by Bienz and Pelham (1987). It is possible that the HSP genes and the napin genes share cis-acting sequences other than the Heat Shock Element (HSE). In heat shock genes, the developmental control elements have been shown to differ from the HSEs (reviewed in Bienz and Pelham, 1987). The link between napin expression, heat treatment and microspore embryogenesis could be indirect, perhaps through a shared transcription factor.

Heat shock of carrot somatic embryos can arrest development if applied during a 2 to 4 hr window of sensitivity which occurs during the mid to late globular stage of embryo development. Globular embryos synthesize little or no heat shock mRNA during exposure to heat shock, unlike embryos of other stages. However, the low level of existing heat shock mRNA is translated at an elevated rate. It has been proposed that the strong bias for translation of HS transcripts at this critical time prevents the production of a protein essential for the continuation of embryogeny (Zimmerman et al., 1989). One can speculate that heat treatment of the microspores similarly prevents the production of a protein or proteins essential for pollen development. In this case, the default pathway would be one of embryogenesis. The levels of HS transcripts and proteins in microspores at this stage are unknown.

The trigger for inducing embryogenesis in B. napus microspores, whether induction or repression of gene expression, or a combination of events, remains to be determined. Napin gene expression may be directly involved in the induction of embryo development but it seems more likely that the presence of napin mRNA is a marker for embryogenic potential, rather than a cause of it. A similar marker role has been suggested for Dc3, a cDNA prepared from carrot somatic embryo RNA (Wilde et al., 1988). Once the microspore embryogenic pathway is initiated, the resultant embryos behave in a manner morphologically and molecularly similar to zygotic embryos.

CHAPTER 6

BRASSICA NAPUS TRANSFORMATION AND PRELIMINARY ANALYSES

6.1. INTRODUCTION

Brassica napus is an important oilseed crop which is amenable to transformation by Agrobacterium tumefaciens, and therefore an obvious target for crop improvement through genetic engineering. Seed-specific genes can be valuable tools in the genetic engineering process because they can be used to localize the expression of a particular trait to the seed. There is an additional advantage when the trait can be expressed in haploid microspore embryos. Homozygosity can be achieved in one step by doubling of the chromosomes by colchicine treatment.

In this chapter, expression of the atar1-GUS constructs was studied in both zygotic and microsporic embryos from transgenic B. napus plants at the level of reporter enzyme activity.

6.II. RESULTS

6.II.1. Brassica napus Transformation

Brassica napus cv. Westar was transformed with five different vectors (nos-GUS, pTCS, pTCL, pTLF1, pTLF1R; see Figures 3.4, 3.5, 3.6). The transformation protocol is outlined in Figure 6.1. Transformants were selected on 40 µg/ml kanamycin. Agrobacterium tumefaciens Ti plasmid pMP90, unlike pGV3850 which was used for N. tabacum transformation, does not carry a carbenicillin gene (Koncz and Schell, 1986). Therefore, carbenicillin, which is less toxic to B. napus tissues than cefotaxime (De Block et al., 1989; Charest, 1988), was used to clear the tissues of A. tumefaciens.

Kanamycin-resistant shoots were transferred to the greenhouse after rooting on selection medium. Flowers were self-pollinated and tagged on the day of anthesis. Seed samples, collected at weekly intervals, as well as somatic tissue samples were tested for GUS enzyme activity. Samples were screened using the same microtiter plate assay described in 4.II.2.a.

Figure 6.1 Scheme for Brassica napus Transformation and
Sample Collection

Km^R , kanamycin-resistant.

Cut 3-4 mm cross-sections from
sterilized internodal stem
pieces and place on 1/10 EM plates

Precondition 4 days, 25°C

Inoculate with 48 hr. culture
of Agrobacterium tumefaciens
containing vector

Place on fresh 1/10 EM plates

Brassica napus stem sections
filter paper
Nicotiana tabacum cell suspension
1/10 EM medium

Incubate 2-3 days, 25°C

Transfer to EM selection plates
containing Kanamycin (Km) and
Carbenicillin (Cb)

Maintain Km^R shoots on selection
(MSB5, 1% sucrose, Km Cb)
until develop roots

Transfer to greenhouse

Tag flowers on day of pollination (anthesis)

Samples collected for initial screening
seeds: 1,2,3,4,5,6,7 wks after pollination; dry
leaf, stem, flower, roots

6.II.2. Summary of Initial Screening of B. napus Plants

The results of the initial screening of Km^R B. napus plants are presented in Table 6.1. Only 72% of the Km^R plants were also GUS-positive. Seed GUS activity levels in the B. napus plants tended to be low. No GUS activity was detected in somatic tissues. The two plants giving the most intense fluorescence in the microtiter plate assay were chosen for further study (TCS-B1, TLF1-B1). Due to the advanced stage of the original plants, subsequent experiments were performed on their progeny.

6.II.3. Subsequent Experiments

A flow chart outlining the samples tested in the remaining experiments can be found in Figure 6.2.

Four plants derived from the seeds of TLF1-B1 and four plants derived from the seeds of TCS-B1 were analysed for the presence of the GUS gene by Southern blotting. All plants contained at least two copies of the gene. The complete set of results is presented in 6.II.4. One plant from each group of four was selected for GUS analysis. Flowers were tagged on the day of anthesis. Seeds at specific time points post anthesis as well as leaf, flower and stem samples were analysed by fluorogenic GUS activity (see 6.II.5.). Histochemical GUS analysis of zygotic embryos

Table 6.1 Summary of Initial Screening of Km^R

Brassica napus Plants

* See Plates 4.1, 4.2 for examples of GUS activity.

ND, not detectable; med, medium; Km^R, kanamycin-resistant.

BREAKDOWN OF RELATIVE GUS ACTIVITY LEVELS * IN VARIOUS TISSUES OF GUS-POSITIVE PLANTS									
VECTOR	Total no. Km ^R plants	Total no. GUS-positive plants	GUS ACTIVITY PROFILE					no. plants with profile	
			seeds	leaf	stem	flower	roots		
<u>nos</u> -GUS	3	1	low	ND	ND	ND	ND	1	
pTCS	1	1	med	ND	ND	ND	ND	1	
pTCL	2	1	low	ND	ND	ND	ND	1	
pTLF1	8	7	med	ND	ND	ND	ND	1	
			low	ND	ND	ND	ND	6	
pTLF1R	4	3	low	ND	ND	ND	ND	3	

Figure 6.2 Summary of Brassica napus Plants and Samples

Based on the results of the initial screening (Table 6.1), two B. napus plants were chosen for further study: TLF1-B1, TCS-B1. All subsequent experiments were performed on progeny obtained by self-pollination of the two original transgenic plants. Both zygotic and microsporic embryos were examined in the progeny of TCS-B1.

TLF1-B1 and TCS-B1: original GUS-positive B. napus plants transformed with vectors pTLF1 and pTCS, respectively.

Lower case letters (a to k) represent individual plants derived from seeds of the two original plants. Plants TCS-B1 e and f were grown under normal greenhouse conditions until producing seed, then they were cut back and transferred to preconditioning temperatures (10°C day/5°C night) for microsporic embryo production. Plants g to k were grown under the standard conditions for microsporic embryo production outlined in Chapter 2. Microsporic embryos of various stages of development were selected for GUS assays. In some cases, cotyledon stage embryos were incubated in the presence of 10 μ M ABA for 48 hr before collection.

ZYGOTIC EMBRYOS

TLF1-B1

leaf samples
for Southern
blot analysis

flowers tagged at time of anthesis

Samples for GUS assay (fluorometer method)

seeds: 10. 18. 23. 25. 28. 35. 45 dpa
leaf. flower. stem

MICROSPORIC EMBRYOS

TCS-B1

PROGENY

plants grown at
25°C day/20°C night
then cut back and
transferred to
10°C day/5°C night

plants grown at
10°C day/5°C night

buds used in microspore culture system

Samples for GUS assays

microsporic embryos +/- ABA

was not included due to the inconsistency and background staining obtained with these samples.

Microsporic embryos from the transgenic B. napus plants were also analysed for GUS expression (6.II.6.). At the time that the decision was made to examine the microsporic embryos, two plants (e and f), which had already flowered, were present in the greenhouse. These plants were cut back and placed in the preconditioning (10°C day/5°C night) growth chamber for several weeks until new buds appeared. In the meantime, more seeds from the same lot were sown. Plants g to k were grown under the standard conditions used to optimize microsporic embryo yield (see Chapter 2).

6.II.4. Southern Blot Analysis of Transformed B. napus Plants.

DNA was isolated from four progeny of TLF1-B1 and four of TCS-B1. The DNA was digested with Hind III (Plate 6.1), which cuts within the polylinker and also within the plant DNA, outside of the inserted T-DNA. The size of the hybridizing fragment will vary depending on the site of segregation of the T-DNA. The GUS probe hybridized with two to five or more bands in the transgenic plant DNA. No hybridization was detected with untransformed Westar DNA. Each set of progeny plants showed segregating hybridization patterns. When the DNA was cut with Xba I and probed

Plate 6.1 Southern Blot Analysis of Transformed

Brassica napus Plants: Hind III Digestion

A. Autoradiograph from Southern blot. Individual plants are listed across the top of the figure. The designations indicate the vector used for transformation and the B. napus plant tested. Molecular weight markers are placed to the right. Westar: untransformed B. napus cv. Westar.

B. Diagram indicating the segment of the GUS gene used to make the radioactive probe for Southern blot analysis. The plant DNA was digested with Hind III which cuts within the polylinker and again at an unidentified site within the plant DNA external to the inserted T-DNA.

A

B

with GUS (Plate 6.2), the numbers of bands were similar but the sizes differed from those obtained with Hind III. This was expected since Xba I also cuts within the polylinker and within the plant DNA. Slight variations in numbers could be due to different fragments of DNA, carrying one or more copies of the gene, migrating to the same position in the gel. Some bands in the autoradiographs appeared much darker than others.

Digestion of the DNA with Hind III and Eco RI releases a fragment internal to the T-DNA which hybridizes to the GUS probe. Unlike the N. tabacum integrations discussed in Chapter 4, there did not appear to be any deletions or rearrangements of the integrated GUS gene in B. napus transformants. Bands of the expected size (2.5 to 2.7 kb) were detected by the GUS probe (Plate 6.2).

6.II.5. GUS Fluorogenic Activity During B. napus Seed Development

The time course of GUS activity during seed development is presented in Figure 6.3. Significant activity was not detected until midway through development, as would be expected for a seed storage protein. Activity levels dropped in older seeds. The activity in plant TLF1-B1a was slightly stronger and slightly earlier than that in plant TCS-B1b but this could be due to plant-to-plant variation.

Plate 6.2 Southern Blot Analysis of Transformed
 Brassica napus Plants:
 Xba I, or Hind III/Eco RI Digestion

A. Autoradiograph from Southern blot. Individual plants and enzymes are placed across the top of the figure. The designations indicate the vector used for transformation and the B. napus plant tested. Molecular weight markers are to the right.

B. Diagram indicating the segment of the GUS gene used to make the radioactive probe for Southern blot analysis. The plant DNA was digested two different ways. Xba I cuts within the polylinker and at a second unidentified site within the plant DNA. The Hind III/Eco RI digest yielded a fragment of approximately 2.6 or 2.7 kb (depending on the vector) that was recognized by the probe.

A

B

Figure 6.3 Fluorogenic GUS Activity in Developing
Brassica napus Seeds

Seeds from 2 to 3 siliques were pooled and homogenized per time point. Extracts were assayed in duplicate and the average plotted.

MU, 4-methylumbelliferone.

untransf. Westar: untransformed B. napus cv. Westar

Unfortunately, GUS activity could not be localized within the zygotic embryo by the histochemical method due to high background staining in both transformed and non-transformed plants.

6.II.6. GUS Activity in Microsporic Embryos

All B. napus cv. Westar microspore cultures were prepared by Nancy Long and Dr. Keith Pomeroy.

6.II.6.a. Histochemical localization of activity

The GUS histochemical assay results observed in microsporic embryos were inconsistent, varying in staining pattern and intensity from preparation to preparation, even when using buds from the same donor plants. Some batches did not stain at all. The staining pattern believed to be associated with the transferred GUS gene is shown in Plate 6.3 C to E. GUS staining was first detected in the lower axis of the embryo (Plate 6.3, C) then it spread both up and down the axis from that point (Plate 6.3, D and E). As observed in N. tabacum embryos, the histochemical GUS staining also spread inwards to the vascular bundle (results not shown). The histochemical staining observed in these preparations from plants TCS-B1, e and f was confirmed by

Plate 6.3 Histochemical Localization of GUS Activity in
Brassica napus Microsporic Embryos

Embryos were collected from microspore cultures, rinsed in 0.1 M NaPO₄ and stained overnight in X-gluc (5-bromo-4-chloro-3-indolyl-glucuronide).

A, early cotyledon stage embryo; B, cotyledon stage embryo; C, D, E, late cotyledon stage embryos. Embryos A to E were collected from microspore cultures of plants TCS-B1 e and f. F, untransformed Westar late cotyledon stage embryo cultured in the absence of ABA; G, untransformed Westar late cotyledon stage embryo cultured in the presence of 10 μ M ABA; H, late cotyledon stage embryo (Plant TCS-B1 j) cultured in the absence of ABA; I, late cotyledon stage embryo (Plant TCS-B1 j) cultured in the presence of 10 μ M ABA. Embryos F to I were obtained from the experiment described in Figure 6.4. Embryos cultured in the presence of ABA were generally smaller than the corresponding embryos cultured without ABA.

Bar: 1 mm.

the detection of fluorogenic GUS activity in the microtiter plate assay (results not shown).

Other staining patterns such as stained axis tips (Plate 6.3, B) were sometimes observed. Diffuse staining in the axis and cotyledons (Plate 6.3, G and H) was seen in microsporic embryos from both transformed and non-transformed plants with or without an ABA treatment. When cultured in the presence of ABA, the embryos took on a more intense generalized staining (Plate 6.3, G and I).

6.II.6.b. Fluorogenic GUS activity

The inconsistencies of the histochemical results were reflected when the samples were analysed for fluorogenic GUS activity. Two out of three microspore preparations tested negative in the fluorometer assay. The results of the third experiment are presented in Figure 6.4. In microsporic embryos from plant TCS-B1 j, low fluorogenic activity was detected when the embryos were exposed to 10 μ M ABA for 48 hr. (In a previous experiment, microsporic embryos from plant j gave negative results, even when treated with ABA.) Plant TCS-B1 k gave rise to embryos which had low fluorogenic activity even in the absence of ABA. Incubation with ABA increased the activity by more than 4-fold. Microspores from other transgenic plants (g,h,i) were not used in this particular experiment. The corresponding

Figure 6.4 Fluorogenic GUS Activity in Brassica napus
Microsporic Embryos

Cotyledon stage microsporic embryos (21 days of culture) were cultured for an additional 48 hr with (+) or without (-) 10 μ M ABA. Embryos were collected and tested for GUS activity using the fluorometer method.

Control: Embryos from untransformed B. napus cv. Westar

j: Embryos from transformed plant TCS-B1 j

K: Embryos from transformed plant TCS-B1 k

histochemical staining of the embryos used for Figure 6.4 appears in Plate 6.3 F to I.

The cause of the inconsistencies in GUS activity is as yet unknown but appears to be linked to the differences between individual microspore preparations rather than the differences between plants from which those microspores are obtained. This question will be discussed further in 6.III.

6.III. DISCUSSION

6.III.1. Brassica napus Transformation and Screening for Transformants

The transformation procedure for B. napus is much more labour intensive than that for N. tabacum. This is directly related to the frequency of transformation, which is at least two orders of magnitude higher in N. tabacum than in B. napus (Charest, 1988). In this work, substantially fewer Km^R plants (Table 6.1) were obtained during B. napus transformation as compared to N. tabacum (Chapter 4), and only 72.2% of these were also GUS-positive. However, at least one GUS-positive representative was found in every vector group of transgenic B. napus plants.

Kanamycin is often used as the selectable marker for B. napus transformation (Charest et al., 1988; Radke et al., 1988; De Block et al., 1989; Moloney et al., 1989; Boulter et al., 1990) but some problems have been noted. Low levels (10 to 15 µg/ml) of Km were found to allow a significant number of non-transformed plants to escape the selection process, but attempts at direct selection at high levels (80 µg/ml) proved to be unsuccessful (Charest et al., 1988; Radke et al., 1988). It is possible that even at 40 µg/ml Km, some non-transformed shoots may have escaped selection. Some shoots did not turn from green to white until several

weeks on Km medium. "Escapes" may account for the high level (27.8%) of Km^R shoots that did not express detectable GUS activity (Table 6.1) as compared to results in N. tabacum. Of course, inactivation or rearrangement of the GUS gene, or differential regulation of the constructs in N. tabacum and B. napus, cannot be ruled out.

Southern blot analysis detected multiple copies of the GUS gene in transgenic B. napus plants. The integrated T-DNA appeared to be intact (Plate 6.1, Hind III/Eco RI lanes). In all N. tabacum plants analysed, at least one of the integrated genes was rearranged (Plate 4.4). Perhaps integrations are more stable in B. napus DNA or there is less degradation or modification of the DNA during transfer prior to integration.

In some cases more than one plant gave rise to a particular pattern of hybridizing bands (for example, TCS-B1a and TCS-B1c; TCS-B1b and TCS-B1d; Plate 6.1). This is not surprising in view of the fact that these plants were all derived from the first generation of seed from the original self-pollinated transgenic. The integrated genes would be expected to segregate in this generation since the original plant would be heterozygous for any particular insertion.

6.III.2. GUS Activity in B. napus Embryos

6.III.2.a. Zygotic embryos

The developmental expression profile for GUS activity in transgenic B. napus plants is presented in Figure 6.3. GUS activity became detectable above background levels between 23 and 28 dpa, and peaked at 35 dpa. These results are similar to those obtained by Crouch and Sussex (1981) for napin accumulation in B. napus embryos. The slight differences in timing and level of GUS expression between the two transgenic plants (TCS-B1b, TLF1-B1a) may have been due to plant-to-plant variation or differences in gene copy number or homozygosity rather than any real differences in vectors. In N. tabacum, plants transformed with pTCS possessed on average, ten times more GUS fluorogenic activity than plants of the pTLF1 group, but activity levels within individual vector groups in N. tabacum varied widely. No comparison in activity levels between the two vector groups in transgenic B. napus can be made based on the results in Figure 6.3.

No GUS activity was detected in the somatic tissues of transgenic B. napus plants, and activity in seeds was low. Only two plants showed activity levels in the microtiter plate assay above that obtained in N. tabacum with nos-GUS (Plate 4.2; Table 6.1). Based on the initial screening and

the results in Figure 6.3 it appeared that GUS activity levels were lower in B. napus, as compared to levels obtained with the same vectors in transformed N. tabacum (Table 4.2; Figure 4.2). However, the small number of B. napus plants and wide range of activity levels among plants transformed with the same vector make a definitive analysis difficult. Low levels of chimaeric gene expression in B. napus transformants has been noted previously with a variety of promoters. Methylation and developmental regulation of the genes were proposed as possible explanations (De Block et al., 1989).

6.III.2.b. Microsporic embryos

GUS expression in microsporic embryos was inconsistent. Activity varied with individual microspore preparations, regardless of the source of the buds. Many factors could contribute to the variation. Some very preliminary experiments looked at embryo density in the cultures. Fewer embryos per plate resulted in large embryos with well-developed cotyledons but GUS activity did not appear to be correlated with cotyledon size (results not shown). Embryos were collected after 3 to 4 wk in culture. Napin protein is not detected by gel electrophoresis in B. napus zygotic embryos until approximately 25 dpa (Crouch and Sussex, 1981). It is possible that some embryos had not matured

enough before collection to produce detectable amounts of the GUS enzyme. A "maturation medium" used to culture embryos up to 7 wk was discussed in Chapter 5. Embryos produced storage protein mRNA during the culture period in this medium but premature germination of the embryos was noted. Further manipulation of the culture conditions would be required in order to achieve a maturation phase in microsporic embryos equivalent to that in zygotic embryos.

Taylor et al. (1990) showed that the accumulation of both storage protein and storage protein mRNA in B. napus microspore-derived embryos was stimulated when the embryos were incubated for 48 hr in 10 μ M ABA. GUS fluorogenic activity was also responsive to ABA (Figure 6.4). The presence of possible ABA consensus sequences in the arabin promoter was noted in Chapter 3.

Histochemical GUS activity was observed in cotyledon stage microsporic embryos. This result was consistent with the detection of endogenous napin mRNA in 21-day cotyledon stage embryos (Plate 5.3). Embryos cultured in the presence of ABA often turned blue when incubated with X-gluc. This staining was seen even in untransformed ABA-treated embryos (Plate 6.3, G) and therefore was considered background staining. Perhaps ABA induces endogenous GUS-like enzymes within the embryos. Other diffuse staining patterns (Plate 6.3, H) were also considered to be background. The background GUS activity in microsporic embryos cultured

without ABA never reached the levels seen in zygotic embryos but the possibility that it could lead to false interpretations of the data cannot be ruled out.

The histochemical staining attributed to the transferred GUS gene occurred as an intense blue colour only in the embryo axis (Plate 6.3, C,D,E and I). Staining was first detected in the lower axis, then progressed through the embryo in a manner similar to that seen in the transgenic N. tabacum embryos (Chapter 4) except that the B. napus cotyledons remain unstained. De Clercq et al. (1990) reported that the A. thaliana arabin gene at2S1 (equivalent to atar1) is expressed in the embryo axis but directed the production of little or no mRNA in the cotyledons. The axis-specific expression seen in A. thaliana and B. napus but not in N. tabacum may reflect the genetic relatedness of A. thaliana and B. napus (both are members of the family Brassicaceae). N. tabacum may lack the appropriate regulatory signals for axis-specific expression of the atar1-GUS construct.

The atar1-GUS constructs functioned as would be expected for a storage protein gene in B. napus zygotic embryos. Expression in microsporic embryos was inconsistent and difficult to interpret. Radke et al. (1990) studied the expression of a B. campestris napin gene (1,2)-GUS construct in transgenic B. napus plants. They also reported faithful expression of the GUS gene in zygotic embryos, but observed

continual GUS expression from the globular through to the cotyledon stage in microspore-derived embryos.

Further studies would be required in order to determine the cause(s) of the inconsistencies encountered when studying gene expression in microspore-derived embryos. These inconsistencies may prove to be valuable clues in the search for the identity of factors affecting embryonic development.

CHAPTER 7

GENERAL DISCUSSION AND SUMMARY

The main objective of the work presented in this thesis was to study the expression of a seed storage protein gene during development. Storage proteins are of interest for several reasons. They accumulate to high levels, and directly contribute to the nutritional quality of seeds. Storage protein genes provide a model for investigating developmental, spatial and temporal regulation of gene expression. An Arabidopsis thaliana 2S albumin storage protein (arabin) gene was selected for study for reasons outlined in the introduction.

The first step in the project was the isolation and sequencing of an arabin gene, designated atar1. The region of atar1 5' to the translation initiation site was analysed for potential cis regulatory elements. Based on this information, transformation vectors were constructed containing fragments of the atar1 5' sequence and the coding region of the GUS reporter enzyme gene. GUS activity was assayed in both N. tabacum and B. napus transformants.

In general, results were consistent with the use of the arabin promoter and upstream sequences. Seed-specific GUS

activity peaked during the last half of embryo development. However, there were some differences observed when the expression pattern in transgenic N. tabacum was compared to that obtained in transgenic B. napus, with the same vector.

In A. thaliana, 2S albumins accumulate primarily in cotyledon stage embryos (Heath et al., 1986). The expression of the atar1-GUS constructs in B. napus was also detected in cotyledon stage embryos and paralleled the time course of endogenous napin accumulation. GUS activity was detected in transgenic N. tabacum much earlier, at the late globular to early heart stage. Spatial differences in expression were also noted. Transgenic N. tabacum embryos expressed GUS initially in the cells of the upper axis and eventually in the cotyledons, whereas GUS activity was only detected in the axis of B. napus embryos, beginning in the lower region. The B. napus GUS staining pattern was observed in microsporic embryos. Extensive background staining occurred in zygotic embryos which made interpretations difficult. It could be argued that the differences in spatial localization of GUS activity may be due to variations induced by tissue culture in the microsporic embryos. However, in situ analysis of at2S1 (equivalent to atar1) in A. thaliana (Guerche et al., 1990) supported the axis-specificity of this gene. The initial sites and progression of activity within the axis itself could not be determined from the in situ study.

From these data, it appears that the expression pattern of atar1-GUS in transgenic B. napus is closer to that of native arabin than the pattern in transgenic N. tabacum. This likely reflects the greater degree of genetic similarity between A. thaliana and B. napus. N. tabacum does not produce an arabin equivalent. Therefore, transcription factors and other regulatory proteins used in the production of the atar1-GUS transcript would have to be "borrowed". These concerns could be circumvented by using an homologous system in which to study gene expression. Although not available at the time that this work was initiated, transformation of A. thaliana has since been reported (Valvekens *et al.*, 1988; Lloyd *et al.*, 1986; Feldmann and Marks, 1987). N. tabacum was originally chosen as a transformation system due to its ease and reliability compared to other systems available at that time. In addition, correct temporal and tissue-specific expression of a Phaseolus vulgaris 7S globulin storage protein (phaseolin) gene had been reported in transgenic N. tabacum (Greenwood and Chrispeels, 1985; Sengupta-Gopalan *et al.*, 1985).

One cannot rule out the possibility that the rearranged copies of the integrated gene constructs detected in N. tabacum contributed to the observed differences in expression in N. tabacum and B. napus. No rearrangements were detected in B. napus transgenic plants.

During the course of this investigation, several papers were published by other laboratories on arabin genes and their expression. The at2S1 gene (equivalent to atar1) was shown to be developmentally and seed-specifically regulated in transgenic N. tabacum. The at2S1 protein was detected in the early stages of N. tabacum seed development (De Clercq et al., 1990) confirming our results with the GUS protein. The at2S1 gene 5' flanking sequences were used to direct expression of a reporter gene or a modified 2S albumin gene in seeds of N. tabacum, B. napus and A. thaliana (De Clercq et al., 1990; Vandekerckhove et al., 1989). In all these cases, the gene constructs contained approximately 1.7 kb of sequence upstream of the at2S1 translation initiation site. We have localized seed-specificity, developmental regulation and ABA responsiveness to a 392 bp fragment (-436 to -44) of the arabin gene atar1.

In situ hybridization showed that at2S1 is expressed only in the A. thaliana embryo axis (Guerche et al., 1990), but the specific sites of mRNA expression throughout the axis were not determined in detail. Histochemical localization of GUS activity (Chapters 4 and 6) revealed that induction of the atar1-GUS construct proceeded through the embryo in a wave-like fashion. What purpose would be served by this pattern of gene induction? Since storage proteins act as nitrogen reserves, perhaps induction is linked to the presence of large quantities of appropriate

amino acids. Vegetative storage protein genes are known to be induced when a plant organ contains excess amino acids (ie. when a target for nitrogen exported from the organ is removed, or, if the plant is grown under high nitrogen conditions) (reviewed in Staswick, 1990). Seed storage protein production may follow the accumulation pattern of amino acids in the developing embryo. The napin protein appears before cruciferin in B. napus (Crouch and Sussex, 1981; Crouch et al., 1984; DeLisle and Crouch, 1989). Perhaps the commencement and peaks of the production of the two storage proteins are staggered to avoid direct competition for the amino acid pools. Of course, there is a long period of overlap in the production of the storage proteins but the "crests" of each wave would reach a particular amino acid pool at different times.

It is unknown if atar1 is shut down throughout the embryo in a similar wave-like fashion. When fluorogenic GUS activity was monitored (Figures 4.2, 6.3) during development, activity peaked then decreased as the seeds matured. Napin transcripts have been shown to decline before those of cruciferin in B. napus, and the mRNAs are undetectable in dry seeds (Crouch et al., 1984). Arabin message also decreases as the seeds mature (Guerche et al., 1990). There was no apparent decrease in GUS activity when the embryos were assayed histochemically. However, the GUS protein and the X-gluc cleavage product are very stable

(Jefferson, 1987a,b). Drying and/or rehydration of the seeds has been proposed as the switch which shuts off embryo-specific genes and induces germination-specific genes (Bewley et al., 1989; Comai and Harada, 1990) but it is evident that the regulation of individual members of the embryo-specific set of genes is fine-tuned by other mechanisms.

Non-transcriptional levels of regulation must also be taken into account. GUS activity depends on the production of a protein, and therefore can be influenced by a number of regulatory steps including post-transcriptional and post-translational modifications and mRNA stability.

Other seed genes have been shown to have similar patterns of expression. B. napus napin and cruciferin mRNAs (Fernandez et al., 1991) as well as soybean Kunitz trypsin inhibitor, β -conglycinin, glycinin, and lectin mRNAs (Goldberg et al., 1989) are accumulated in a wave pattern in developing embryos. How does the embryo achieve a pattern of gene expression that is coordinated but allows for some temporal and spatial variation? One possible mechanism would involve a diffusible factor such as a hormone or protein. Coordination could be achieved through interaction with a cis element shared by the seed protein genes. Cis- and trans-acting elements specific for each gene could modify the shared regulatory signal. The lack of expression of a particular gene in a particular tissue could be the result

of tissue-specific negative regulatory elements or post-transcriptional mechanisms such as mRNA transport and stability or post-translational modifications. The timing of seed protein gene induction could be influenced by the susceptibility of the gene to the diffusible factor. Genes could have a low affinity for the factor or could require multiple molecules of the factor to be bound to achieve induction. Expression of the gene would be delayed until the gradient concentration of the factor reached a critical level. Alternately, there could be several diffusible factors that work on the same principle but are tailored to specific seed genes.

What is the source of the inductive signal? GUS activity was initially detected in ground meristem cells of the upper embryo axis in transgenic N. tabacum. In B. napus, the first cells to produce functional GUS molecules were in the lower axis of the embryo. Fernandez et al. (1991) have postulated that a B. napus embryo maturation program is regulated by a factor or factors centred at the shoot apex. Perhaps the source or the target varies with the species. Factors could diffuse away from more than one source. The inductive signal(s) appear to reside within the embryo itself since microsporic embryos, which develop in the absence of endospermal, seed coat or sporophytic influences, also express GUS in a wave-like manner.

It is possible that the expression of seed protein genes is not regulated by a diffusible factor, but is an intrinsic trait of the cells themselves. The state of gene expression may reflect the maturity of individual cells within the embryo.

Why are seed protein genes expressed in this manner? Perhaps it is more efficient for the embryo to have a common regulatory mechanism for this group of genes, or perhaps the mechanism is related to the particular physiological state of the embryo. These genes are developmentally regulated but it is unknown if they in turn, influence development. Since these proteins are expressed in the later stages of development, it seems unlikely that there is any direct effect on the developmental program. In B. napus, the production of napin mRNA is correlated with the embryogenic potential of microspore populations, but the role of napin, if any, in the induction and progression of embryo development is unknown.

Many questions remain to be answered. There are some obvious suggestions for follow-up experiments. Deletion analysis of the 5' flanking sequences (and 3' flanking sequences) will allow for more detailed mapping of the cis regulatory elements. That study could be expanded to search for trans-acting factors which bind to the cis elements. Is there a common factor which interacts with all seed protein genes expressed in a wave-like fashion?

The investigation of arabin gene expression presented in this thesis has provided a tool with which to study a regulatory mechanism that operates on a broad scale in the developing embryo.

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