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Exordium a novel gene in Arabidopsis identified by promoter trapping

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EXORDIUM
A NOVEL GENE IN *ARABIDOPSIS*
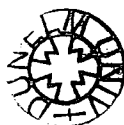# IDENTIFIED BY PROMOTER
TRAPPING

Thesis submitted for the degree of
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by

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ABSTRACT

The aim of the project was to characterize the expression of a GUS promoter trap in a transgenic line of *Arabidopsis thaliana*, and to investigate the function of the tagged gene. GUS expression in the transgenic line, designated line EM2, was found to be localized to embryos, and to the regions of most active cell division in the seedling, notably the apical meristems and young leaves. The tagged gene was named *EXORDIUM* (*EXO*). Line EM2, which contains a single copy of the promoter trap T-DNA, was found to have no obvious phenotype when homozygous for the T-DNA insertion, and when grown under a range of nutritional and hormonal conditions *in vitro*. It was found that the expression of the GUS gene and of the native *EXO* gene, was each up-regulated by exogenous auxin and down-regulated by exogenous cytokinin. The cloned *EXO* promoter was introduced as a GUS fusion into transgenic plants of *A. thaliana* and found to be expressed ion the same tissues as EM2, and additionally in the root vascular tissues, leaves and siliques, although to different extents in different transgenic lines. It was found that the *EXO* mRNA abundance accumulated in seedlings treated with hydroxyurea, which induces cell-cycle arrest at the G1-S transition. Further analysis demonstrated that *EXO* mRNA is preferentially abundant during M-phase of the cell cycle. Transgenic plants were produced containing sense and antisense versions of the *EXO* gene under the transcriptional control of the CaMV35S promoter. One antisense line exhibited an abberant phenotype, characterized by a reduced size and abnormal shoot branching pattern. EXO encodes a predicted protein of 314 amino acids, of unkown function.

DECLARATION

The material contained in this thesis has not been submitted for a degree in this or any other university.

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ACKNOWLEDGEMENTS

ABBREVIATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
$AgNO_3$	silver nitrate
ATP	adenosine triphosphate
bp	base pair (length of a single nucleotide)
BSA	bovine serum albumin
CaMV35S	cauliflower mosaic virus 35S RNA gene
cDNA	complementary DNA
cpm	counts per minute
DAG	days after germination
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTPs	2'-deoxynucleotide 5'-triphosphates
EDTA	ethylenediaminetetraacetic acid
EM2	the promoter trap line EM2
EXO	the *EXORDIUM* gene
GA_3	gibberellic acid (A_3)
GUS	β-glucuronidase
gusA (*uidA*)	gene encoding β-glucuronidase
HCl	hydrochloric acid
HU	hydroxyurea
IAA	indoleacetic acid
IPCR	inverse PCR
kb	kilobase (length of 1000 nucleotides)
KOH	potassium hydroxide
LB	Luria-Bertani medium
$MgCl_2$	magnesium chloride

mRNA	messenger RNA
½ MS10	half strength Murashige and Skoog medium containing 10g/l sucrose
MU	4-methylumbelliferone
MUG	4-methylumbelliferone-β-D-glucuronide
NAA	naphthaleneacetic acid
NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen orthophosphate
NaH_2PO_4	sodium dihydrogen orthophosphate
NaOH	sodium hydroxide
NPA	N-1-naphthylphthalamic acid
NPTII	neomycin phosphotransferase
nptII	gene encoding neomycin phosphotransferase
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	double stranded RNA interference
rpm	revolutions per minute
sdH_2O	sterile deionised water
SDS	sodium dodecyl sulphate
SE	standard error of the mean
T-DNA	transferred DNA
Ti plasmid	tumour-inducing plasmid
Tris	tris(hydroxymethyl)aminomethane
X-gluc	5-bromo-4-chloro-3-indoyl β-D-glucuronic acid

CONTENTS

INTRODUCTION

1.0 Introduction

Plants make up the majority of the world's biomass. They are responsible for maintaining atmospheric oxygen levels and they form the basis of all but the most extraordinary of known ecosystems. The relationship between humans and plants has shaped our history, from the first cultivation of crops, millennia of selective breeding, the discovery and exploitation of artificial crossing in the eighteenth century through to modern genetic engineering. Plants have been exploited not only for food but also for building materials, clothing, tools, medicines and for their aesthetic value. However, it is only recently, with the advent of molecular biology, that we have been able to study the mechanism by which plants achieve their diverse properties, namely through the interaction between their genes and the environment, via hormonal and other signalling pathways.

1.1 Plant Growth and Development

1.1.1 Embryogenesis

Plant development can be divided into two phases: pre- and post-germination. The first of these, more commonly termed embryogenesis, is the process by which the zygote divides and grows in a highly regulated manner to become a mature embryo with the capability to develop into a mature plant.

Unlike animal embryos, which at maturity resemble a miniature version of the adult, plant embryos are morphologically simple, consisting of a shoot meristem, one or two cotyledons, a hypocotyl and a root meristem. A radial pattern is also laid down, consisting of epidermis, ground tissue and vascular tissue (Laux & Jürgens, 1997). The majority of structures characteristic of the adult plant (including leaves, stems and roots) develop post-embryonically by differentiation of tissues formed at the meristems. In contrast to animals, plant reproductive organs develop entirely post-embryonically through reprogramming of the vegetative shoot meristem (Goldberg, 1988). The plant embryo represents a simple model with which to study the relationship between gene expression and *de novo* organogenesis.

Embryogenesis is a tightly controlled process. Cell division and differentiation must be regulated in such a way as to give rise to an organised pattern in which tissues and organs are correctly positioned (Jürgens, 1994). Embryogenesis in *Arabidopsis* is a rapid process, a mature seed being formed within 14 days of fertilization. The first division that occurs following fertilization is asymmetric, giving rise to a large basal cell and a smaller apical cell. This establishment of polarity is a key event in embryogenesis as the apical cell is the basis of the embryo proper while the basal cell forms the suspensor, a structure that connects the developing embryo to the maternal tissue. The apical cell then undergoes two vertical divisions at right angles to one another followed by a transverse division to generate the 'octant' stage embryo. The boundary between the two tiers of cells is known as the 'O' boundary with the cells above it being destined to become the cotyledons and shoot apex, and those below becoming the hypocotyls. The root forms from the hypophysis, the distal cell of the suspensor. Thus, positional information is laid down very early in the proembryo. Anticlinal divisions then occur to generate a 16-cell embryo with a protoderm (the outer cells). The central cells divide both longitudinally and transversely to create regular tiers, and the protoderm cells divide anticlinally to form the epidermis. Following this cell differentiation, bilateral symmetry is established as the globular embryo produces distal lobes (the precursors of the cotyledons) and becomes heart shaped. At this stage further cell differentiation occurs to generate distinct zones that will go on to become the ground tissue and vasculature. This is also the stage at which the root meristem becomes active, producing tiers of root tissues from the initials above the quiescent centre and layers of root cap from the initials below. Axial elongation produces the torpedo stage embryo in which the hypocotyls and cotyledons are clearly distinguishable. These organs continue to develop and fold back in the cotyledonary stage until they arrest as a mature embryo consisting of cotyledons, shoot apical meristem, hypocotyl, radicle and root meristem (Lindsey & Topping, 1993, Dolan *et al.*, 1993).

Post-embryonic root development in *Arabidopsis* continues in a regular manner to such a degree that cell lineages may be traced back to specific positions in the embryo, allowing the construction of a 'fate map' (Scheres *et al.*, 1994). Cells do not however maintain identity based on their origin but rather adopt the identity indicated

by positional signals as has been demonstrated using laser ablation experiments (van den Berg *et al.*, 1998).

1.1.2 Embryonic mutants

Despite the morphological simplicity of the plant embryo, studies aiming to elucidate the genetic control of embryogenesis have revealed the great complexity that exists at the molecular level. Goldberg *et al.* (1989) present data suggesting that an excess of 20,000 genes are expressed during soybean seed development. Many of these genes may be responsible for housekeeping functions in addition to those that are involved in embryo-specific processes. Mutant studies have distinguished genes responsible for embryo morphogenesis, and these can be divided into different groups representing the different processes involved in embryogenesis, namely apical-basal pattern formation, radial pattern formation, and correct development of shape (Mayer *et al.*, 1991). Mutants belonging to the first group (apical-basal patterning mutants) include *monopteros*, which has no root or hypocotyl; *fackel*, which has no hypocotyl; and *gnom/EMB30*, which has no root and reduced or no cotyledons (Mayer *et al.*, 1991). Mutants of this class are often termed seedling-lethal mutants as they are usually unable to develop past the seedling stage due to their extreme phenotypic abnormalities. Mutants of the second class (radial pattern mutants) have been identified through analysis of root mutations in *Arabidopsis* seedlings. These include *wooden leg*, which has impaired organization of the vascular tissue; and *scarecrow*, in which the endodermal and cortical layers are affected (Scheres *et al.*, 1995). Although these mutations were identified from screens for root abnormalities, the layer-specific phenotypes are also apparent in the hypocotyls and can be traced back to the embryo. The third class of mutations are those in which despite all pattern elements being present, the embryo and seedling are morphologically abnormal. Genes in this category are interesting as they demonstrate that aspects of pattern formation may be uncoupled from correct morphological development and so indicate that these processes are independently regulated. Such genes include *hydra* (Topping *et al.*, 1997), which has a variable number of cotyledons, a short, wide hypocotyls and a much reduced root system; and *fass* (Torrez-Ruiz & Jürgens, 1994), which like *hydra* differentiates all elements of body pattern but is compressed in the apical-basal axis and has a circumferentially enlarged hypocotyl. Both these mutations appear to be due to incorrect cell expansion and orientation of cell divisions indicating that the control

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of these processes is vital for correct morphogenesis. Further evidence that overall pattern can be maintained in the absence of correct cell division activity comes from the *tangled-1* mutant of maize (Smith *et al.* 1996). Like *hydra* and *fass*, *tangled* mutants have cells that divide in abnormal orientations (in this case during leaf development) and yet overall leaf pattern is maintained. Mutant leaves are shorter than those of wild-type, presumably due to the lack of orientated elongation of cells, again similar to the situation in *hydra* and *fass*. This suggests that processes of embryo development are similar to those of post-embryonic development.

In many cases mutations in the embryo are followed by mutations in the adult plant, even where this is not a direct effect of the embryonic mutation. For example, *fass* flower organs are compressed in their proximal-distal axis suggesting that the fass gene has a similar role throughout plant development and is not restricted to embryogenesis (Torrez-Ruiz & Jürgens, 1994). The *cup shaped cotyledon* mutant, which has fused cotyledons in the embryo also has fused flower parts, suggesting a common mechanism exists for organ separation in the embryo and flower (Aida *et al.*, 1997). Many of the genes cloned to date are expressed late in embryogenesis, however in order to understand the early events it is important to isolate genes that are expressed much earlier.

1.1.3   Hormonal control of plant growth and development

Auxin is implicated in many aspects of plant development, both during embryogenesis and post-embryonic growth. Some embryo mutants previously classed as shape or pattern mutants have since been discovered to be impaired in auxin regulation. *fass* mutant seedlings have average auxin levels over 2.5 times higher than those of wild-type seedlings (Fisher *et al.*, 1996), and a range of phenotypes reminiscent of many of the pattern mutants isolated are generated by *in vitro* treatment of developing wild-type *Brassica juncea* embryos with IAA (Hadfi *et al.*, 1998). *fass* seedlings also evolve more ethylene than wild-type seedlings, maybe due to the auxin up-regulation of ACC synthase, and ethylene treatment has been demonstrated previously to cause lateral expansion of growing cells (Eisinger 1983). Furthermore, *fass* seedlings grown on an inhibitor of ethylene synthesis show a sharp decrease in width and an increase in length (Fisher *et al.*, 1996). These results

strongly indicate the involvement of ethylene, produced perhaps as a result of aberrant auxin regulation, in the generation of the *fass* phenotype.

The polar transport of auxin is also important for various aspects of development. Excised globular shaped *Brassica juncea* embryos cultured *in vitro* on polar auxin transport inhibitors show an inability to establish bilateral symmetry, as is shown by the development of cylindrical fused cotyledons (Liu *et al.*, 1993). *Arabidopsis bodenlos* mutants (Hamann *et al.*, 1999) do not develop a primary root meristem and are resistant to 2,4-D. The mutation can be traced back to the two cell embryo in which the apical daughter cell divides horizontally in the mutant instead of vertically, and subsequently the cell destined to become the hypophysis divides abnormally and fails to generate a quiescent centre or root cap (Hamann *et al.*, 1999). The mutation is postulated to link the role of auxin-mediated apical-basal pattern formation with the formation of the primary root meristem. Interestingly, the mutant is capable of producing normal post-embryonic roots and develops into a fertile, although phenotypically abnormal, adult plant, demonstrating that initiation of secondary roots is regulated differently from that of the primary root. Lateral root development has also been shown to be dependent on polar auxin transport. Application of polar auxin transport inhibitors to the shoot-root junction of *Arabidopsis* caused a decrease in the number of lateral roots formed in a dose-dependent manner, and exogenous auxin application rescued the inhibition (Reed *et al.*, 1998). The *tir3* mutant of *Arabidopsis* that has reduced polar auxin transport also shows a deficiency in lateral root production.

One of the more dramatic phenotypes attributed to impaired polar auxin transport is that of the *Arabidopsis pin-formed* (*pin*) mutants. These mutants have abnormal inflorescence axes terminating in either a naked pin-like structure or in some cases a single, highly abnormal, flower structure. Leaves are also abnormal, being of errant shape and in some cases positioned incorrectly (Okada *et al.*, 1991, Gälweiler *et al.*, 1998). Gibberellin application was demonstrated not to complement the phenotype (Goto *et al.*, 1987). Okada *et al.* (1991) demonstrated that polar auxin transport is much reduced in the *pin1* mutant with respect to wild-type, and that the mutant also has dramatically reduced amounts of free IAA. The reduction of polar auxin transport in the mutant was concluded to be the cause of the inflorescence mutation as

exogenous application of polar auxin transport inhibitors also distorted the formation and development of floral meristems, generating wild-type plants with phenotypes resembling those of the *pin1* mutants. Sections taken from pin1 inflorescences in the vicinity of auxin-synthesising cauline leaves revealed extensive xylogenesis, consistent with an accumulation of auxin proximal to these organs due to impaired basipetal transport of the hormone (Gälweiler *et al.*, 1998). Subsequent cloning of *PIN1* and localization of the protein led to the proposal that PIN1 may be a catalytic auxin efflux carrier protein active in basipetal auxin transport (Gälweiler *et al.*, 1998).

Gibberellic acid mutants often display dwarf phenotypes, and GA induces stem growth in many rosette plants and dwarf mutants. The growth is a combined effect of enhanced cell division activity in the meristem and increased cell elongation (Kende & Zeevaart, 1997).

Relatively few cytokinin mutants have been isolated to date. One *Arabidopsis* mutant, *amp1*, has elevated cytokinin levels and has accelerated leaf and inflorescence development, and short roots. Mutants often produce more than two cotyledons, suggesting that the mutation acts during embryogenesis as well as in post-embryonic development. Dark-grown *amp1* seedlings do not form an apical hook, have a shortened hypocotyl relative to wild-types and form true leaves (Chaudhury *et al.*, 1993). Increased cytokinin production was subsequently found to induce the expression of the root meristem homeobox genes *KNAT1* and *STM*, providing a role for cytokinin in the shoot apical meristem (Rupp *et al.*, 1999). The inhibition of root and hypocotyl elongation by elevated cytokinin might not be a direct effect but rather may be mediated through cytokinin-induced ethylene production as exogenously applied ethylene inhibitors partially block the effects of cytokinin. Support for this idea comes from the cytokinin *ckr1* mutant, which is also resistant to ethylene (Carey et al., 1995). Ethylene also induces root hair development and so is implicated in the specification of cell differentiation (Tanimoto *et al.*, 1995).

1.1.4   Cell cycle

Plant growth is achieved through cell cycle activity at the meristems which gives rise to all the tissues and structures of the adult plant. Control of the cell cycle, specifically which cells (where and how many) divide, the direction in which they divide and the

regulation of their expansion is considered to be responsible for correct organogenesis (Meyerowitz 1996).

The eukaryotic cell cycle involves the replication of the chromosomes and the division of the mother cell into two daughter cells. The mitotic cell cycle is characterised by four phases, G1, S, G2 and M. Immediately following a cell division is the first of two growth phases (termed G1 and G2 respectively) during which the cell increases in size and protein content. It is at this stage that the cell commonly rests and the exit from G1 represents a major regulatory step in the cycle. Should conditions be favourable and the cell enter a new round of division, the next phase, S phase, is the period during which DNA is synthesised and the chromosomes are duplicated. Following S phase there is a second growth phase (G2) when the cell increases in size and accumulates ATP and other high-energy phosphates. The exit from G2 is a second point at which regulation occurs. The final phase in the cycle is mitosis (M phase) when the cell divides, forming two new cells (Alberts *et al.*, 1994). The cell cycle is highly conserved among eukaryotes with a common class of heterodimeric serine/threonine protein kinases (histone H1 kinases) driving its progression. The catalytic cyclin dependent kinase (CDK) subunit is activated by association with a cyclin subunit at certain points of the cell cycle (Mironov *et al.* 1999).

Due to the sessile nature of plants, their development is a highly plastic process, strongly influenced by environmental cues. Mechanisms must exist by which plants can control meristematic cell cycle activity in order to mediate developmental changes. For example, elevated CO_2 levels lead to increased rates of shoot meristem cell proliferation in *Dactylis*. This is achieved by a shortening of the G1 phase and an increase in the number of actively dividing cells (Kinsman *et al.* 1996, 1997). The nature of the mechanisms involved remain largely unknown, but there is evidence for hormonal control of the cell cycle (den Boer & Murray 2000b, Burssens *et al.* 1998, Frank & Schmülling 1999, Huntley & Murray 1999).

It has long been known that both auxin and cytokinin supplements are required for cell proliferation in culture, but only recently has any molecular basis for their action in the cell cycle been elucidated. Using excised pith parenchyma from *Nicotina*

tobacum Zhang *et al.* (1996) demonstrated that auxin alone induced expression of a $p34^{cdc2}$-like histone kinase but that cytokinin was required for its activity. Cytokinin-starved cells lacking $p34^{cdc2}$-like histone kinase activity arrest in the G2 phase of the cell cycle, whereas auxin is required at both G1 and G2. $p34^{cdc2}$ is a key catalyst in the cell cycle and is regulated in various ways. At the G1 checkpoint the availability of cyclin subunits, as well as the decline of cyclin dependent kinase inhibitor (CKI) proteins controls $p34^{cdc2}$ activation whereas at the G2 checkpoint mitotic cyclins are usually in excess and the $p34^{cdc2}$/cyclin B complex is activated by the removal of an inhibitory phosphate. Cytokinin is supposed to cause this dephosphorylation via the phosphoprotein phosphatase Cdc 25, thus allowing the cell to enter mitosis (Zhang *et al.* 1996).

A further requirement for cytokinin in the *Arabidopsis* cell cycle has been reported to occur at the G1-S transition (Riou-Khamlichi *et al.* 1999). This requirement is mediated through cytokinin-induced expression of cyclin D3 (cycD3) and cell cultures overexpressing cycD3 do not require cytokinin to proliferate. Different regulatory mechanisms have been hypothesised to be active in different cell types and it is possible that the apparent discrepancy in cytokinin requirement reported is due to the use of cell cultures derived from distinct cell types. The mechanism by which cytokinin induces cycD3 is unknown but is independent of protein phosphorylation and so may involve a phosphorelay (Soni *et al.* 1995, Riou-Khamlichi *et al.* 1999). Interestingly, cycD3 transcription is also up-regulated by brassinosteroids, and cytokinin can be substituted by epi-brassinolide in cell cultures (Hu *et al.* 2000). Brassinolide also induces expression of Cdc2b (the second of two CDKs identified in *Arabidopsis*) in dark conditions, but not in the light (Yoshizumi *et al.* 1999). Additional hormonal control of the cell cycle is exemplified by the tissue-specific accumulation of Cdks and cdc2-activating kinase (CAK) in response to gibberellins in rice (Sauter *et al.* 1995, 1997) and the induction by ABS of a protein kinase inhibitor (ICK1) demonstrated to interact with cdc2 in *Arabidopsis* and inhibit CDK-like kinase activity (Wang *et al.* 1997, 1998).

Evidence is accumulating that regulation of the cell cycle represents a target for the control of plant development. As cell cycle progression is driven by the activation of CDK catalytic subunits by regulatory cyclin subunits, the transcription and proteolysis

of these proteins are good candidates for manipulation. Few conclusive experiments have been conducted to date, but a cyclin has been suggested to be a limiting factor in *Arabidopsis* root development as ectopic expression of cyc1AT under the control of the constitutively expressed cdc2aAt promoter caused an acceleration of root growth (Doerner *et al.* 1996). Another cyclin, (cycD3), caused extensive leaf curling, disorganised meristems, increased leaf number, late flowering and delayed senescence when overexpressed in *Arabidopsis* (Riou-Khamlichi *et al.* 1999). Cdc2aAt plays a key role in the cell cycle and its expression is considered to be critical in the regulation of meristem activity (Martinez *et al.* 1992). Cell proliferation, orientation of division and cell size control were compromised in tobacco plants with reduced cdc2aAt activity, although overexpression did not result in an increase in cdc2 activity and no aberrant phenotype was observed in these plants (Hemerly *et al.* 1995). Furthermore, *Arabidopsis* cdc2a mutants produce distorted embryos with seedlings defective in leaf growth and phyllotaxis suggesting that cell divisions are critical in embryogenesis and seedling morphogenesis (Hemerly *et al.* 2000). Overexpression of the CDK inhibitor ICK1 in *Arabidopsis* results in both a reduction in CDK activity and significant inhibition of plant growth, with both size and morphology being affected (Wang *et al.* 2000). Antisense plants with reduced levels of cdc2b expression exhibit a short-hypocotyl and open-cotyledon phenotype when grown in the dark. This reduction in hypocotyl length is due to a reduction in the size and not the number of the cells, and cell division in the SAM is also unaffected (Yoshizumi *et al.* 1999). Overexpression of the mitotic inducer cdc25 from fission yeast in tobacco produced plants with aberrant leaf morphology and premature flowering (Bell *et al.* 1993) and also altered frequency of lateral root formation (McKibbin *et al.* 1998). Cdc25 is suggested to mediate its response through activation of cdc2 and so a double mutant overexpressing both cdc25 and cdc2 might be expected to generate an extreme phenotype.

1.2 *Arabidopsis thaliana* as a model organism

Arabidopsis thaliana is a small weedy member of the Brassicacea. It has been adopted as the model genetic plant species and is now widely used as a study organism. There are many advantages to working with *Arabidopsis* and these can be grouped into three main areas.

1.2.1 Development

Arabidopsis plants consist of a rosette of small leaves and, following bolting, a raceme inflorescence with the oldest flowers at the bottom and successively younger flowers up the stem to the indeterminate floral meristem at the top. The flowers consist of four sepals, four white petals, six stamens (2 short, 4 long) and a bicarpellate ovary. Development in *Arabidopsis* has been well characterised from the embryo onwards, and demonstrated to be simple and highly uniform (Scheres *et al.*, 1994, Berger *et al.*, 1998, Scheres & Wolkenfold, 1998).

Owing to its small size, many *Arabidopsis* plants may be grown in a limited space and they may also be grown in aseptic conditions on agar-based medium of defined composition in a Petri dish. This is an important characteristic as it allows large-scale mutant screening studies in closely controlled conditions. Additional advantages to growing plants on agar include the ability to study root development, an area which until recently has been largely ignored due to the difficulty of studying an underground organ, and the growth of seedling mutants which may not survive in the relatively harsh environment of the greenhouse. Plants reach a height of approximately 30-40cm and when grown under long day conditions reach maturity in approximately six weeks. Short day conditions result in a delayed vegetative stage with the rosette accumulating more leaves prior to bolting. This relatively short life cycle facilitates experiments requiring successive generations such as mutagenesis, transformation and genetic crosses. *Arabidopsis* flowers self-pollinate naturally but may be cross-pollinated by artificial manipulation making them ideal for genetic studies. Each plant has a high seed set, depending on environmental conditions a single plant may produce thousands of seeds. Seeds are very small, less than a millimetre in diameter, allowing storage of thousands of seeds in a 1.5ml microcentrifuge tube. Seeds remain viable for many years following maturation, provided they remain desiccated. Germination generally occurs within 48 hours of imbibition and can be made uniform by a 4-7 day cold treatment at $4^{o}C$ (Meyerowitz & Somerville, 1994).

1.2.2 Genetics

Arabidopsis has a very small genome size in relation to many plant species (the haploid nuclear genome is approximately 70,000 kilobase pairs, Meyerowitz 1989) spread over five pairs of chromosomes and contains very little interspersed repetitive DNA (Pruitt & Meyerowitz 1986).

Arabidopsis is amenable to both mutagenesis and transformation by *Agrobacterium*-mediated T-DNA insertion. Large-scale mutagenesis programmes are possible by exposing seed to either chemical mutagens such as ethyl methanesulfonate (EMS) or X-rays, and various transformation methods are available, including root (Lindsey & Wei, 1996), and flower (Clough & Bent, 1998).

1.2.3 Model Plant Status

The third reason that working with *Arabidopsis* is now so attractive is that there is a huge volume of knowledge and resources available to aid research. Not only is every stage of *Arabidopsis* development well characterised and the entire genome sequence released (The *Arabidopsis* Genome Initiative, 2000), but there are also a wealth of mutants, knockouts, and computer databases available (see Somerville & Somerville, 1996).

1.3 Genetic Manipulation

1.3.1 Conventional Breeding

Genetic manipulation of plants by humans has occurred for thousands of years through selective breeding. By repeatedly sowing and selecting the seeds of plants with desirable traits, over generations great improvements were achieved. In many cases carbon partitioning has been altered in crop plants so that the desirable organ (be it fruit, root, stem or leaves) acts as a greater sink for resources than it did in the wild population. Other traits have also been selected, for example modern cereals do not shed their seeds and so are easier to harvest, and members of the Solanaceae have lost their toxicity.

Although conventional breeding has brought about great improvements, it is an extremely arduous process and has a number of disadvantages:

• Conventional breeding is restricted to parent plants that are sexually compatible, which limits the number of traits that may be introduced. Many modern crop species are now reaching the thresholds of yield increase that may be achieved by this method.

• In selecting parents for a single desirable trait, the rest of the parent genome is also selected and so undesirable, as well as desirable, traits are passed on to successive generations. Outcrossing to remove such undesirable traits is labour intensive and time consuming.

• In-breeding of crop species to retain their desirable characteristics results in a reduced genetic variation, often with loss of hardiness or pest resistance.

1.3.2   Genetic engineering

In recent years an alternative form of genetic manipulation has become possible where a single gene responsible for a desirable or undesirable trait may be introduced or knocked out (either by mutation followed by selection, or using antisense or RNAi) respectively without the merging of two entire genomes. This technology is known as genetic engineering and offers additional advantages over traditional methods. By genetic engineering genes responsible for desirable traits may be transferred across species barriers, even in some cases to organisms of a different kingdom (Lindsey, 1992). This introduces possibilities for breeding never before available, such as the use of bacteria to generate products previously derived from animals, the most famous example of which is the production of artificial insulin, and plants too may be engineered to produce novel products such as biodegradable plastic.

Genetic engineering also represents a valuable research tool by which to study gene action. Gene function may be analysed by studying phenotypic mutants resulting from gene mutations, and gene expression and its regulation may be analysed by employing reporter genes. By furthering our understanding of plant genetics we may discover further novel ways in which plants may be manipulated.

1.3.3   Reporter genes

Reporter genes are employed in order to visualise gene expression. Not only are discrete populations of mRNAs transcribed in both temporally and spatially discrete

patterns during plant development, but plant plasticity is, in part, mediated by the activation of specific genes in response to environmental and biochemical signals. It is desirable to visualise the patterns in which genes are expressed in order to elucidate their regulation and function. Direct visualisation of specific mRNAs by *in situ* hybridisation is laborious and therefore unsuitable for many experiments. Reporter genes express either a product that may be visualised directly or an enzyme that produces a visible product in the presence of a substrate. In this way gene expression patterns may be determined, and regulation of the gene may be analysed by observing any alteration in reporter gene expression in response to different conditions. However, since reporter genes represent an indirect method for visualising gene expression, they cannot be assumed to represent the exact expression pattern of the native gene.

The majority of experiments involving reporter genes in plants have employed the *uidA* gene which encodes β-glucuronidase (GUS, Jefferson *et al.*, 1987). GUS is a bacterial enzyme which has almost no background activity in plants, and can be localised histochemically by its ability to cleave a supplied chromogenic substrate. Quantitative analysis is also possible by using substrates that can be converted to fluorescent reaction products. Low abundance transcripts and those expressed in a spatially restricted pattern, such as the *COLUMELLA* gene of *Arabidopsis* (Topping & Lindsey, 1997) may be visualised using GUS as a reporter as the detection is highly sensitive. The main disadvantage of GUS as a reporter is that assays are destructive to the plant material, allowing analysis of only a single developmental stage. This prevents GUS being used to study gene expression over time in a single plant and means it cannot be used to screen a primary population of transformants. In recent years other reporter systems have been developed.

The firefly luciferase gene has been used successfully as a reporter. Upon addition of the substrate luciferine, the enzyme produces a bioluminescent product that can be detected in vivo using a non-destructive video imaging system. Unlike the GUS product, the luciferase product is unstable, allowing both increases and decreases in gene expression to be analysed in real-time. Millar *et al.* (1992) used the firefly luciferase gene as a reporter to investigate the circadian clock. Unfortunately

measurements of luciferase activity must be performed in darkness which interferes with plant physiology. Another disadvantage is that, like GUS, luciferase requires a substrate to be introduced inside the plant (Quaedvlieg *et al.*, 1998).

Another reporter gene recently developed for use in plants is the green fluorescent protein (GFP) of the jellyfish *Aequeoria victoria*. GFP is not toxic and can be visualised directly *in planta* without the addition of exogenous substrates or cofactors, although the high levels of auto fluorescence in various plant organs and calli make it a less sensitive reporter than GUS (Quaedvlieg *et al.*, 1998). The small size of GFP (238 amino acids, Chalfie *et al.*, 1994) makes it ideal for protein fusion experiments. The GFP reporter has successfully been used, for example to study virus movement during plant infection (Baulcombe *et al.*, 1995).

1.3.4   Gene Isolation by promoter trapping

In order to undertake genetic engineering it is first necessary to isolate genes and clone them. Numerous methods have been developed in order to isolate genes of intrest, and these can be divided into two broad categories: 1) isolating the genes responsible for a known mutation and 2) isolating novel genes. Methods used to isolate genes causing a known mutation that may have arisen either naturally or by chemical mutagenesis or irradiation include chromosome walking, differential screening of cDNA libraries and targeted transposon tagging. In recent years emphasis has moved towards facilitating the isolation of novel genes, particularly by insertional mutagenesis using either transposon-tagging or T-DNA-mediated insertional mutagenesis ((Topping & Lindsey, 1995).

Plants displaying a mutant phenotype are isolated, and cloning of the disrupted gene is facilitated by the presence of a section of DNA of known sequence (gene tagging). A great number of genes have been isolated using these techniques, including *EMB30* (Shevell *et al.*, 1994) and *AUX1* (Bennett *et al.*, 1996) which were cloned from *Arabidopsis* via T-DNA mediated gene tagging, and *PHANTASTICA* (Waites & Hudson, 1995) cloned from Antirrhinum via transposon tagging.

The method employed by this laboratory has been one of T-DNA-mediated promoter tagging. A population of *Arabidopsis* was transformed with a promoterless *uidA*

Fig. 1.1   pΔgusBin19 (Topping *et al.* 1991)

Plasmid used to generate the EM2 promoter trap line. Includes nptII (kanamycin resistance) gene for selection of transformants and a promoterless gusA gene (promoter trap)

SacI
EcoRI
Kpn
ATG
Sma
gusA
1.8 kb
nos3'
EcoRI
(filled in)
250 bp
Bam
Xba
HincII
Pst
Sph
HindIII

LB
E
H
lacZ3'
229 bp
lacZ5'
157 bp
nos3'
260 bp
npt-II
1 kb
nos5'
310 bp
SstII
RB

440 bp
from mp19
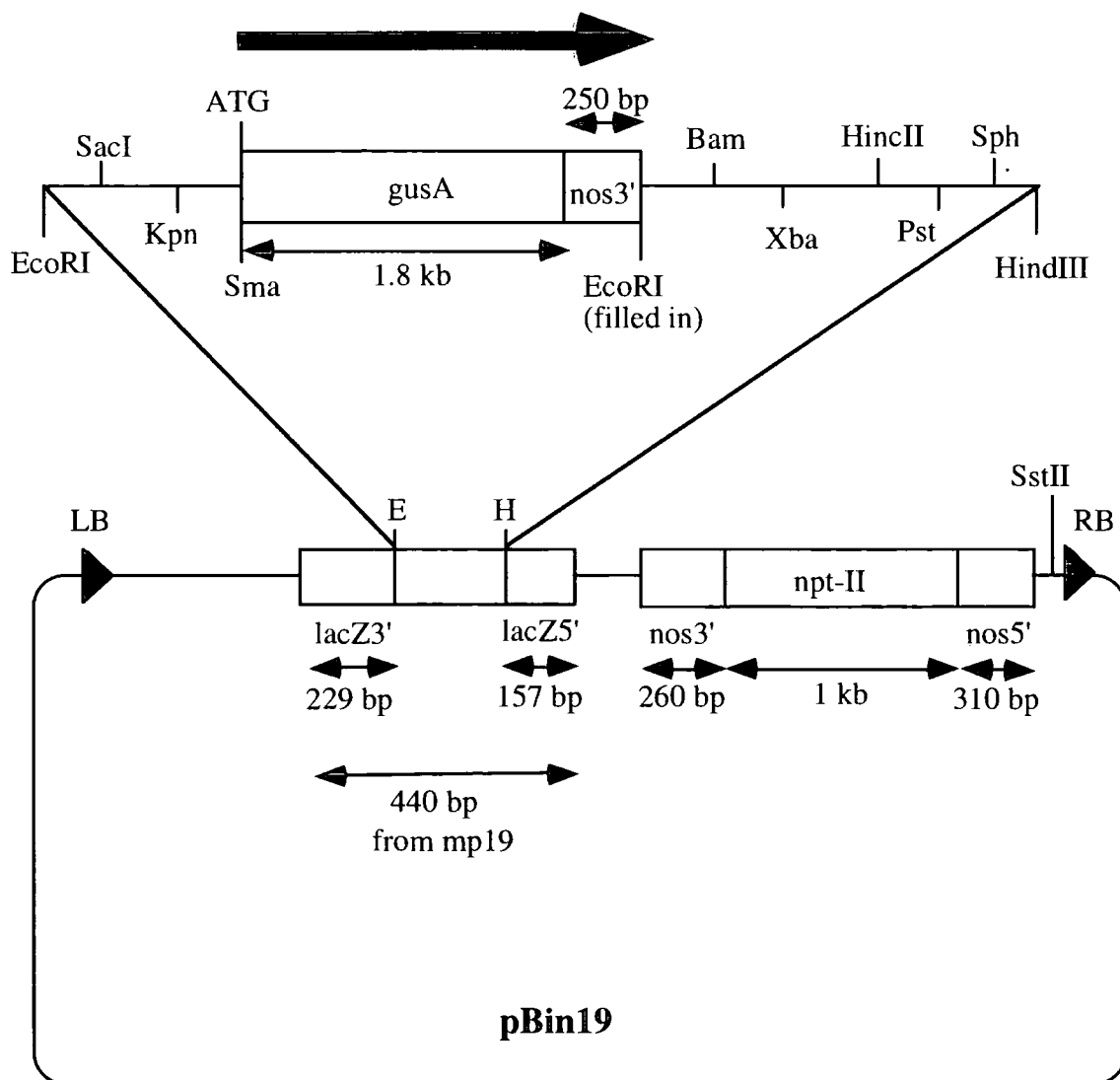
pBin19

pΔgusBin19

12 kb

(GUS reporter) gene by *Agrobacterium*-mediated gene transfer. The T-DNA vector used (pΔgusBin19, Topping *et al.*, 1991) contained an *nptII* gene under the control of a constitutive promoter that allowed selection of transformed plants by their ability to grow on kanamycin (Fig. 1.1). The transformed (kanamycin-resistant) population was then screened for GUS activity, the rationale of the technique being that GUS activity should only be detected following integration of the T-DNA downstream of a native gene promoter in such a way that it may drive GUS expression (Lindsey *et al.*, 1993). Promoter trapping has two advantages over insertional mutagenesis. Firstly promoter trapping is not dependent on the generation of an obvious mutant phenotype. This is important as there are several reasons a phenotype may not be observed: the insertion event may not disrupt gene function; the gene may be redundant; or the phenotype may only be observed under certain environmental or other conditions. The promoter trap T-DNA may however act as an insertional mutagen, and aberrant phenotypes may be screened for. Secondly, following detection of reporter gene activity, the expression of the reporter gene should reflect the pattern of the native gene expression. This has been demonstrated in *Arabidopsis* by Kertbundit *et al.* (1991). Analysis of the reporter gene expression under different conditions may suggest a function for the native gene without further cloning.

Hundreds of lines transformed with pΔgusBin19 were generated in this laboratory and screened for GUS activity. Lines showing activity were further analysed for restricted patterns of GUS expression (Lindsey *et al.*, 1993).

1.3.5   Isolation of the EM2 line

The promoter trap line EM2 was isolated from a screen for GUS activity in *Arabidopsis* embryos (Topping *et al.*, 1994). Promoter trapping is an ideal technique for the isolation of genes involved in embryogenesis. Many genes that are important in embryogenesis cause lethal mutations when knocked out, and it is difficult to isolate mRNA from the developing embryo. A tagging method that allows visualisation of gene expression is therefore highly advantageous. The majority of embryogenesis genes cloned are involved in the late stages of embryogenesis, however it is the early stages that are of most interest to the developmental biologist,

because it is early in embryogenesis that many of the critical events of pattern formation occur.

GUS activity in the EM2 line was found to be constitutive throughout the embryo from the four cell stage onwards. Activity was restricted to the embryo proper, with none detectable in the suspensor, embryo sac, testa or silique wall. GUS expression in the mature embryo was strongest in the developing radicle with weaker staining in the cotyledons. GUS activity in the seedling was found in the young cotyledons and leaves, and most strongly in the root tips (Fig. 1.2). Segregation analysis was performed to assess the number of T-DNA copies present in the line. Of 155 seeds from a single primary transformant germinated on kanamycin, 76.8% were kanamycin resistant, indicating that the line contained a single T-DNA insert. This was confirmed by Southern analysis (Topping *et al.*, 1994). IPCR generated a DNA fragment which was successfully used to screen a cDNA library, confirming that the promoter trap had tagged a transcribed sequence (Topping *et al.*, 1994). Due to the presence of GUS activity in the very early embryo, the tagged gene was named *EXORDIUM*.

1.4   Aims and Objectives

The overall aim of this work is to further our understanding of the processes that occur in early embryogenesis through the characterisation of a gene tagged by a promoter trap T-DNA.

Specific objectives of the work include: developing an understanding of the factors regulating GUS expression in the promoter trap line EM2; determining whether GUS expression in the EM2 line is representative of *EXO* expression in the wild-type line; and defining a function for the *EXO* gene.

1.5   Summary

The importance of studying the interaction between plant genes and development has been described, with reference to the use of the model plant species *Arabidopsis thaliana*. The importance of isolating novel genes and methods by which this may be achieved have been introduced, with particular reference to promoter trapping.

Subsequent chapters will describe the methods used (Chapter 2.0), and the results of genetic, phenotypic and molecular characterisation of the EM2 line and the *EXO* gene. Chapter 6.0 will discuss the results obtained and assess their biological significance.
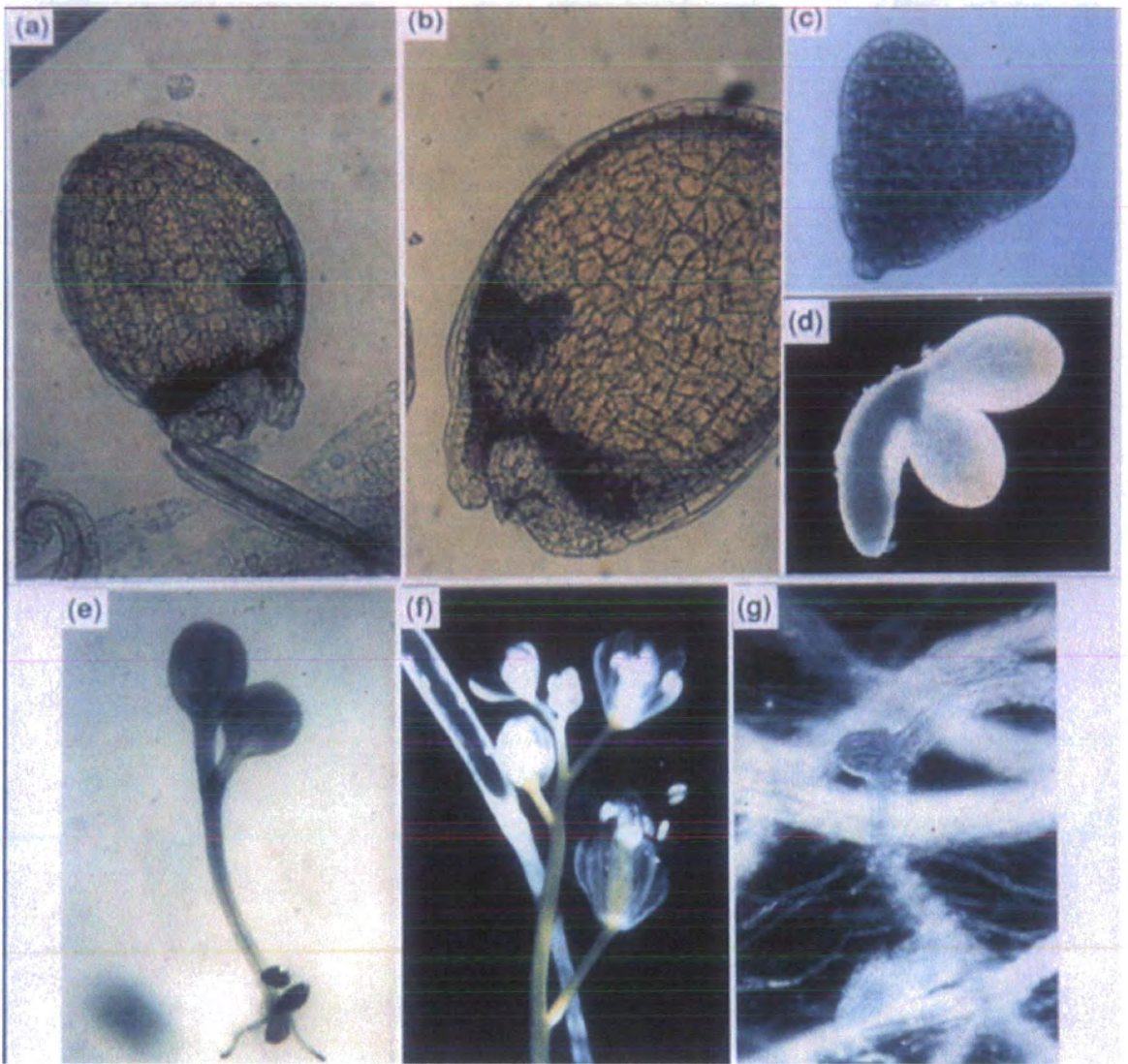
MATERIALS AND METHODS

2.0   Materials & Methods

2.1   Materials

2.1.1   Chemicals

All chemicals used were obtained from Sigma-Aldrich (Poole, UK), Fisher Scientific, (Loughborough, UK), ICN Pharmaceuticals Ltd. (Basingstoke, UK), Merck Ltd. (Poole, UK), Fisons Scientific Equipment (Loughborough, UK), Difco Laboratories (Detroit, USA) or Bio-Rad Laboratories (Hemel Hempstead, UK) unless otherwise stated.

X-Gluc was obtained from Melford Laboratories (Suffolk, UK), vancomycin-HCl from Duchefa (Haarlem, The Netherlands) and agarose from Helena Biosciences (Sunderland, UK). Intercept was obtained from Levington (Ipswich, UK) and dNTPs were obtained from Bioline (London, UK).

2.1.2   Radiochemicals

Radiolabelled nucleotides were obtained from ICN Pharmaceuticals Ltd (Basingstoke, UK). $[^{32}P]\alpha$-dCTP was supplied at a concentration of 10mCi/ml and a specific activity of 3000Ci/mmol.

2.1.3   Enzymes

Restriction endonucleases, T4 ligase and RNase-free DNase were obtained from Promega Ltd. (Southampton, UK). *Taq* polymerase was from Bioline (London, UK), Expand™ High Fidelity PCR System was from Boehringer Mannheim (Indianapolis, USA) and Shrimp Alkaline Phosphatase was from Sigma-Aldrich (Poole, UK).

2.1.4   Oligodeoxynucleotides

Oligodeoxynucleotide primers used in PCR reactions were obtained from MWG-Biotech (Ebersberg, Germany).

Primers used were as follows:

Promoter start:5'- **GCT CTA GAG** CGC GTG ATA ATC ACG TGA C -3'

tp stop: 5'- **CGG GAT CCC** GCT TGA TGA AAC AGA AAG AG -3'

fp stop:	5'- **CGG GAT CCC** GGT GAA ATA GAA GGT TGT G -3'
EXOf:	5'- **GCT CTA GAG** CCC CAT CAC ACT CTC -3'
EXOr:	5'- **GTC CTA GAG** CGA CCA AAC ATT GGA C -3'
sORFf:	5'- GTT GTC GGA CTC GAA ACT GAG -3'
sORFr:	5'- GAT GTA TGG AGT GTG ATG GGG C -3'

Bases shown in bold are recognition sequences for restriction endonucleotides to facilitate further cloning. Promoter start, EXOf and EXOr have an Xba1 site, and tp stop and fp stop have a BamH1 site.

2.1.5 Kits

The Total RNA Isolation kit was from Advanced Biotechnologies (Epsom, UK), Prime-It II Random Primer Labelling kit was from Stratagene Ltd. (La Jolla, California). The Nucleon® Phytopure Plant DNA Extraction kit (Nucleon Biosciences, Lanarkshire, UK), the Wizard™ Plasmid Purification kit from Promega (Southampton, UK). The Geneclean kit was from Bio 101 Inc. (La Jolla, California), the High Pure PCR Cleanup kit from Boehringer Mannheim (Indianapolis, USA), the TOPO-TA Cloning® kit and the Plasmid Mdi kit used for isolating plasmids from *Agrobacterium* were from Qiagen Ltd. (Surrey, UK).

2.1.6 Bacterial Strains

E.coli strain XL1-blue MRF' (Stratagene, La Jolla, California) was used to prepare electrocompetent cells and as a plasmid host. *Agrobacterium tumefaciens* strain C58 (Dale *et al.*, 1989) was used for plant transformations. The strain has been modified so as not to cause crown gall disease whilst retaining the virulence factors required for T-DNA insertion into plant genomic DNA. C58 was selected for with 25mg/l nalidixic acid.

2.1.7 Plasmids

pCR® 2.1 TOPO (Invitrogen, Groningen, The Netherlands) was used to clone DNA fragments generated by PCR. pDH51 (Fig. 2.1, Pietrzak *et al.*, 1986), pGUS-1 (Fig. 2.2, kindly donated by Dr. J. Topping, University of Durham) and pCIRCE (Fig. 2.3, Bevan 1994, 1998), were used to create novel plasmid constructs for plant transformation. pDH51 contains the CaMV 35S promoter and terminator and was

Fig. 2.1   pDH51 (Pietrzak *et al.* 1986)

Plasmid containing 35S constitutive promoter used for sense and antisense constructs

EcoR1
Nco1

35S Promoter

EcoRV

Kpn1
Sma1
BamH1
Xba1
Sal1
Pst1
Sph1

ori

CaMV polyA

Kpn1
EcoR1
Hind111

pDH51
3.42 Kb

AMP
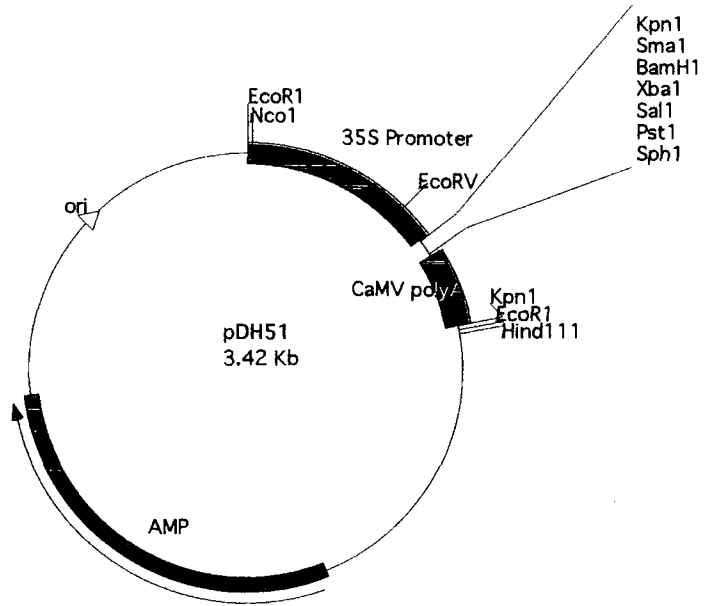
Fig. 2.2   pGUS-1 (Topping *et al.* 1991)

Plasmid containing the gus gene used for promoter::gus constructs

HindIII.SphI.PstI.SalI.Xba1.BamHI.Sma1

pGUS
5700 bp

GUS

Ap

NosTer

Sst1 2341

EcoR1 2600

BgII
LB
XhoI
PstI
Nos prom.
NPT II
SphI
ClaI
pCIRCE
11.00 Kb
Noster
LacZ
NPT II
RB
BgII
ColEI ori
oriV

HindIII
SphI
PstI
SalI
HincII
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
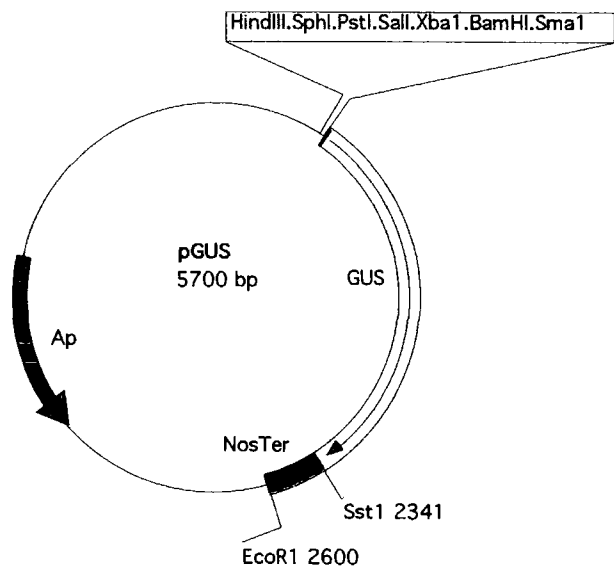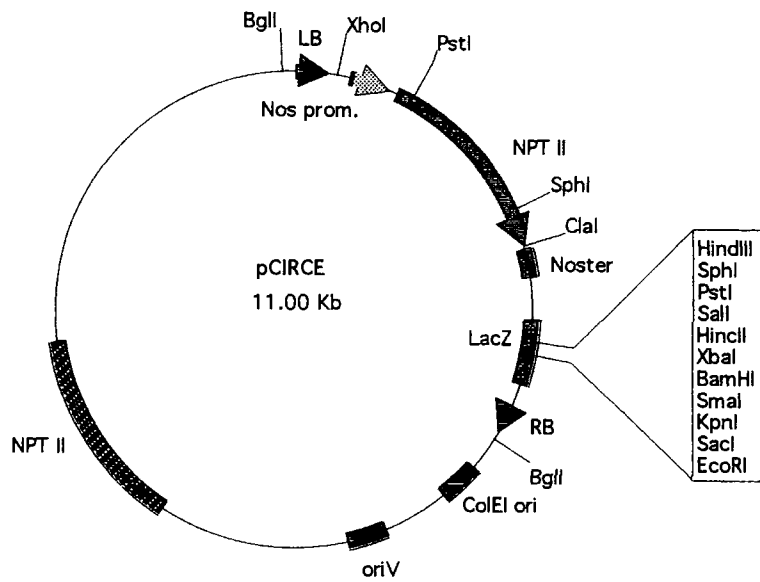
Plasmid name: pCIRCE
Plasmid size: 11.00 kb
Constructed by:
Construction date:
Comments/References: pCIRCE is a derivative of pBIN19 in which the T-DNA   NPT
II gene has been corrected. Part of the M13/lacZ region duplicated in pBIN19 has   been
deleted and the NPT II gene moved next to the LB unstead of the RB as in pBIN19

used to create sense and antisense constructs. pGUS-1 was used to create promoter-GUS constructs. pCIRCE was used as a wide host range binary cloning vector for *Agrobacterium*-mediated plant transformation. PRK2013 was used to provide the mobilisation function during triparental mating.

2.1.8 Bacterial culture media

Luria-Bertani (LB) medium: 10g/l Bacto-tryptone, 5g/l Bacto-yeast extract, 5g/l NaCl, autoclaved for 20 minutes at 121°C.

LB agar: As for LB medium with 15g/l Bacto-agar added prior to autoclaving.

SOC medium: 20g/l Bacto-tryptone, 5g/l Bacto-yeast extract, 5.84g/l NaCl, pH6.8-7.0, autoclaved. 10mM $MgCl_2$, 10mM $MgSO_4$, 2g/l glucose filter sterilised through 0.2 μm acrodiscs (Gelman) and added after autoclaving.

2.1.9 Plant material

Arabidopsis thaliana var. C24 and Columbia and the transgenic line EM2 were supplied by Prof. K. Lindsey (University of Durham).

Arabidopsis mutant lines used were: *Aux1-7* (Columbia, Maher & Martindale, 1980), *Axr1-12* (Columbia, Estelle & Sommerville, 1987), *pin-1* (Enkheim, Okada *et al.*, 1991), *rooty* (Columbia, King *et al.* 1995), *amp-1* (Col-0, Chaudhury *et al.* 1993). These were obtained from the Nottingham *Arabidopsis* Stock Centre.

2.1.10 Plant culture media

$^1/_2$MS10: 2.2g/l Murashige and Skoog medium (Sigma #M5519), 10g/l sucrose adjusted to pH7.4 with 1M KOH, 8g/l agar added and autoclaved at 121°C for 15 minutes.

2.1.11 Hormones

Hormone stocks were filter sterilized using 0.2μm acrodiscs (Gelman) and added to molten $^1/_2$MS10 (cooled to approximately 60°C) and mixed thoroughly prior to pouring.

2.2 Bacterial Growth Conditions

E. coli was grown in LB media (with shaking) or on LB agar plates at 37°C.

A.tumefaciens was grown in LB or SOC media (with shaking) or on LB agar at 30°C.

Long term storage of bacteria was achieved using glycerol stocks: 0.3ml 50% glycerol mixed with 0.7ml of an overnight liquid culture frozen at -80°C.

2.3 Plant Growth Conditions

2.3.1 Germination and seedling growth

Plants grown to maturity for collection of seed and crossing were grown in 4:1 Levingtons Multipurpose compost: silver sand (for enhanced drainage) under long day conditions (16 hours light; 8 hours dark) at 22°C with regular watering as necessary. Soil was treated with a systemic insecticide called Intercept (Levington, Ipswich, UK). Seed collection was facilitated by the use of Aracon tubes (Beta Tech, Belgium).

Prior to germination, all seeds were cold-treated at 4°C in the dark for 4-7 days to increase germination frequency and ensure relative uniformity of germination timing.

Seedlings for physiological studies and RNA extraction were grown on $^1/_2$MS10 medium in sterile Petri dishes at 22°C under continuous light conditions.

For studies of root length, seedlings were grown on $^1/_2$MS10 in a square Petri dish, supported at an angle of approximately 70° from horizontal.

2.3.2 Seed Sterilisation

Seeds for growth on $^1/_2$MS10 were surface-sterilised to remove any bacteria, yeast or fungi that would otherwise thrive on the nutrient-rich medium.

Seeds were immersed in 70% (v/v) ethanol for 30 seconds to partially de-wax the seeds. The ethanol was then removed and replaced with 20% bleach for 20 minutes, after which time the seeds were rinsed thoroughly in sdH$_2$O to remove any residual bleach which may inhibit germination.

2.3.3 Crossing

Since *Arabidopsis* flowers are self-pollinating, buds from the female (acceptor) plant must be selected which are as close to maturity as possible but in which the anthers have not yet dehisced and no pollen has been released.

Fine watchmaker's forceps were used to manipulate floral buds under a stereomicroscope and remove immature anthers from the acceptor flower. Mature pollen was then physically transferred from a male (donor) parent to the isolated stigma.

Lines in which the homozygous mutant has impaired or complete loss of fertility may only be crossed in the heterozygous state. As the heterozygotes are often indistinguishable from the wild type, it is desirable to screen for a 1:3 ratio of mutant embryos in mature siliques prior to selecting a plant for crossing. Seeds were removed from the silique using a sharp pointed needle, suspended in 0.1M KOH and crushed under a coverslip to release the embryos. Mutant embryos were identified using a stereomicroscope.

Not all mutations are apparent in the embryo. Where it is not possible to identify heterozygotes in this manner a larger number of crosses must be performed as only 2/3 of plants will carry the mutation.

2.4 Determination of GUS activity

2.4.1 Histochemical localisation of GUS activity

(After Jefferson *et al.*, 1987).

GUS activity can be visualised by incubating samples in solution containing the substrate 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (x-gluc). The effect of GUS activity on this substrate is to cause oxidative dimerisation to form an insoluble blue dye. The addition of potassium ferricyanide and ferrocyanide (oxidation catalysts) to the solution acts to reduce the diffusion of a soluble reaction intermediate into surrounding tissues (Mascarenhas & Hamilton 1992).

X-Gluc (dissolved in a small volume of NN-dimethyl formamide) was diluted to a final concentration of 1mM in histochemical buffer (100mM phosphate buffer, pH 7.0, 10mM EDTA, 0.1% v/v Triton X-100, 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide).

Samples were incubated in this solution at 37°C for a suitable length of time (30 minutes-24 hours), and then photographed directly after having been cleared of chlorophyll by incubation in 70% v/v ethanol.

2.4.2 Quantification of GUS activity by Fluorimetric assay

GUS activity can be quantitatively measured by fluorimetric assay for 4-methylumbelliferone (MU), which is the product formed from the cleavage of the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) (Jefferson et al. 1987).

Tissue was ground with white quartz sand (Sigma) in 200μl GUS fluorimetric buffer (50mM $NaH_2PO_4.H_2O$, 10mM β-mercaptoethanol, 0.1% (v/v) Triton X-100, 1mM EDTA, pH 7.0) in a microfuge tube. The homogenate was vortexed and then centrifuged for 10 minutes at 12,000 rpm in a microfuge at room temperature.

20μl of the supernatant was transferred to a microtitre plate well containing 140μl GUS fluorimetric buffer and equilibrated for 5 min at 37°C. Reactions were carried out in a Fluoroskan Ascent fluorimeter (Labsystems) at 37°C. MUG (Sigma), dissolved in GUS buffer, was added as a substrate to a final concentration of 1mM and the fluorescence measured at an excitation λ of 365 and an emission λ of 455nm at time zero and at every 20 minutes for 3 hours.

GUS activities were expressed with respect to the protein content of the samples in order to normalise the data. Protein concentrations were determined using the Bradford reagent (BioRad) according to manufacturer's instructions. 10μl of the crude extract supernatant was mixed with 2ml diluted Bradford reagent and the absorbance at 595nm was measured. A standard curve was produced using known amounts of Bovine Serum Albumin (Promega). GUS enzyme specific activities were expressed as pmol MU produced per mg protein per minute of reaction.

2.5   Microscopy and photography

Low magnification observations and photography were performed using an Olympus SZH10 stereo microscope. Photographs were taken on tungsten-balanced ISO-160 film (Kodak) using an Olympus SC35 35mm camera. Crosses were performed under a Zeiss STEMI SV8 stereo microscope.

2.6   RNA isolation

Total RNA was extracted from plant tissue using the Total RNA Isolation Reagent kit (Advanced Biotechnologies, Epsom, UK). This is a rapid, simple method based on a solution of guanadine salts and urea which act as denaturing agents. The method is reliable and produces consistent results. The sample is homogenised with the reagent and separated into two phases by extraction with chloroform. The total RNA remains exclusively in the aqueous phase while DNA and proteins are extracted into an organic phase and interphase. The total RNA is precipitated by addition of isopropanol, washed in ethanol and dissolved in DEPC treated water (2ml DEPC in 1l sdH$_2$O, mixed vigorously, stirred overnight and autoclaved prior to use).

1. Homogenisation

Approximately 1g (fresh weight) of tissue was frozen in liquid nitrogen and ground to a fine powder in a pestle and mortar. This powder was added to 10ml RNA Isolation Reagent in a sterile 50ml tube and homogenised using a polytron.

2. RNA Extraction

The homogenate was incubated for 5 minutes at 4°C to permit complete dissociation of nucleo-protein complexes. 2 ml was then added and the samples shaken vigorously for 15 seconds before being stored on ice for 5 minutes. Following centrifugation at 12,000xg for 15 min at 4°C the homogenate formed two phases. The upper, aqueous phase (40-50% total volume) containing the RNA was removed to a fresh 50ml tube, with care taken not to disturb the interphase.

3. RNA Precipitation

An equal volume of isopropanol was added and the samples stored for 10 minutes at 4°C. Samples were then incubated at 12,000xg for 10 minutes at 4°C to form a white pellet of RNA at the bottom of the tube.

4. RNA Wash

The supernatant was removed and the pellet was washed twice with 10ml 75% ethanol by vortexing and subsequent centrifugation for 5 minutes at 4°C. Finally the pellet was briefly dried under a vacuum for 5-10 minutes to remove as much ethanol as possible and resuspended in 50-100µl DEPC-treated water by vortexing for 1 minute. RNA samples were stored at -80°C.

5. Quantification

RNA concentration was determined spectrophotometrically at 260nm and quantified using the following equation:

$$(\text{dilution factor} \times 40 \times \text{abs260})/1000 = \mu g/\mu l \text{ RNA}$$

2.7 Northern blotting

2.7.1 RNA formaldehyde gel electrophoresis

Formaldehyde gels were prepared by dissolving 1.5g agarose in 123ml H_2O by heating in a microwave oven and allowing to cool to approximately 70°C before adding 12ml formaldehyde and 15ml 10x MOPS (10mMEDTA, 0.5M MOPS, pH7.0, DEPC treated). The gel was poured in a fume hood and allowed to cool for approximately 1 hour. The gel was extremely fragile, and care was taken during handling to avoid damage.

RNA samples were mixed with an equal volume of freshly made up running buffer (200µl 5x MOPS, 120µl formaldehyde, 100µl deionised formamide) and heated to 70°C for 10 minutes in a microfuge tube with holes punched in the lid to allow evaporation of solutes. Samples were cooled on ice and 0.1 volume of loading dye added. To a single, extra, sample, and to 5µl RNA markers (Promega), 1µl (5ml/ml) ethidium bromide was added.

The gel was pre-run at 80-100 volts in 1x MOPS running buffer for approximately 30 minutes prior to addition of RNA samples.

After electrophoresis the gel was quickly photographed with a ruler and rinsed 2 or 3 times in sdH$_2$O to remove formaldehyde.

2.7.2   RNA transfer

The northern blotting apparatus was assembled in a reservoir of transfer buffer (10x SSC: 1.5M NaCl, 0.15M sodium citrate) by placing the gel onto filter paper wicks supported on a platform above the buffer. The transfer of the RNA from the gel to the membrane is by capillary flow of transfer buffer through the gel and membrane and into a stack of absorbent pads. To ensure capillary action only through the gel, its edges were surrounded by clingfilm. Nylon membrane (Zeta-Probe GT, Bio-Rad) cut to the size of the gel and pre-soaked in distilled water was laid on the gel surface and any bubbles carefully removed. Three layers of filter paper, pre-wetted with transfer buffer, were laid on top of the membrane, followed by a stack of nappy booster pads (Boots plc, Nottingham, UK) and the whole stack was weighed down slightly from above to ensure contact without compressing the gel. Transfer occurred over 16-20 hours during which time the reservoir of buffer was replenished as necessary.

Following transfer all nappy pads and filter paper were removed, the position of the wells marked carefully on the membrane with a pencil along with any additional information required for identification and orientation. The membrane was rinsed in 2x SSC (0.3M NaCl, 30mM sodium citrate), blotted dry between filter paper, and allowed to air dry before being baked at 80°C for 30 minutes between two layers of filter paper to fix the RNA to the membrane. The gel was checked under UV prior to discarding to confirm complete transfer of RNA had occurred.

Membranes were stored flat between two layers of filter paper sealed in a plastic bag.

2.7.3   Preparation of radiolabelled probes by oligolabelling

High specific activity probes can be produced by the use of random sequence oligonucleotides as primers in the labelling of DNA. Random hexanucleotides anneal

at multiple sites along the length of a DNA template, the resulting primer-template complexes being a substrate for the Klenow fragment of *Escherichia coli* DNA polymerase 1. New DNA strands are synthesised by the enzyme by the incorporation of nucleotide monophosphates at the free 3'-OH group of the primer. By substituting a ^{32}P-radiolabelled nucleotide, dCTP, for the non-radioactive version in the reaction mixture the DNA probe synthesised is radiolabelled along its entire length, producing probes with high specific activities (10^8-10^9 cpm/μg).

The labelling was carried out using a Prime-It®II Random Primer Labelling Kit (Stratagene, La Jolla, California) according to the manufacturer's instructions. 25ng of the DNA template to be labelled, 10μl random oligonucleotide primers and high quality distilled water to a final volume of 34μl were added to a microfuge tube and boiled for 5 minutes to denature the DNA, before being transferred to ice to cool and then briefly centrifuged to collect any fluid that had condensed in the lid. To this mixture was added 10μl 5x Buffer, 5μl α-^{32}P-dCTP and 1μl EXO(-)Klenow (5U/μl in buffered glycerol solution). The final mixture was carefully mixed and briefly centrifuged before being incubated at 37°C for 1 hour after which time 2μl Stop Mix (0.5M EDTA pH 8.0) was added (EDTA chelates magnesium ions which are an essential co-factor for the enzyme).

Probes were purified using NuncTrap Push® Columns (Stratagene, La Jolla, California) that separate unincorporated nucleotides from radiolabelled DNA probes by the passage of the probe through a resin column to which the unincorporated nucleotides are bound.

Purified probes were stored in lead pots at -20°C if not used immediately. Before use, the probe was denatured by boiling for 5 minutes and snap-cooled on ice.

2.7.4 Hybridisation of a radiolabelled DNA probe with immobilised nucleic acid

Hybridisation was carried out in Techne (Cambridge, UK) bottles in a Techne hybridisation oven. In order to prevent non-specific binding of the DNA probe to the

nylon membrane it is necessary to include in the prehybridisation and hybridisation buffers a blocking agent, in this case, SDS.

Solutions used:

50x Denhardt's solution: 10g/l ficoll, 10g/l polyvinylpyrrolidene, 10g/l BSA, pentax fraction V, autoclaved

20x SSC: 175.3g/l NaCl, 88.2g/l sodium citrate, pH 7.0, autoclaved

20x SSPE: 174g/l NaCl, 27.6g/l $Na_2PO_4.H_2O$, 7.4g/l EDTA, pH 7.4, autoclaved

The nylon membrane, with bound nucleic acids, was placed in a Techne bottle with 25ml prehybridisation solution (12.5ml deionised formamide, 2.5ml 50x Denhardts, 0.25ml 10% SDS, 0.25ml 10mg/ml denatured herring sperm DNA, 0.25ml 10 mg/ml poly(A), 6.25ml 20x SSPE, 3.0ml sdH_2O) and incubated at 42°C for 4-24 hours. The prehybridisation solution was then drained and replaced with 9.5ml hybridisation solution (5ml deionised formamide, 0.4ml 50x Denhardt's solution, 0.1ml 10% SDS, 2.5ml 20x SSPE, 0.1ml poly(A), 0.2ml denatured herring sperm DNA, 1.2ml sdH_2O) and 50µl denatured probe and incubated at 42°C for 26-48 hours.

Following hybridisation the membrane was washed to remove any unbound probe. The hybridisation solution was removed (hybridisation solution may be stored and reused) and replaced by 50ml wash solution 1 (2x SSC, 0.1% SDS) and incubated at room temperature for 10 minutes. This was then repeated once with wash solution 1 and twice with wash solution 2 (1x SSC, 0.1% SDS). The membrane was washed a further four times for ten minutes each with wash solution 3 (0.1x SSC, 0.1% SDS) twice at 42°C and twice at 55°C.

Following the final wash the membrane was blotted between filter paper and sealed in Saran Wrap (Dow Chemical Company)

2.7.5 Detection and visualisation

The detection of sites of hybridisation of radiolabelled probe to RNA on the membrane was by autoradiography. The membrane was exposed to x-ray film (HyperFilm™, Amersham, UK) in a light-tight cassette with intensifying screens at -80°C for an appropriate length of time (4 hours-4 weeks).

Films were developed using an automated developing machine (Compact X4 Automatic Developer, X-ograph Imaging Systems, Malmesbury, UK)

Following exposure the membrane was stripped in preparation for reuse. The membrane was washed twice for 20 minutes each time in a large volume of 0.1x SSC, 0.5% SDS at 95°C before blotting between filter paper and sealing damp between Saran Wrap (Dow Chemical Company).

2.8   Electrophoresis

2.8.1   DNA agarose gel electrophoresis

DNA can be separated, purified and quantified by electrophoresis through an agarose gel matrix. Migration of linear nucleic acid molecules through the gel is inversely proportional to the length of the molecule and so by loading markers of known size alongside samples it was possible to determine their sizes. Likewise by running markers of known quantities of DNA alongside samples, estimates of amount could be made. The range of size of DNA molecules separated can be altered by varying the agarose concentration; more concentrated gels retard the movement of nucleic acids and so are beneficial for analysing smaller sized molecules while less concentrated gels are used for slow moving large molecules. Unless stated otherwise all gels used were 1% agarose in TAE buffer.

The position of DNA within the gel following electrophoresis was visualised through the addition of ethidium bromide to the gel. This dye interchelates between bases of nucleic acid and the complexes produced fluoresce under UV light.

1% (w/v) agarose (Helena Biosciences, Sunderland, UK) was dissolved in TAE buffer (4.84g/l Tris-base, 1.142ml/l glacial acetic acid, 20ml/l 0.5M EDTA pH 8.0) by heating in a microwave oven. The molten agarose solution was allowed to cool to approximately 60°C, ethidium bromide was added to a final concentration of 0.5 µg/ml and the molten gel was then poured into an appropriately sized gel tray, a comb added and the gel allowed to set at room temperature.

DNA samples were mixed with 0.1 volume loading dye (0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 15% Ficoll®400, 10mMTris-HCl (pH 7.5) and 50mM EDTA (pH 8.0, Promega) to aid loading and to allow the extent of DNA migration to be assessed during electrophoresis. The gel was immersed in TAE buffer in a gel tank. DNA samples and markers (Hyperladder IV, Bioline (London, UK), unless otherwise stated) were added to the wells and subjected to a constant electrical voltage of 1.0-10.0 v/cm.

Following electrophoresis the DNA was visualised on a UV transilluminator using Molecular Analyst® software (BioRad) and digitally recorded or printed on thermal printer paper (Mitsubishi).

Gels for Southern blotting were photographed alongside a ruler to enable estimation of DNA fragment sizes following hybridisation.

2.8.2   Purification of DNA fragments from agarose gels using the Geneclean II kit

DNA fragments produced by restriction enzyme digestion or PCR were purified from TAE-agarose gels using the Geneclean II kit (Bio 101 Inc., La Jolla, California). Following electrophoresis the slice of gel containing the DNA fragment was cut from the gel and placed in a microfuge tube along with 3 volumes (w/v) of NaI stock solution and incubated at 55°C until the gel had dissolved. 10µl Glassmilk suspension was added, the solution was mixed by gentle inversion and then incubated on ice for 10 minutes with regular mixing to allow binding of the DNA to the Glassmilk. Following centrifugation at 12,000rpm in a benchtop microcentrifuge for 10 seconds the supernatant was removed and the pellet was washed three times by resuspension in 200µl NEW Wash and centrifugation at 12,000rpm for 10 seconds. Following the final wash all traces of NEW Wash were removed, the pellet was resuspended in 10µl sdH2O and heated to 55°C for 2 minutes to allow dissociation of the DNA from the Glassmilk before centrifugation for 30 seconds at 12,000rpm in a microcentrifuge. The supernatant containing the DNA was removed to a new microfuge tube, recentrifuged to remove any traces of Glassmilk and the resulting DNA solution was stored at -20°C.

2.9 Extraction of Plasmid DNA

Small scale purifications of plasmid DNA from bacteria (minipreps) were carried out using the Wizard™Plus Miniprep DNA Purification Systems kit (Promega). The method is rapid, simple and reliable and it is possible to perform multiple minipreps at the same time. The resulting plasmid DNA was suitable for DNA sequencing and all cloning purposes.

Minipreps were carried out according to the kit manufacturer's instructions. The bacteria harbouring the plasmid were grown overnight at 37°C with vigorous shaking in 5ml LB medium containing the appropriate selective antibiotic. The bacteria were pelleted by centrifugation at 10,000xg for 10 minutes, the supernatant was removed and the cells were resuspended in 300µl of Cell Resuspension solution which contains RNase A to degrade cellular RNA. The cells were transferred to a 1.5ml microfuge tube, 300µl Cell Lysis Solution was added and the contents were mixed thoroughly by repeated inversion of the tube, causing the cell suspension to clear immediately (some bacterial cells are resistant to lysis and may require incubation for 3-5 minutes for efficient lysis). 300µl of neutralisation solution was added to the lysed cells and mixed by inversion of the tube, precipitating the cell debris and genomic DNA. The lysate was centrifuged at 10,000xg in a microcentrifuge for 10 minutes to pellet the precipitate. Plasmid purification was achieved using the Wizard™ Miniprep Column. For each miniprep one 5ml syringe was inserted into the Luer-Lok® extension of each Minicolumn and the plunger removed. Into the barrel of the syringe was pipetted 1ml DNA Purification Resin and the cleared lysate supernatant from the previous step. The plunger was then returned to the syringe and the slurry slowly and carefully pushed into the minicolumn. DNA binds instantly and is thereby retained on the column as the mixture is passed through. 2ml Wash Solution was then added to the syringe barrel and pushed through the column. The minicolumn containing the plasmid DNA was removed from the syringe, transferred to a microfuge tube and centrifuged at 10,000xg in a microcentrifuge for 2 minutes to remove any residual Column Wash Solution. Finally the Minicolumn was transferred to a new microfuge tube, 50µl sdH₂O added and centrifuged at 10,000xg in a microcentrifuge for 20 seconds to elute the plasmid DNA. The Minicolumn was discarded and the DNA stored at -20°C.

2.10 PCR

2.10.1 Standard PCR

The Polymerase Chain Reaction (PCR) results in the amplification of a segment of DNA between two regions of known sequence, catalysed by the *Taq* polymerase enzyme from the bacterium *Thermus aquaticus*. Oligonucleotide primers are designed which are complementary to the regions of known sequence, and the reaction allowed to proceed through several cycles of denaturation, primer annealing and polymer extension. DNA template (which may be 10-100ng genomic DNA, a cloned fragment of DNA in a plasmid or a bacterial colony) plus primers to a final concentration of 0.2μM, *Taq* polymerase (Bioline, London, UK), the supplied 10x reaction buffer, dNTPs to a final concentration of 1mM, $MgCl_2$ to a final concentration of 1.5mM and sdH_2O were added to a 0.5ml PCR tube (the enzyme added last) and mixed thoroughly. The contents were overlaid with a drop of mineral oil to prevent evaporation of the components during thermocycling and the reactions were carried out in a Perkin-Elmer Thermal Cycling Machine.

The reaction mixture was first incubated at 94°C for $2^1/_2$ minutes to ensure complete denaturation of the template DNA, and then underwent 30 cycles of: denaturation at 94°C for 30 seconds, annealing at approximately 60°C (depending on the optimum for each primer combination) for 1 minute, and extension at either 68 or 72°C for 1-2 minutes, depending on the length of the expected product. Following the final cycle there was an additional incubation at 68 or 72°C for 10 minutes to ensure all DNA molecules were fully extended.

PCR products were sized by electrophoresis alongside known DNA standards and cloning of PCR fragments was carried out as described in section 2.13.

2.10.2 Purification of PCR products

PCR products were either run out on an agarose gel and purified using the Geneclean kit (Bio 101, La Jolla, California) as described in section 2.9.2 or purified directly using the High Pure PCR Clean-Up kit (Boehringer Mannheim). Nucleic acids bind rapidly and specifically to glass surfaces in the presence of a chaotrophic salt. The bound material can then be separated from salts, proteins and other impurities by

washing. Small oligonucleotides, free nucleotides and dimerised primers from PCR do not bind and so are also removed. Nucleic acids elute from the glass surface in a low salt buffer or water. The method was carried out according to the manufacturer's instructions.

500μl Binding Buffer (containing guanidine-thiocyanate) was added to a 100μl PCR reaction, mixed well, added to a High Pure Filter Tube within a collection tube and centrifuged at 12,000 x g for 30s in a benchtop microcentrifuge. The filter harbouring the nucleic acids was then washed twice, first with 500μl Wash Buffer, then 200μl Wash Buffer and subsequent centrifugation. The filter was then removed to a clean 1.5 ml microfuge tube, 50μl sdH$_2$O was added to the filter and it was recentrifuged. An aliquot of the purified PCR product was run out on a gel with DNA markers to assess size and concentration and the remainder stored at -20°C.

2.11 DNA cloning into a plasmid vector

2.11.1 Digestion of DNA with restriction endonucleases

Restriction enzymes (restriction endonucleases) are isolated from a variety of bacteria and are widely used to cleave DNA molecules, both plasmid or genomic. They recognise a short palindromic DNA sequence, typically 4, 5 or 6 nucleotides, at which they cleave the molecule. Restriction enzymes have specific requirements for optimal reaction conditions, being particularly sensitive to the concentration of certain ions. Restriction enzymes and optimised 10x reaction buffers were obtained from Promega and reactions were carried out according to the manufacturer's instructions.

Approximately 1μg DNA, 2μl 10x buffer, 2μl BSA (1mg/ml), 1μl enzyme 1 (10u/μl), 1μl enzyme 2 (10u/μl) (if required) were added to a microfuge tube and the volume made up to 20μl with sdH$_2$O. The reaction mixture was then incubated at the appropriate temperature for 1-4 hours (overnight for genomic DNA).

Following digestion vector DNA was dephosphorylated prior to ligation and insert DNA was either purified using the High Pure PCR Clean Up kit (Boehringer Mannheim) or run out on an agarose gel and purified using the Geneclean II kit.

Vector DNA digested with a single enzyme was subsequently treated with shrimp alkaline phosphatase (Sigma) to dephosphorylate the 5' ends to prevent recircularisation of the vector and ligation between vector DNA molecules. 1µl shrimp alkaline phosphatase was added to the restriction digest mixture and incubated at 37°C for 30 minutes, followed by 10 minutes at 70°C to inactivate the phosphatase. Linearised DNA was purified from agarose gels as detailed previously in section 2.12.

2.11.2 Ligation

The enzyme T4 ligase joins DNA fragments together by catalysing the formation of a covalent phosphodiester bond between a 5'-phosphoryl group and an adjacent 3'-hydroxyl group. T4 ligase and ligation buffer were obtained from Promega and reactions were carried out according to the manufacturer's instructions.

50-200ng vector DNA (cut with suitable restriction enzymes), an equal (or greater) molar amount of insert DNA (with compatible sticky ends), 1µl 10x ligation buffer and 1µl T4 DNA ligase were added to a microfuge tube and the volume made up to 10µl with sdH$_2$O. The contents were mixed and incubated at room temperature for ten minutes before transformation into competent bacterial cells.

2.11.3 Transformation of *E. coli* with plasmid DNA by electroporation

1. Preparation of competent cells

A single colony of *E. coli* (strain XL1-blue MRF') from a fresh LB plate was inoculated into 400ml sterile LB medium and grown up at 37°C with rapid shaking to an optical density of A$_{600}$ 0.3-0.4. The culture was centrifuged at 2500xg for 10 minutes, the pellets resuspended in 200ml ice cold sdH$_2$O and henceforth stored on ice at all times. The cells were recentrifuged twice at 2500xg for 10 minutes at 4°C, resuspended first in 200ml ice cold water and then in 50 ml ice cold 10% glycerol. The cells were finally spun at 2000xg for 10 minutes at 4°C and resuspended in 1.2ml ice cold 10% glycerol. 50µl aliquots were added to pre-chilled microfuge tubes and cells not used immediately were flash frozen in liquid nitrogen and stored at -80°C.

2. Electroporation

Cells were thawed on ice, 2µl of ligation mixture (or plasmid DNA) was added, and were transferred to ice cold electroporation cuvettes (0.2cm electrode gap, Bio-Rad). Transformation was carried out using a Bio-Rad Gene Pulser set at 2.5V, 25µF and Pulser Controller apparatus set at 200Ω. Immediately following electroporation the cells were removed to 1ml LB medium and incubated with gentle shaking for 1 hour at 37°C. 50-200µl of the cells were spread on LB plates containing suitable selective antibiotics and incubated overnight at 37°C. Only bacteria harbouring circularised plasmids containing the relevant antibiotic resistance genes are expected to grow.

3. Analysis of colonies

Two methods were employed for the analysis of resulting colonies: the first is a PCR method for the rapid primary analysis of a large number of colonies and the second involves isolating the plasmid DNA for restriction analysis. Multiple colonies were routinely screened by the first method, and those yielding positive results were further analysed using the second method.

For colony PCR a mastermix of all components except template DNA (see section 2.11 for details of PCR) was made up and 50µl aliquots added to 20 numbered PCR tubes. Individual colonies were selected with a toothpick, streaked onto a numbered grid on a fresh selective LB plate, and the toothpick, harbouring bacteria, was introduced to the corresponding PCR tube. During thermocycling the bacteria lyse and plasmid DNA becomes accessible as template. Primers specific to the insert DNA will only cause amplification from colonies where the vector and insert have ligated. PCR products should be run out on an agarose gel next to DNA markers to check that positives are of the correct size and not artefactual.

Colonies which yielded PCR products of the predicted size were grown up overnight at 37°C in LB medium containing suitable selective antibiotics and the plasmid isolated as described in section 2.10. Restriction analysis (described in section 2.13) was carried out to confirm the correct plasmid was present.

A single colony showing bands of predicted sizes following restriction analysis was sent for automated sequencing to check for any base errors before continuing.

2.11.4 DNA cloning into a plasmid vector using the TOPO TA Cloning® kit

PCR products amplified using *Taq* polymerase (section 2.11) may be directly cloned into a plasmid vector using the TOPO TA Cloning® kit (Invitrogen, Groningen, The Netherlands). *Taq* polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearised plasmid vector (pCR®2.1-TOPO) supplied in the kit has single, overhanging, 3'deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. TOPO® Cloning exploits the ligation activity of topoisomerase I by providing an 'activated' linearised TA vector. Ligation of the vector with a PCR product containing 3' overhangs is very efficient and occurs spontaneously within 5 minutes at room temperature. Cloning and subsequent transformation was carried out according to the manufacturer's instructions.

1. TOPO® Cloning reaction

To 1µl pCR®-TOPO® vector was added 0.5-4µl fresh PCR product and sdH$_2$O to a final volume of 5µl. This was gently mixed for 5 minutes at room temperature after which time 1µl 6x TOPO® Cloning Stop Solution (0.3M NaCl, 0.06M MgCl$_2$) was added, mixed for 10 seconds and the reaction cooled on ice. Addition of the Stop Solution creates conditions that favour the dissociation of the topoisomerase from the DNA and thus increases the yield of transformants.

2. One Shot® Chemical Transformation

2µl of the TOPO® Cloning reaction was added to a vial of One Shot® Cells (Invitrogen), mixed gently without pipetting, and incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42°C without shaking, and immediately transferred to ice, 250µl of room temperature SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl$_2$, 10mM MgSO$_4$, 20mM glucose) was then added and the tube shaken horizontally for 30 minutes. 10-50µl from each transformation were spread onto selective plates (50mg/l

ampicillin) and incubated overnight at 37°C. This method generates hundreds of colonies, and typically 10-20 were picked for analysis.

2.12 DNA sequencing

DNA sequencing was performed by the DNA Sequencing Lab at the University of Durham using an ABI 373 DNA Sequencer and Dye Terminator Labelling Reactions (Perkin Elmer Applied Biosystems).

2.13 Plant transformation

2.13.1 Transformation of *Agrobacterium tumefaciens* with plasmid DNA

Two methods were employed to transform *Agrobacterium*: triparental mating and electroporation.

1. Triparental mating

Triparental mating is a conjugation procedure in which a helper strain of bacteria provides the transfer functions to mobilise a plasmid from *E.coli* to *Agrobacterium tumefaciens*. This method is after Topping *et al.*(1993).

Two days before the mating, 10ml of LB medium containing 25mg/l nalidixic acid was inoculated with *Agrobacterium* strain C58 and incubated at 30°C with rapid shaking for 48 hours. The following day two 10 ml cultures of *E.coli* were set up, one harbouring the helper plasmid pRK2013 and the other harbouring the kanamycin resistant pCIRCE construct to be introduced. 100μl aliquots were taken from each of the 10ml cultures, mixed in a sterile microfuge tube (the remaining liquid cultures were stored at 4°C) and centrifuged at 12,000rpm in a benchtop microcentrifuge for 5 minutes. The pellet was resuspended in 10μl 10mM MgSO$_4$ and the droplet pipetted onto an LB agar plate and incubated at 30°C for 48 hours to allow movement of the plasmid. The bacteria were then streaked out on an LB plate containing 100mg/l kanamycin and 25mg/l nalidixic acid to select for *Agrobacterium* harbouring the pCIRCE plasmid and incubated overnight at 30°C. Each of the parental strains were streaked out on a duplicate plate as a control.

2. Electroporation

This method is from Shaw (1995). Two 1.5ml aliquots of an overnight culture of *A. tumefaciens* (strain C58) were centrifuged for 30 seconds at 12,000rpm in a benchtop microcentrifuge, the pellets were resuspended in 0.5ml ice cold 1mM HEPES/KOH pH 7.0 and recentrifuged. This was repeated twice and the pellets were resuspended in 0.5ml ice cold 10% glycerol (the glycerol was filter sterilised as autoclaving leads to the formation of aldehydes which inhibit electroporation). The cells were recentrifuged, resuspended in 20µl 10% glycerol and the two tubes combined to give 40µl total. The plasmid DNA was added (approximately 1µg DNA/40µl cells for optimum results) and the tube incubated on ice for 2 minutes. The cells were electroporated in an ice cold cuvette using a Bio-Rad Gene Pulser set at 2.5V, 400Ω and 25µF. The cells were then removed to 1ml SOC broth (section 2.1.8) and incubated for 4-6 hours before 100µl aliquots were plated out on selective LB plates.

2.13.2 Plasmid Isolation from *Agrobacterium* using the Qiagen Plasmid Isolation kit

This kit was used to isolate plasmid DNA from *Agrobacterium*. Following alkaline lysis of the cells, plasmid DNA is bound to the QIAGEN Anion-Exchange Resin under conditions of low salt and pH. Impurities (including RNA, proteins, dyes) are removed by a medium-salt wash and plasmid DNA is eluted in a high-salt buffer. Isopropanol precipitation concentrates and desalts the plasmid DNA.

Cells were grown overnight in 100ml LB medium to a density of approximately 1×10^9 cells/ml and harvested by centrifugation at 6,000 x g for 15 minutes at 4°C. All traces of supernatant were removed and the pellet was completely resuspended in 4ml Buffer P1 to which RNaseA had previously been added. 4ml Buffer P2 was added and the contents of the tube mixed gently by inversion of the tube. This step causes lysis of the cells and care was taken not to shear genomic DNA. Cell debris, including genomic DNA, proteins and SDS was then precipitated by addition of 4ml Buffer P3, followed by gentle mixing of the tube contents by inversion and incubation on ice for 15-20 minutes. The tube was then centrifuged at 20,000 x g for 30 minutes at 4°C to pellet the debris, and the supernatant containing the plasmid DNA was promptly removed to a fresh tube and recentrifuged at 20,000 x g for 15 minutes at 4°C to

remove all suspended and particulate material. Meanwhile a QIAGEN-tip 100 was equilibrated by addition of 4ml Buffer QBT. The tips work by gravity flow; solutions are added to the top of the tip, move through the resin, and flowthrough is collected underneath. The supernatant was then added to the QIAGEN-tip and allowed to run through before two washes of 10ml Buffer QC each to remove all remaining contaminants. Following the second wash, the DNA was eluted with 5ml buffer QF and transferred to 1.5ml microfuge tubes, 0.8ml eluate per tube. DNA was precipitated by addition of 0.56ml room temperature isopropanol, mixing, and immediate centrifugation at 15,000 x g for 30 minutes at 4°C. The supernatant was washed with 2ml room temperature 70% (v/v) ethanol and recentrifuged for 10 minutes at 15,000 x g. The ethanol was removed, the pellet air-dried for 5-10 minutes and redissolved in a suitable volume (50 - 100μl) sdH$_2$O. Plasmid DNA was stored at -20°C.

2.13.3 Plant transformation by *Agrobacterium* using the dipping method.

This technique was developed by Clough and Bent (1998) based on Bechtold *et al* (1993).

Arabidopsis thaliana var. Columbia were sown on soil in plant pots covered with netting and grown for 3-4 weeks. Primary bolts were removed and the plants grown until the secondary bolts were 10-15 cm tall and displaying a number of immature flower buds. *Agrobacterium tumefaciens* harbouring the required construct were grown for 48 hours at 30°C in 200ml LB medium. The culture was pelleted by centrifugation and resuspended in 1 litre of a freshly made 5% sucrose solution containing 0,05% (V/V) Silwett-77 (Lehle Seeds, Round Rock, USA). Plants were dipped into the solution and gently agitated for 10-15 seconds before removal to a shaded high humidity environment for incubation overnight. The following day plants were returned to normal greenhouse conditions. A second dipping was performed 7 days after the first. Plants were then left to grow and set seed. Mature seed was collected, surface sterilised and germinated on ½MS10 with antibiotics to kill any remaining *Agrobacterium* (850mg/L vancomycin), and select for transformants (35mg/L kanamycin). Antibiotic resistant plants were transferred to soil and seed from these plants was tested for segregation on selective plates.

RESULTS

3.0 The promoter trap line EM2

This chapter describes the investigation of the promoter trap line EM2. GUS activity in the line is due to a single T-DNA insertion and has been shown previously to be localised to areas of cell cycle activity: throughout the embryo from the 4 cell stage onwards, shoot and root apices, and the young cotyledons and leaves (Topping *et al.* 1994). The T-DNA has been shown previously to be inserted 782bp upstream, presumably in the promoter, of a native gene. Due to GUS expression being detectable in the early embryo in the EM2 line, the tagged gene was named *EXORDIUM* (*EXO*). No obvious abnormal phenotype is observed in the EM2 line, which is homozygous for the T-DNA. One possible reason for this is that there may be a conditional phenotype, observed only by screening plants under certain conditions. Alternatively, the gene may be functionally redundant, or the T-DNA may not have altered the expression of the *EXO* gene. Plant structure and function are a result of interactions between genetic information and environmental stimuli, the two being co-ordinated by various means including hormonal control. EM2 plants were grown under various environmental and hormonal conditions and analysed for changes in GUS activity pattern and any phenotypic deviation from the wild type. Plants were also crossed into mutant backgrounds and the offspring likewise analysed.

For meaningful comparisons between treatments it was important to use a standardised GUS histochemical staining procedure (described in section 2.4.1). In order to establish what length of time it was appropriate to stain for, plants were grown to 7 DAG and stained for various lengths of time: 0, 0.5, 1, 2, 6, 24 h (Fig. 3.1A). Staining was observed as early as 0.5 h at the root tips in an area corresponding to the meristem, by 6h it was visible in petioles and in the elongation zone of the root tip, and by 24 hours staining could also be seen in the cotyledons and young leaves. This indicates that *EXO* is expressed to high levels in the root tips, with a gradient of expression through the elongation zone, and with low levels of expression occurring in young cotyledons and leaves. There is no staining observed in the hypocotyl or root (other than the tip or site of a newly emerging lateral) suggesting there is either no expression in these tissues, or extremely low levels of expression. A standard staining time of 2 hours was used for all subsequent experiments as this represented a

Fig. 3.1 GUS activity in the EM2 line

A EM2 seedlings grown to 7 days and stained histochemically for GUS activity for various lengths of time: i) 0h, ii) 1h, iii) 2h, iv) 6h, v) 24h

Staining was visible in the root tip at 1h, in petioles at 6h and in cotyledons and young leaves at 24h

Scale bar = 1cm

B Root tips of EM2 seedlings grown to 7 days and stained histochemically for various lengths of time: i) 0h, ii) 0.5h, iii) 1h, iv) 2h, v) 6h, vi) 24h

Staining was first observed at 0.5h in the root tip in an area corresponding to the meristem. At 6h staining was observed in the distal part of the elongation zone. At 24h staining was observed strongly throughout the root tip but not in differentiated root cells

A 2h staining time was used in all subsequent experiments

Scale bar = 0.1cm
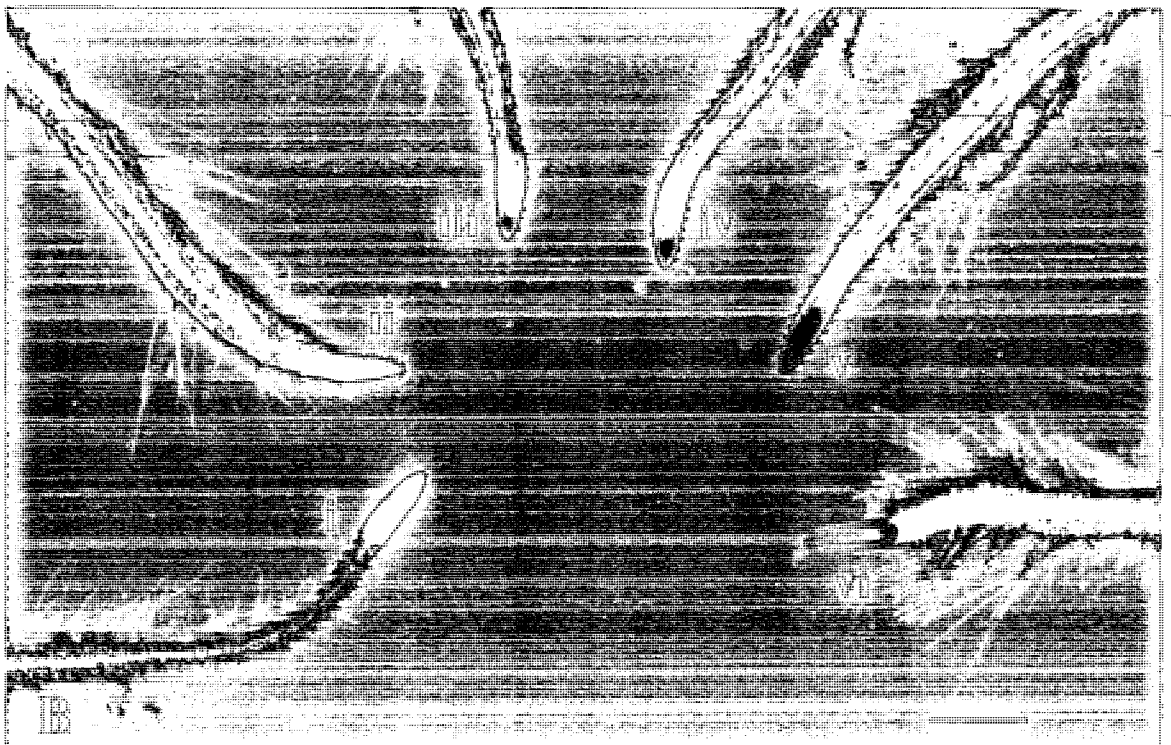
convenient timepoint at which both up- and down-regulation of GUS activity could be expected to be observed at the root tip (Fig. 3.1B)

## 3.1 Effect of environmental conditions on the EM2 line.

Being sessile and unable to avoid adverse environmental conditions, plants have evolved numerous mechanisms by which they are able to survive (Schiefelbein & Benfey 1991). Such plastic responses are often mediated by modifying the growth and development of the plant. The following experiments were designed to investigate whether a range of nutritional and environmental factors play a role in the regulation of GUS expression or reveal a phenotype in the EM2 line.

3.1.1 Dark

Of all the exogenous factors influencing plant growth and development, light is one of the most important. Numerous developmental processes are controlled by light, including: stem elongation, hypocotyl hook unfolding, leaf expansion, seed germination, flower initiation, phototropism, stomatal opening and initiation of chloroplast development (Jensen et al. 1998). Dramatically different plant phenotypes arise from growth under different quantities and qualities of light. Seedlings germinated in the dark show a well-characterised etiolation response which enables the cotyledons to reach light and so begin photosynthesising before the seed reserves are exhausted. EM2 plants germinated in the dark and grown to 7 DAG show ectopic GUS expression in the elongating hypocotyl in addition to the usual staining at the root tip. Little or no expression is observed in the unexpanded cotyledons (Fig. 3.2). Plants were measured to assess any difference in seedling length between the transgenic line and the wild type, but there was no significant difference, see Table 3.1.

3.1.2 Cold

Plants germinated and grown at 4°C showed greatly retarded development. At 8 weeks plants reached a stage similar to that achieved in approximately 10 days under normal conditions. There was no ectopic GUS expression in EM2 plants grown at 4°C, root tips showed GUS activity comparable to untreated plants (Fig. 3.3).
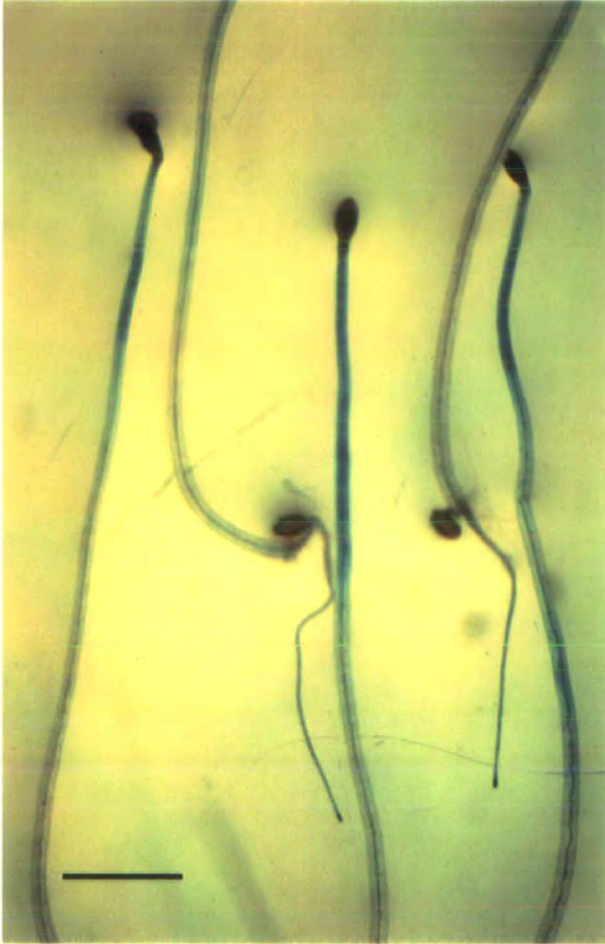
Fig. 3.2
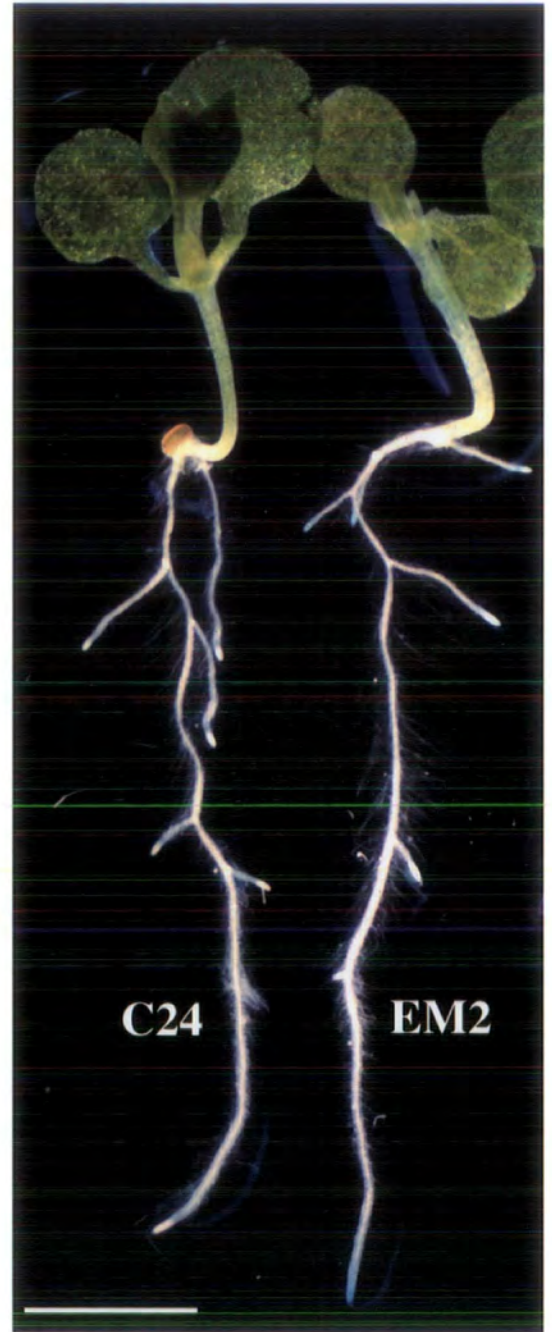
C24   EM2

Fig. 3.3

Table 3.1. Average C24 wild-type and EM2 dark-grown seedling lengths (mm), n=21. See Appendix for raw data.

|	C24	EM2
Root length	7.57 (s.d. 2.1)	7.14 (s.d. 2.2)
total seedling length	25.62 (s.d. 2.2)	25.33 (s.d. 2.3)

3.1.3 Nutrient availability

Nitrate, phosphate and other nutrients are essential to plant growth and function for such processes as protein synthesis, ATP production, DNA synthesis, phosphorylation *etc.* Nitrate availability has been demonstrated to have a major effect on root architecture, with many plant species producing a proliferation of lateral roots preferentially in areas rich in nutrients (Zhang *et al.* 1999, Zhang & Forde, 2000). Nutrient starvation leads to altered gene expression and metabolism as the plant decreases activities involving nutrient usage and access reserves.

In order to establish whether *EXO* expression is regulated during these processes, plants were grown on media containing varying amounts of nutrients: $^1/_2$MS10, $^1/_4$MS10, 0MS10 for 7 days and analysed for alteration in GUS staining pattern. Comparisons were also made with wild-type plants grown under the same conditions to determine whether EM2 showed any phenotypic variation. Plants grown under low nutrient conditions were smaller than control plants (Fig. 3.4A + B) but there was no obvious phenotypic difference between EM2 and C24; both being of similar height and root length and showing similar amounts of branching and lateral root formation. GUS staining was observed in the same pattern in treated and untreated EM2 plants. GUS staining at the root tip of plants grown on $^1/_4$MS10 appeared to be less restricted than that in the control but this was not the case for the 0MS10 treatment (Fig. 3.4 C + D).

3.1.4 Sucrose concentration

Sucrose has been demonstrated to be a factor in determining root growth rate. Hauser *et al.* (1995) observed that root growth rate is reduced when plants are grown in media containing low concentrations of sucrose. Dickinson *et al.* (1999) suggest that there is
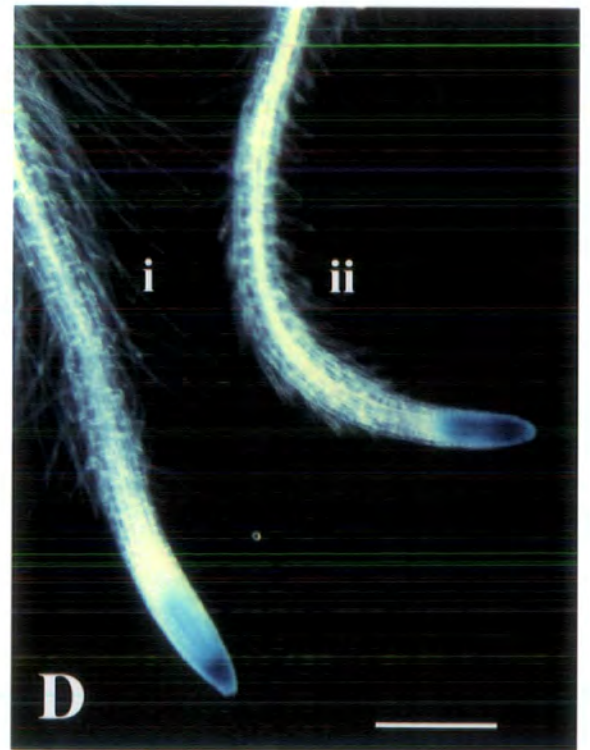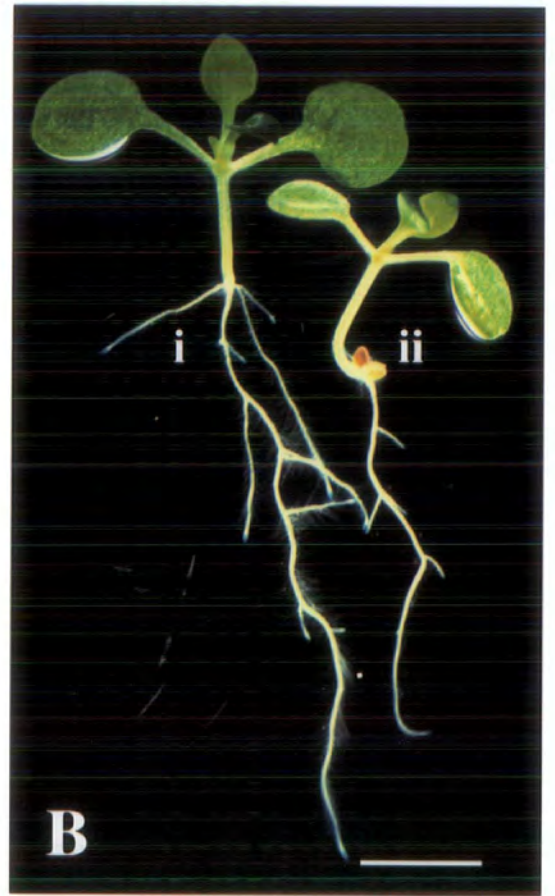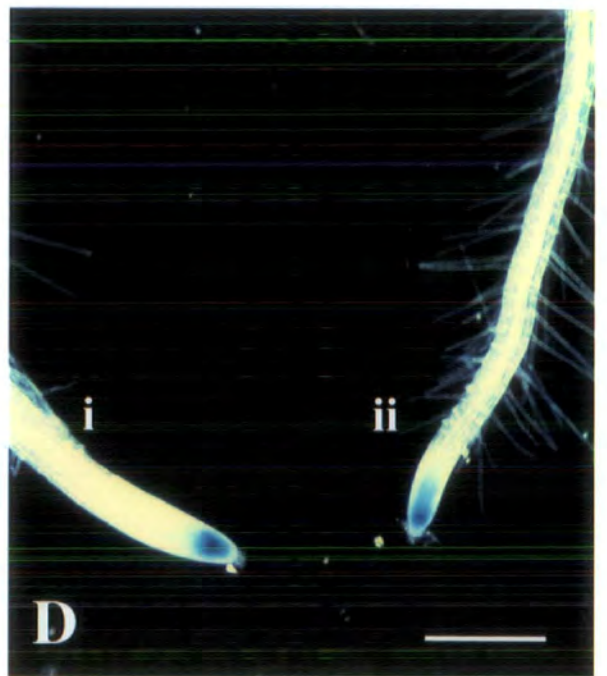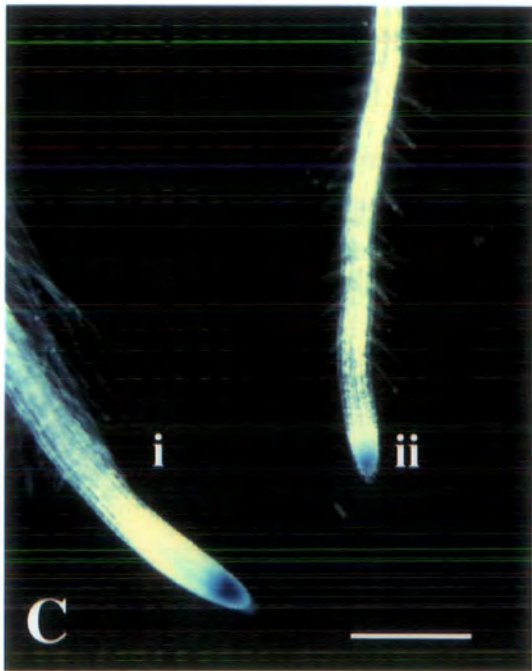
cross talk between the sugar sensing and cell cycle signalling pathways in *Saccharomyces cerevisiae* which enables the yeast to arrest cell division under conditions of nutrient deprivation, and plant roots may be similarly regulated as root growth is dependent on a continuous carbon supply that allows respiration activities and the building of new tissues (Müller *et al.* 1998a, and references therein).

EM2 and C24 plants were grown to 7 days on media containing various amounts of sucrose: $^1/_2$MS10 (1% sucrose), $^1/_2$MS1 (0.1% sucrose), $^1/_2$MS0 (no sucrose) and analysed for any aberrant GUS expression. Wild-type controls were also subjected to the same treatments in order for phenotypic comparisons to be made. As predicted, plants grown on low levels of sucrose showed a dramatic retardation of root growth and development in general (Fig. 3.5A + B) but there was no difference between the transgenic and wild-type lines. GUS staining, whilst remaining in the same pattern, was less intense in plants grown on low levels of sucrose, with plants grown on zero sucrose showing the greatest downregulation (Fig. 3.5C + D).

3.2 Hormone treatments

Hormones have been demonstrated to be strongly involved in various aspects of plant growth and development as described in the Introduction. To investigate whether hormones influenced GUS expression in the promoter trap line EM2, plants were grown on various hormones, hormone precursors and inhibitors of hormone action and analysed for alteration in GUS expression. For all treatments a comparison was made between the transgenic line and a wild-type control to determine whether a conditional phenotype might be revealed.

EM2 plants, which are homozygous for the T-DNA insertion, were also crossed into various mutant backgrounds compromised in some aspect of hormonal regulation and analysed for both alteration in GUS expression pattern, and any phenotype that might arise from a double mutation.

3.2.1 Auxin

All aspects of root development are profoundly affected by plant hormones, particularly auxins, cytokinins and ethylene. Several auxin-resistant mutants show root growth abnormalities including agravitropism, elongation and a decrease in the
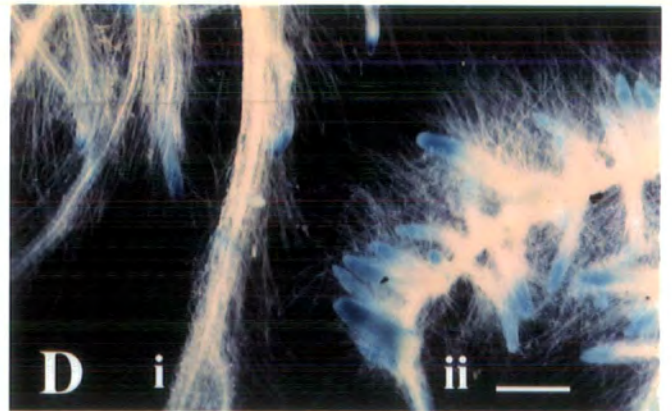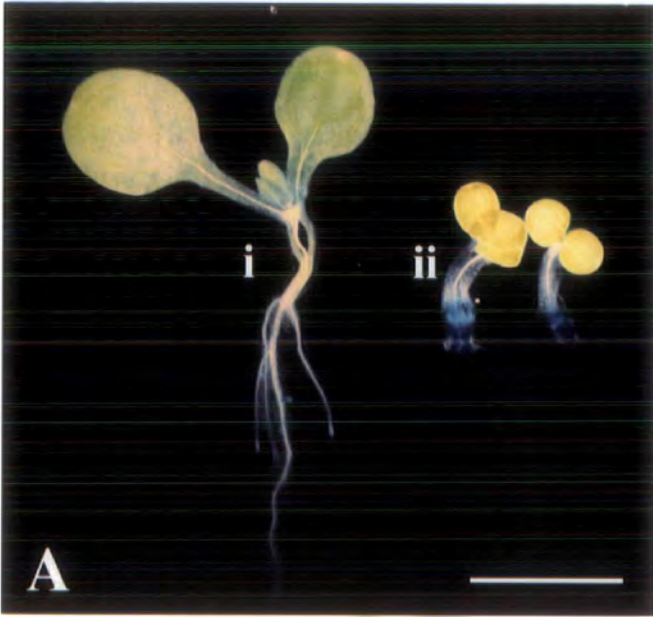
number of laterals formed, strengthening the connection between auxin and root development (Schiefelbein & Benfey, 1991, Hobbie & Estelle, 1995, Bennett *et al.*, 1996, Müller *et al.*, 1998b, Marchant *et al.*, 1999). To investigate whether GUS expression in the EM2 line is regulated by auxin, a series of experiments was performed.

Plants were grown to 5 and 10 days on 10μM 1-naphthalene acetic acid (NAA, a synthetic homologue of the naturally occurring indole acetic acid, IAA), and compared with plants grown for the same period of time on $^{1}/_{2}$MS10. 10μM NAA represents a concentration at which callus is not induced to form yet auxin induction of genes occurs. A dramatic increase in GUS expression is observed at the root tips in the NAA treated plants. The results are shown in Fig. 3.6A and B respectively. Plants germinated and grown for 5 days on $^{1}/_{2}$MS10 before transfer to 10μM NAA for a further 5 days show a similar upregulation with respect to untreated plants (Fig. 3.6C). Figure 3.6D shows a comparison between two EM2 plants, one grown to day 11 on $^{1}/_{2}$MS10, the other transferred to 10μM NAA at day 7 for a further 4 days. GUS activity is upregulated in the NAA-treated plant with respect to the untreated plant with expression occurring in a larger area of the root tip.

NAA was used for these experiments as it is more stable than the natural auxin IAA which is broken down *in planta*, and therefore more suitable for long-term growth experiments. In order to test whether IAA causes the same upregulation of GUS expression in the EM2 line, plants were grown on 10μM IAA and 10μM NAA for 7 days and compared. The results are shown in Figure 3.7A and B respectively. Both auxin treatments caused an upregulation of GUS expression. IAA treatment resulted in a lesser increase in expression than did NAA treatment, consistent with the observations of other auxin studies (Müller et al., 1998b).

Plants (both EM2 and C24) grown on 10μM NAA routinely produce adventitious roots from the hypocotyl and cotyledons. GUS expression in the EM2 line is observed in the root tips of these adventitious roots in the same pattern as it is in primary and lateral roots (shown in Figure 3.7C), indicating that GUS expression is associated with all root growth activity regardless of position and origin.
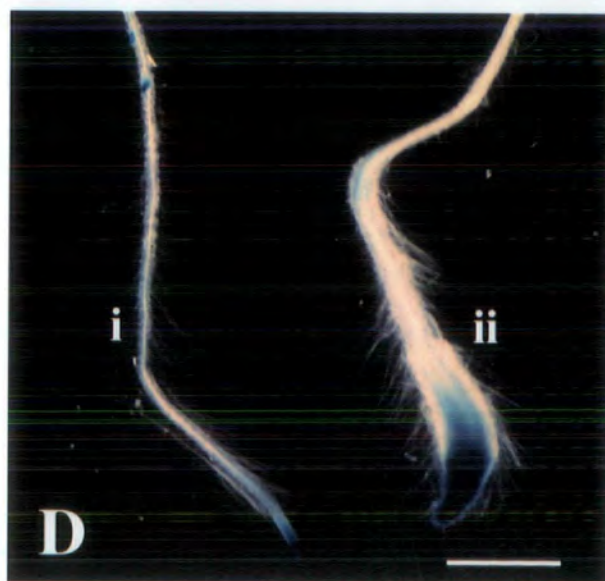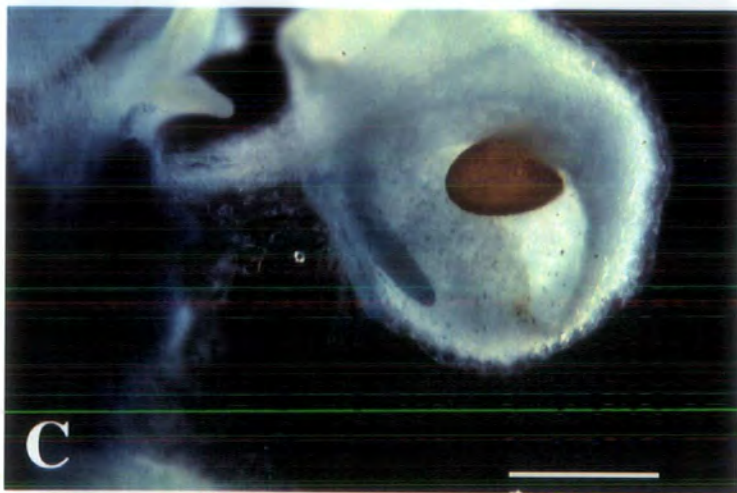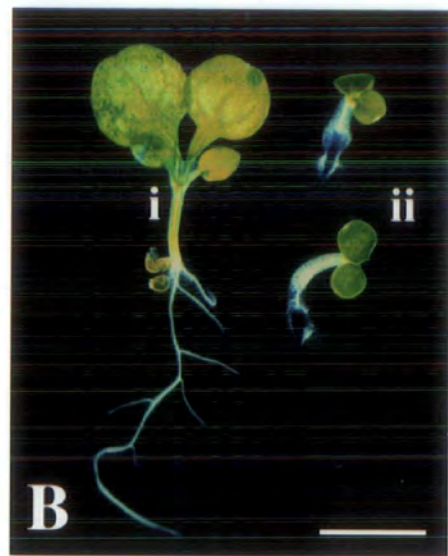
Fig. 3.8   GUS activity in 14 day old EM2 whole seedlings grown to 7 days on ½MS10 and transferred to different concentrations of NAA

Bars represent the standard error of the mean

EM2 GUS activity

y-axis: nmoles MU/mg protein/min.

15.00

10.00

5.00

0.00

x-axis: 0   0.5   1.5

NAA treatment (mg/l)
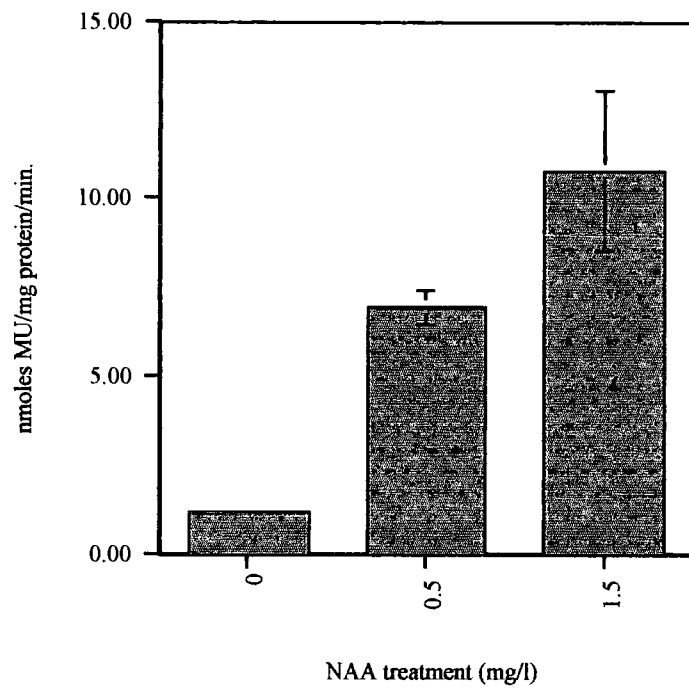
N-1-naphthylphthalamic acid (NPA) is an inhibitor of endogenous auxin transport within the plant. Treatment with NPA causes significant alteration in root morphology, resulting in a swelling at the root tip as seen in Fig. 7D. It has been suggested that NPA causes pooling of auxin in the root tip by restricting its transport back up the root from the tip via the cortex and epidermis, as proposed by Müller *et al.* (1998b). EM2 plants grown to 7 days on 10μM NPA show strong upregulation of GUS expression in the root tip (Fig. 3.7d) which is consistent with this hypothesis.

In order to gain quantitative information about the upregulation of GUS expression in the EM2 line with auxin treatment, fluorimetric GUS analysis (described in section 2.4.2) was performed.

Plants were grown to 7 days post germination, transferred to NAA plates of different concentrations (0, 0.5, 1.0, 1.5mg/l NAA) and harvested after a further 7 days. Lower concentrations were used than for previous experiments in an attempt to minimise the phenotypic effect of the treatment. The assay results, shown in Figure 3.8, reveal that increased NAA concentration causes increased GUS activity in the EM2 line. Plants grown on 1.5mg/l NAA (approximately 7μM) show a six-fold increase in GUS activity with respect to untreated plants.

In addition to subjecting plants to exogenous auxin treatments, EM2 plants were crossed into mutant backgrounds compromised in some aspect of auxin production, perception or signalling. The mutants used represent recessive mutations and so the phenotype is only observed in homozygous individuals. Homozygous EM2 plants were crossed with homozygous mutants where possible, but in cases where the mutants are infertile in the homozygous state, heterozygotes were used. In some cases heterozygotes can be screened for mutant embryos in their siliques as described in section 2.3.3 to ensure wild-type plants are not being used. Where there is no phenotype in the embryo it is impossible to know whether the plant is heterozygous or wild type for the mutation (without molecular analysis) and so greater numbers of crosses must be performed to ensure heterozygous parents have been used. The first generation of plants produced by crossing (the f1 generation) are all heterozygous for both the T-DNA and the mutant locus and are allowed to self-pollinate to generate the

Fig. 3.9   EM2-GUS expression in the root tips of auxin mutant backgrounds
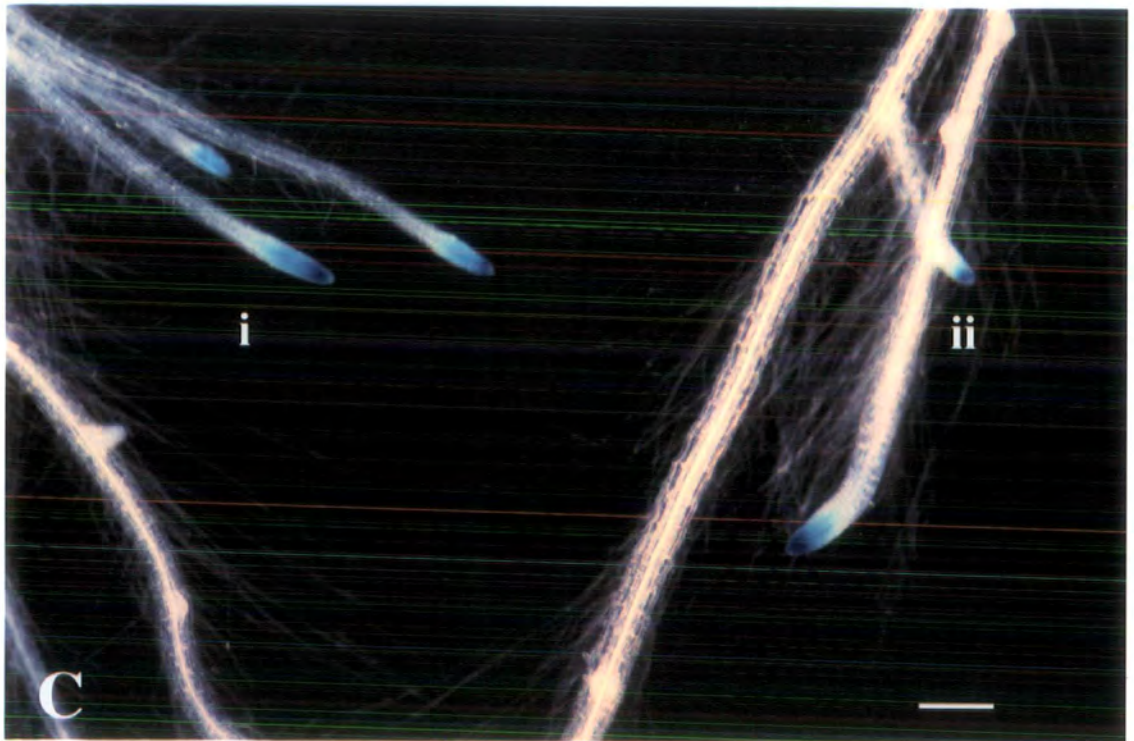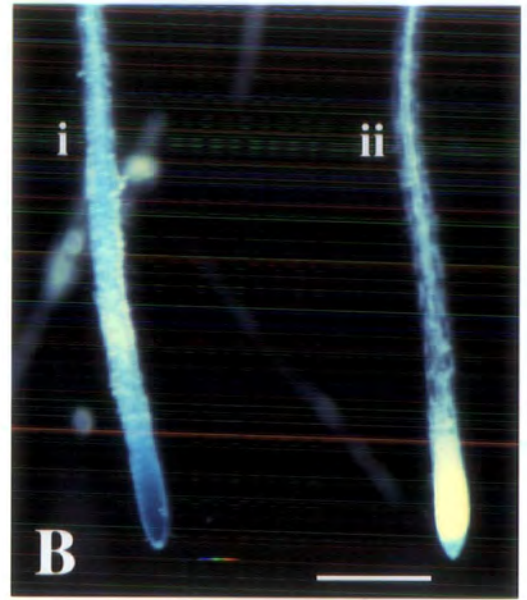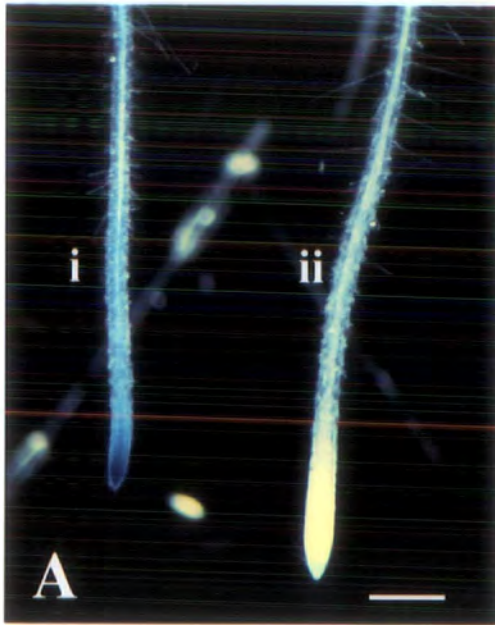
A   i) EM2
     ii) EM2/*Aux-1*

B   i) EM2
     ii) EM2/*Axr-1*

GUS expression was downregulated to very low levels in both the *Aux-1* and *Axr-1* auxin-resistant backgrounds

C   i) EM2
     ii) EM2/*pin-1*

GUS expression was unaffected in the *pin-1* mutant background

Scale bars = 0.1cm

f2 generation which is visually analysed for plants displaying the mutant phenotype of the non-EM2 parent. Of the plants showing a mutant phenotype there is an expected 1:2:1 ratio of plants wild-type:heterozygous:homozygous for the T-DNA. Histological staining revealed the GUS expression pattern which was analysed for change in the mutant background with respect to the EM2 line. Of the GUS-positive mutant plants, one in three is expected to be a double mutant homozygous for both the T-DNA and the recessive mutation. Plants showing both mutant phenotype and GUS expression were analysed for any additional mutant phenotype suggestive of a double mutant effect.

Aux1-7 (Maher & Martindale, 1980) is a mutant isolated from a screen for root growth on 2,4-D. It is resistant to both ethylene and auxin and is agravitropic. The mutant is hypothesised to have an impaired component of the auxin influx carrier (Bennett et al., 1996) as the phenotype can be restored by membrane-permeable synthetic auxins such as 1 naphthaleneacetic acid (NAA, Marchant et al., 1999). GUS activity at the root tip of EM2::*aux1* plants is much reduced with respect to that in EM2 (Fig. 3.9A)

Axr1-12 (Estelle & Sommerville, 1987) is an auxin-resistant mutant first identified by growth on 2,4-D. The resistance has been shown not to be due to altered uptake or metabolism of 2,4-D. The *AXR1* gene is be required for most, if not all, aspects of auxin action in all tissues and so plays an important role in plant development. The predicted protein is related to the E1 ubiquitin-activating enzyme but lacks a cysteine residue essential for E1 activity. As with the *aux-1* cross, GUS expression at the root tip is diminished in the *axr-1* mutantbackground with respect to EM2 (Fig. 3.9B).

The *pin1* mutant (Okada *et al.*, 1991, Gälweiler *et al.*, 1998) has impaired basipetal auxin transport and the protein is suggested to be a component of the auxin efflux carrier. Mutant plants show a highly aberrant phenotype where bolts are restricted to a single pin-like structure with no cauline leaves or flowers. The mutation appears to be restricted to the upper parts of the plant and not to affect root development in the same way as NPA treatment, as mutant plants show no swelling at the root tip and there is no upregulation of GUS expression at the root tip of EM2::*pin1* plants (Fig. 3.9C).

3.2.2 Cytokinin

Cytokinins are another group of phytohormones that have profound effects on root growth. Kinetin (a synthetic cytokinin) is known to be required for maintenance of cell division activity in cell suspension cultures (Kende & Zeevaart 1997). To investigate whether cytokinins are involved in the regulation of GUS expression in the EM2 line, various experiments were performed.

Plants were grown to 7 days on $10\mu M$ kinetin and compared with plants grown for the same time on $^1/_2$MS10. Plants were also grown to 14 days on $10\mu M$ kinetin. The results (shown in figure 3.10A and B respectively) reveal that this concentration of kinetin has a severe inhibitory effect on root development. EM2 plants grown to day 5 on $^1/_2$MS10 and transferred to $10\mu M$ kinetin for a further 5 days (Fig. 3.10C) show that there is a decrease in GUS expression in the root tips with respect to plants grown to day 10 on $^1/_2$MS10. Figure 3.10D shows two EM2 plants, one grown to day 11 on $^1/_2$MS10 and the other transferred to $10\mu M$ kinetin at day 7 for a further 4 days. It can be seen that GUS expression at the root tip is greatly diminished in the cytokinin treated plant, both in intensity of staining and area stained. No phenotypic difference between EM2 and the wild-type control was observed under any of these treatments.

In order to gain quantitative information about the regulation of GUS expression by cytokinin in the EM2 line, fluorimetric GUS analysis was performed as described in section 2.4.2. Plants were grown to 7 days post germination, transferred to kinetin plates of different concentrations (0, 0.5, 1.5mg/l) and harvested after a further 7 days. Lower concentrations were used than for previous experiments in an attempt to minimise the phenotypic effect of the treatment. The assay results, shown in Figure 3.11, reveal that increased kinetin concentration causes decreased GUS activity in the EM2 line. Plants grown on 1.5mg/l kinetin (approximately $7\mu M$) show GUS activity at about 70% that of untreated plants.

3.2.3 Other hormones

The three remaining 'classical' plant hormones; ethylene, gibberellic acid (GA) and abscisic acid (ABA) were also tested for effects on GUS activity in the EM2 line.
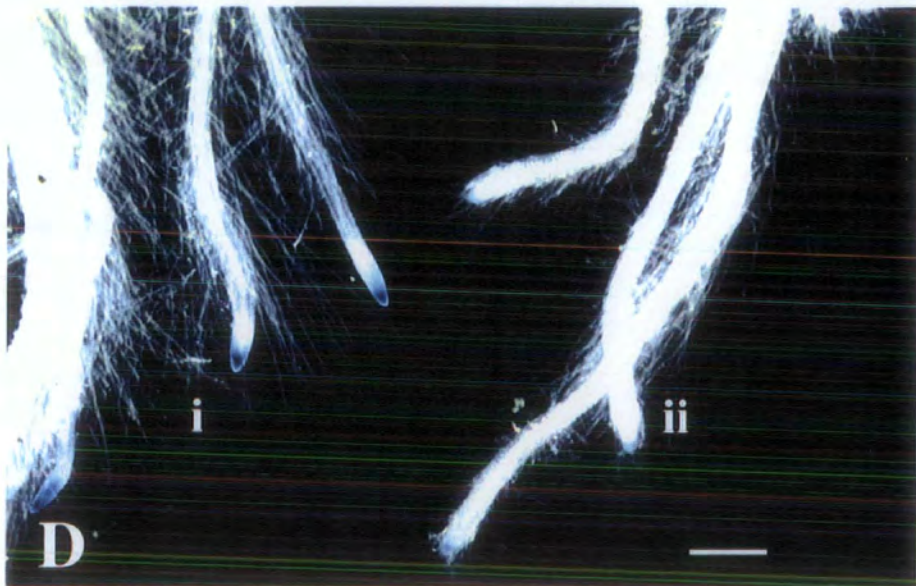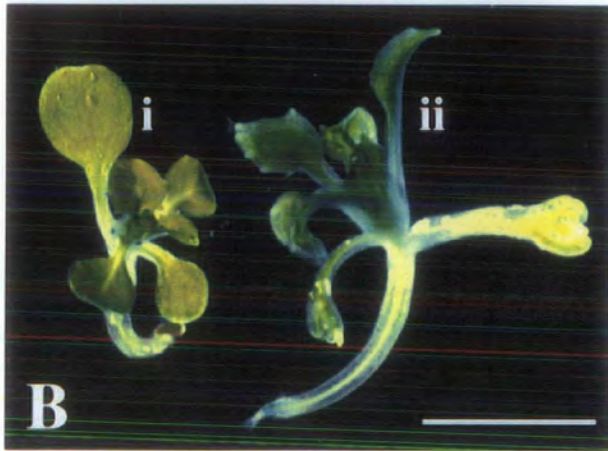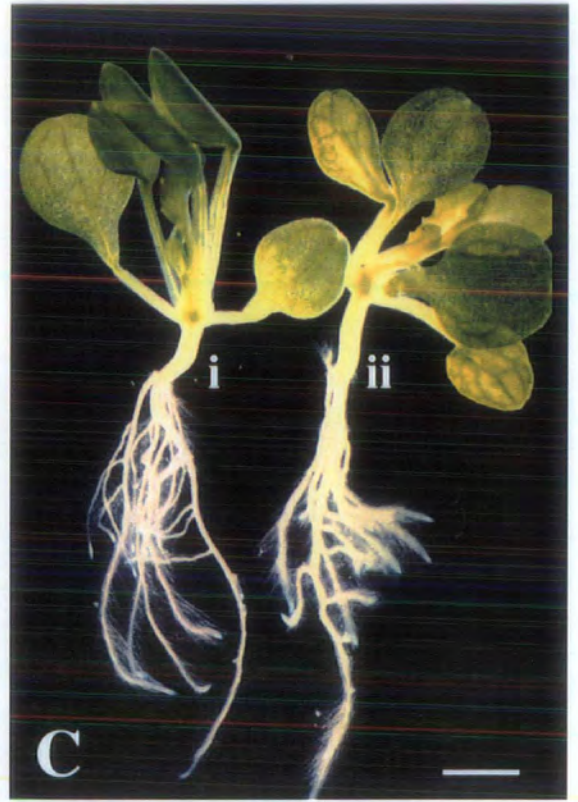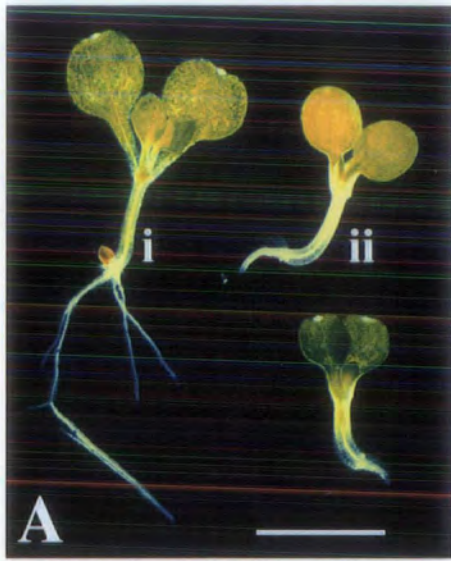
Fig. 3.11   GUS activity in 14 day old EM2 whole seedlings grown to 7 days on ½MS10 and transferred to different concentrations of kinetin

Bars represent the standard error of the mean

EM2 GUS activity

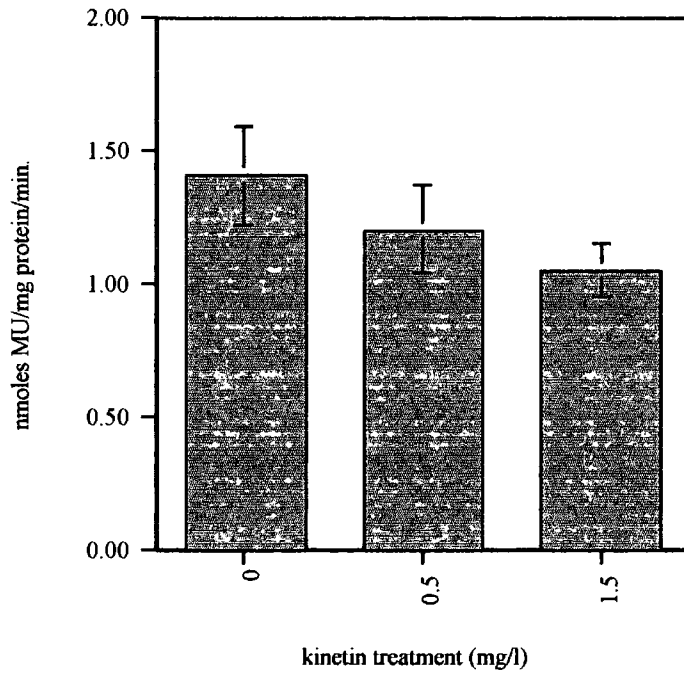Ethylene can both promote and inhibit cell growth, depending on the plant species and cell type, but is generally considered to have an inhibitory effect in *Arabidopsis* (Smalle *et al.* 1997). Plants were grown to 5 days on $^1/_2$MS10 and then transferred to plates containing 100μM ACC (the immediate biosynthetic precursor of ethylene, Fig. 3.12A) or 10μM AgNO$_3$ (which blocks ethylene perception, Beyer et al., 1976. Fig. 3.12B) for a further 5 days. ACC treatment causes ectopic GUS expression up the root in a diffuse pattern. AgNO$_3$ treatment results in a slightly more diffuse GUS staining at the root tip than that seen in the controls but the pattern of expression is unchanged.

ABA prevents precocious germination in the seed and incubating mature seeds in solutions of ABA prevents radicle extension (Bewley 1997). Seedlings germinated on 10μM ABA remain undeveloped by day 10 (Fig. 3.12C) and GUS activity pattern is restricted to the unextended radicle tip. Plants germinated on $^1/_2$MS10 for 5 days and transferred to 10μM ABA plates show a reduction in GUS activity at the root tip (Fig. 3.12D).

GAs are important for germination, both its initiation and its maintenance (Bewley 1997). GA is considered to promote cell elongation in newly divided cells and induces stem growth in many rosette plants and dwarf mutants. It has been suggested that ethylene-induced elongation of semi-aquatic plants upon submergance may be mediated through an increased sensitivity to GA (Kende & Zeevaart 1997). Plants were grown to 5 days on $^1/_2$MS10 before transfer to 10μM GA$_3$ for a further 5 days. Ectopic GUS expression is seen in the root vascular tissue with GA$_3$ treatment (Fig. 3.12E).

3.3   Summary

The results of the experiments described in this chapter indicate that the level of GUS expression in the EM2 line is modulated by auxin and cytokinin, which act antagonistically to increase and decrease expression respectively.

Some alteration in GUS activity pattern was observed under certain conditions; ectopic expression was observed in the hypocotyl of etiolated seedlings and in root

vascular tissue of GA treated plants. In none of the experiments described was an aberrant phenotype observed in the EM2 line with respect to the wild type.

Fig. 3.12   Treatment of EM2 seedlings with ACC, ABA and GA$_3$

A   10 day old EM2 seedlings
        i) Control plant grown on ½ MS10
        ii) Plant grown to 5 days on ½MS10 and transferred to 100μM
        ACC

B   10 day old EM2 seedlings
        i) Control plant grown on ½ MS10
        ii) Plant grown to 5 days on ½MS10 and transferred to 10μM
        AgNO$_3$

Scale bars = 1cm

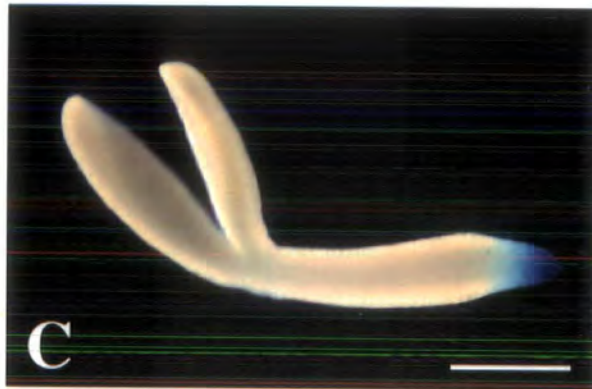C   10 day old EM2 seedling germinated on 10μM ABA

Scale bars = 0.1cm

D   10 day old EM2 seedlings
        i) Control plant grown on ½ MS10
        ii) plant grown to 5 days on ½MS10 and transferred to 10μM ABA

Scale bar = 2cm

E   10 day old EM2 seedlings
        i) control plant grown on ½ MS10
        ii) plant grown to 5 days on ½MS10 and transferred to 10μM GA$_3$

Scale bar = 1cm

4.0   The *EXORDIUM* gene

In the previous chapter, GUS expression in the promoter trap line EM2 was analysed under various environmental and hormonal conditions. In this chapter the expression of the *EXORDIUM* (*EXO*) gene in the wild-type background is analysed and compared with *EXO* expression in the EM2 line. The gene is demonstrated by northern analysis to be regulated in association with the cell cycle and Southern analysis reveals that the gene is exclusive to higher plants. Sequence analysis of the *EXO* gene, its promoter and predicted protein are described.

4.1   Analysis of *EXO* expression.

Previous work revealed that the left border of the T-DNA in the EM2 line is inserted 782bp upstream of the *EXO* gene and so the GUS expression in that line may not reflect the true expression pattern of the native gene. Northern analysis was performed to establish whether the presence of the T-DNA in the EM2 line (see Fig. 4.1) affects expression of the native *EXO* gene.

4.1.1   Comparison of *EXO* expression in the wild-type with *EXO* expression in the EM2 line.

Wild-type C24 and transgenic EM2 plants were grown to 7DAG on $^1/_2$MS10 when they were transferred onto either 1.5 mg/l NAA and 2 mg/l kinetin plates. Seedlings were harvested at times 0, 5h, 24h, 48h, 72h, 96h (0, d1, d2, d3, d4, d5) and total RNA was extracted as described in section 2.6. Equal amounts of the RNA (determined by measuring absorbance at 260nm) were run out and blotted as described in section 2.7. The blots were probed using 0.9kb of the *EXORDIUM* cDNA and the results are shown in Figure 4.2A + B. For both C24 and EM2, steady state mRNA levels increase within 5 hours of transfer to NAA and continue to increase for 24 hours. By 48 hours however levels have returned to approximately the same as before treatment. Following transfer of C24 to kinetin there is no discernible difference in mRNA levels after 5 hours but by 24 hours a decrease is observed. This decrease continues to a minimum at 72 hours, followed by a return to normal (untreated) levels by 96 hours. *EXO* transcript levels in the EM2 line were too low to determine whether the kinetin treatment caused the same pattern in this line. All blots

T-DNA (GUS)

720 bp

T-DNA promoter

1.5 kb

cDNA promoter

sORF

exordium cDNA
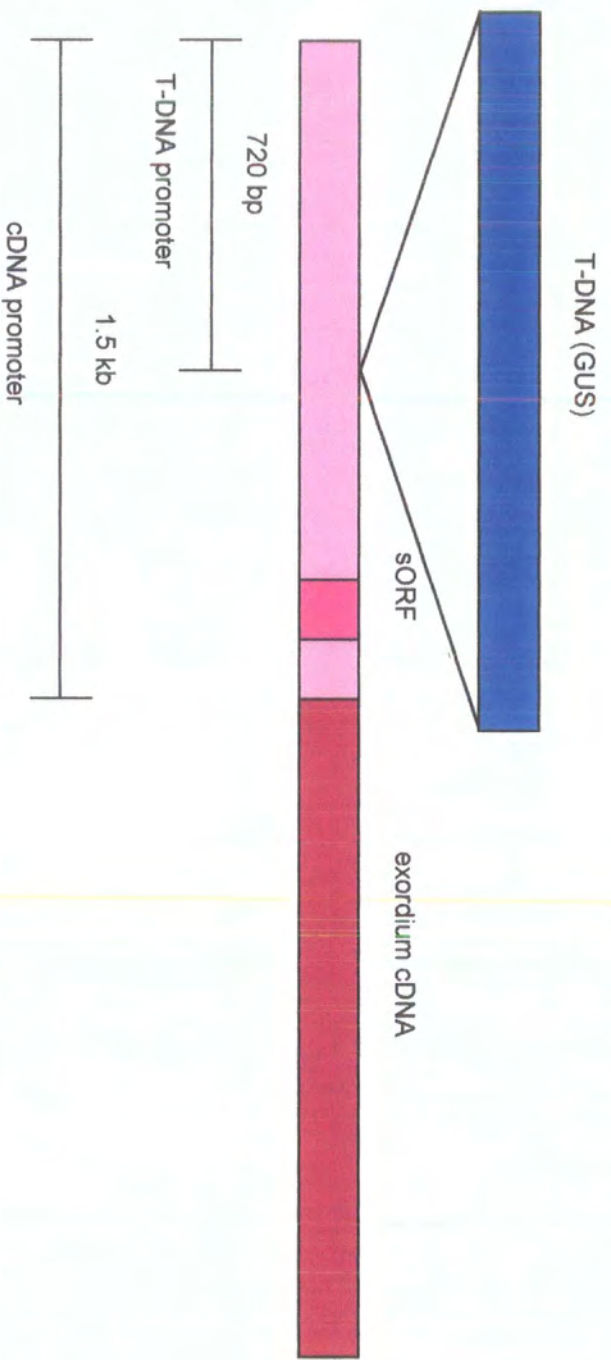
were reprobed with the constitutively expressed actin gene to ensure equal loading had been achieved.

It can be seen from these results that a) the pattern of *EXO* mRNA levels in response to auxin and cytokinin treatment is similar in wild-type and EM2 plants and b) the insertion of the T-DNA in the EM2 line has caused a decrease in steady state mRNA levels with respect to wild-type. Low levels of expression are detectable in the transgenic line and the pattern of expression in response to NAA treatment is similar to that seen in the wild-type.

4.1.2   Cell Cycle regulation of *EXORDIUM* expression.

GUS expression in the EM2 line is observed in areas of cell cycle activity. In order to investigate possible regulation of *EXO* expression during the cell cycle, plants were treated with hydroxyurea (HU) which blocks cell cycle progression at the G1/S transition. C24 plants grown to 7DAG on $^1/_2$MS10 were transferred to 100μM HU plates, sampled at times 0, 24h, 72h, 120h (0, d1, d3, d5) and total RNA extracted as described in section 2.6. Equal amounts of RNA from each timepoint (9μg) were run out on a gel and blotted, and the blot was then probed with a 0.9kb fragment of the *EXO* cDNA as described in section 2.7. Treatment with HU results in an accumulation of *EXO* mRNA, reaching a maximum at 72 hours post transfer before returning to nearly untreated levels by 120 hours. This result suggests that *EXO* may be regulated during the cell cycle (Fig. 4.3A). In order to test this hypothesis, further northern analysis was performed.

Previous work in the laboratory studying the modulation of cyclin transcript levels in *Arabidopsis* cell cultures (Fuerst *et al.* 1996) generated total RNA taken from synchronised cells every four hours post release from the cell cycle inhibitor cycloheximide. The doubling time of the cells was calculated to be 22h. Equal volumes of RNA obtained from times 0, 4h, 8h, 12h, 16h, 20h and 24h were run out and blotted, and probed with 0.9kb of the *EXO* cDNA. The results of this experiment (Fig. 4.3B) show clearly that *EXO* mRNA levels accumulate and diminish in a cell cycle dependent manner, reaching a minimum at 4h and a maximum at 16h post release from cycloheximide. 16 hours post-release was determined to correspond to

Fig. 4.2   Hormonal regulation of *EXO* expression

Ai   *EXO* mRNA levels at various times following transfer of 7 day old C24 and EM2 plants onto 7.5µM NAA. In both lines *EXO* mRNA accumulates following NAA treatment, reaching a maximum at d1 (= 24h) post-transfer. *EXO* transcript levels are much reduced in the transgenic line with respect to wild-type.

Aii   actin mRNA levels. The blot shown in 4.2Ai was reprobed with actin to ensure equal loading had been achieved.

Bi   *EXO* mRNA levels at various times following transfer of 7 day old C24 and EM2 plants onto 10µM kinetin. *EXO* mRNA levels decrease in the C24 line following kinetin treatment, reaching a minimum at d3 (= 72h) post-transfer. *EXO* transcript levels are much reduced in the transgenic line with respect to wild-type.

Bii   actin mRNA levels. The blot shown in 4.2Bi was reprobed with actin to ensure equal loading had been achieved.

Fig. 4.2
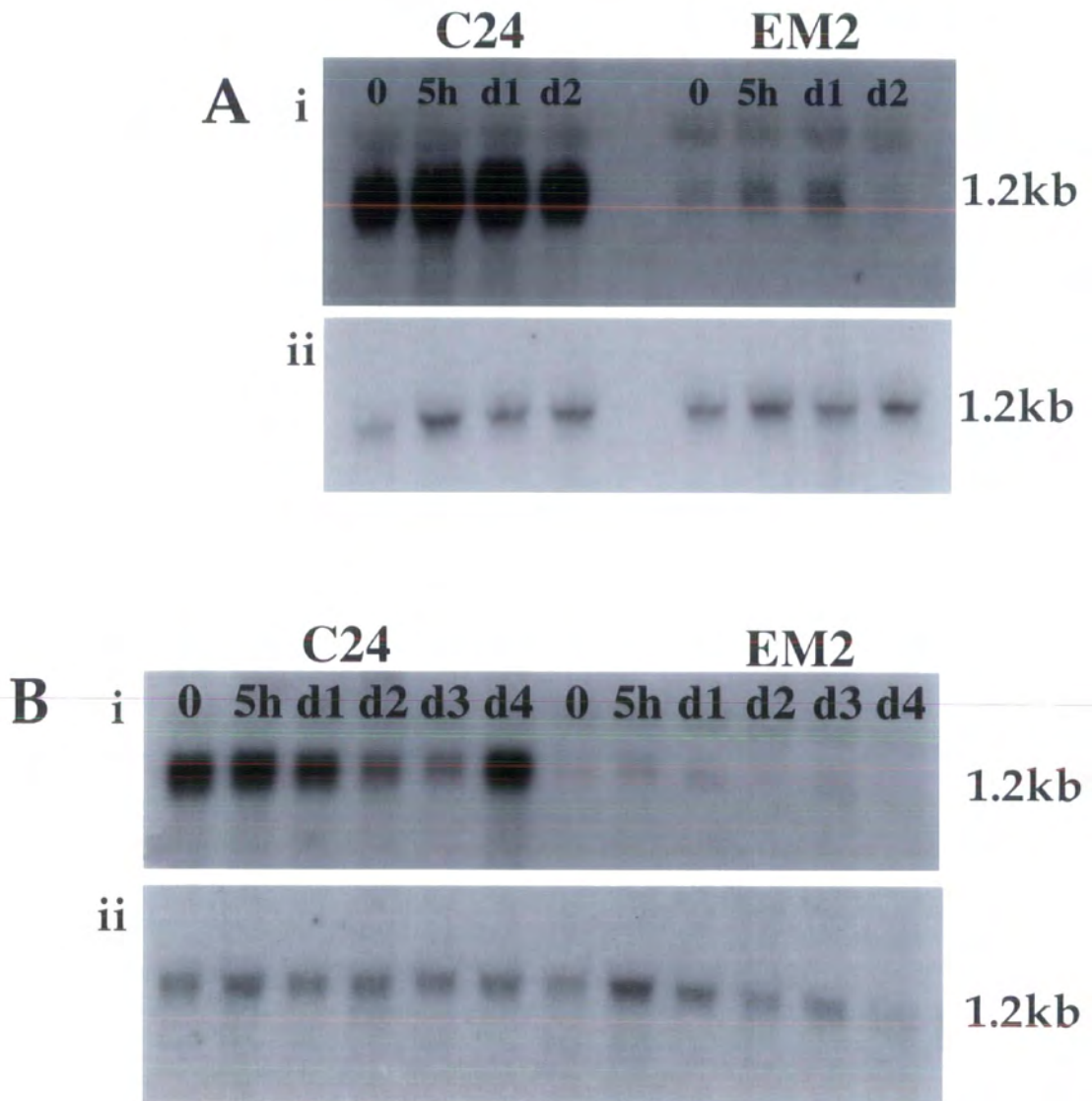
Fig. 4.3   Cell cycle regulation of *EXO* expression

Ai   *EXO* mRNA levels at various times following transfer of C24 plants onto 100μM hydroxyurea. *EXO* mRNA levels increase following treatment, reaching a maximum at d3 (= 72h) post-transfer

Aii   actin mRNA levels. The blot shown in 4.3Ai was reprobed with actin to ensure equal loading had been achieved

Bi   Cell cycle dependent accumulation of *EXO* mRNA levels. Total RNA was extracted from wild-type *Arabidopsis* cell suspension cultures at various times post release from the cell cycle inhibitor cycloheximide. *EXO* mRNA levels reach a minimum at 4h post release and accumulate to a maximum at 16h post release (M phase)

Bii   actin mRNA levels. The blot shown in 4.3Ai was reprobed with actin to ensure equal loading had been achieved

Fig. 4.3
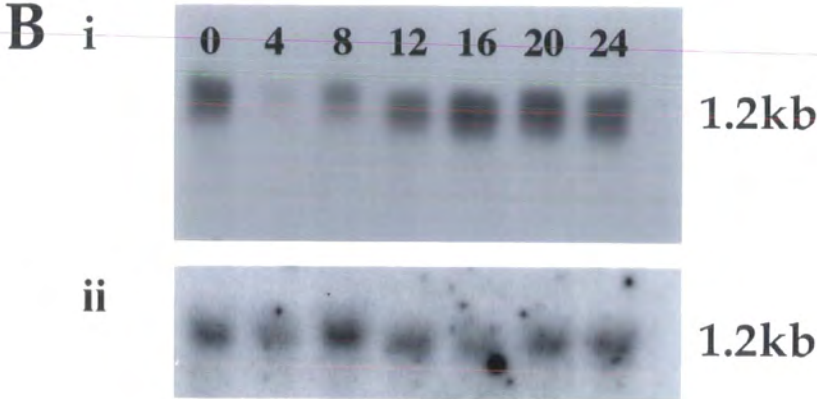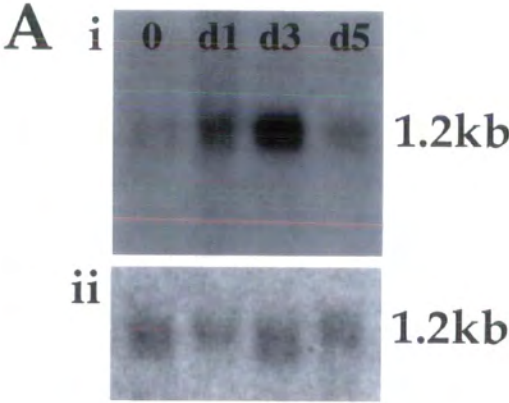
the mitotic phase of the cell cycle (Fuerst *et al.* 1996) and so this result demonstrates that the *EXO* transcript accumulates to a maximum level at M phase of the *Arabidopsis* cell cycle. In light of this it is possible that the results obtained for the hormone regulation of *EXO* (section 4.1.1) may be influenced by any net regulation of the cell cycle in the plant, for example by photosynthetic activity associated with circadian rhythm. Samples were taken at the same time each day for uniformity and to minimise the effect of any environmental factors associated with time of day. Repetition of the experiment to include an untreated control at each timepoint would be desirable in order to confirm that the results obtained are due solely to the hormone treatment.

4.1.3 Analysis of the upstream open reading frame

Analysis of the *EXO* locus reveals a small (104bp) putative open reading frame (ORF) 328bp upstream of the main ORF of the gene. There have been reports of small upstream open reading frames having a regulatory effect on the downstream gene (Futterer & Hohn, 1992, Wang & Wessler, 1998) and so northern analysis was performed determine whether the 104bp ORF is transcribed. PCR primers (section 2.1.4) were designed to amplify the small ORF (sORF) to generate a fragment that could be used as a molecular probe (Fig. 4.4). The blot with the highest RNA loading (the kinetin blot) described in section 3.2.1 was reprobed with the purified product as described in section 2.6. This experiment indicated that the sORF is either not transcribed, or is transcribed at levels too low to allow detection. Computer searches revealed that there are no ESTs on the BLAST database with any significant homology. It was therefore considered unlikely that a transcriptional or translational product of the sORF plays any role in the regulation of the *EXO* gene.

4.2 Sequence analysis of the *EXORDIUM* gene

4.2.1 Homology searches

Database searches revealed that *EXO* has no introns, is located on chromosome IV and is a member of a small gene family of predicted proteins in *Arabidopsis*, all of unknown function. Two of the predicted proteins are of a similar length to EXO (AB008268, AL391712), there are another three proteins of similar length but with lower homology, and a protein which shares some homology with *EXO* at the C

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   1 ACTTAAAACA AAATTAATGA CAACGTGTAC CGCGCGTGAT AATCACGTGA
  51 CAAATTGATG ACGTGGAAGC GCGTAGTAAC ACGTGCCGAC ATAGTGTCAG
 101 TAAATGCTCT CAAATTACCT ATGGATAGGA ATTGTAGGAT TACTCTCTCA
 151 TCTTCCTCTA TCTACTAACT TGTGCCAGCT GTACACTCTT CCCCATATTT
 201 TCCAGCCTGT CCAAAAAAGT CCTCCCAACG TCTCTCAGAA GTTTCACATT
 251 TTTTCTTTAA CTGCCCACGC GCTTTACCCA CAGAAGGAAA AAAAAACACA
 301 GCTTGCATAA GGGATTTACG TCATTAGATC TGTAACTAAG CTGTATATTC
 351 TTCAACACAT TTTTATCGAT ATGGTCAATT TTGAGTCCTC TTTTTAATCA
 401 ATTTTTTATT AGCTGTATGT AACTATTGCT TAATCTGACA CAATTAAATC
 451 TTTATTAATT GTTTTATAGT TTATATAATA CATATATAAA CACTAATTAT
 501 TCCTTAAATA TTTATTTAGA TTACCACTAG CAACTCTTTT TTATTGGTCC
 551 AACTTGATTT CATTTTCTAA TTTGTAGTAT TAAATAAAAA CATCCACAAC
 601 ATGTACAGCT TTTACAACAG GAATCCTATG TCTCATTCTA GCCTCCATGA
 651 GTAGTCTTAT CCAAATCCAA ATATATTCAA AATATTTGTA TATATTTAAA
 701 AATAAAATTA TACTTAGCAT TATATAATAC TCTTTCTGTT TCATCAAGAT
 751 TGATGTTATA G*GTTTTTTTT CTTATTCCAA AAAGTTGATG TTTTAGATCA
 801 ATATTTTTTT TTACTTTTAT TTGTAACTAA TAGATAGAGC AGAGAAGAGA
 851 GAAATAGTGT CTGATATTAT TAAACAAATA AAAAATAAAA AAATAAAAAA
 901 ATTAATTTTT GTGAAAAACC TACAACATCA AACTTTATGG AACGGATGGA
 951 GTATTTATAT GAAGTTAAAC ATAGCATTAT ACATATTTTT ATGAAAACTT
1001 TTGATGAAAA TCATTAAAGA AACTGAGGAA TTTATAATGA CATTTTCACA
1051 TTATAACCAT AACATTGAAA AATAAGAATT TTAAACCAAA GTTGTATGTA
1101 ATGAATAAGT TGTCGGACTC GAAACTGAGT AAATTATAAT GACATTTGAC
1151 TATATGACCA TAACATAATC AAGAAAATTC AAATTTTGAC ATAAACCAAA
1201 AGTTGTGTAG AACTATGGAT CATTCGTTAG CTGGCAACAG TACAATGGTC
1251 CATTACTCCT TTGTATCTTT TCTTTCAATT TCTCTGCACC TTCCCTTTCT
1301 CTTTCGCTTT AAAGCATAAT AATAACATAC CTCTCTCTTT TTCCACCTTT
1351 ATTATTTAAA AATCATATTC AAAATCTAAT TTCCATTTTT AATAAAAATA
1401 AGTCCACTCT GAGAGAGGCA TATATATAGC CCCATCACAC TCCATACATC
1451 TCACAAACCA ATCTAAACAT CAAAAGACAA AAACCAAAGC TCTTTCACTA
1501 TTACACAACC TTCTATTTCA CTTTTCCTCA AAAAAAAGAA AAATG
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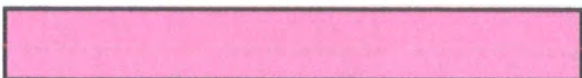
EXO

PHI-1

AB008268

AL391712

AC018363

AB018109
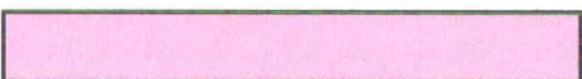
AC007127

AC004667
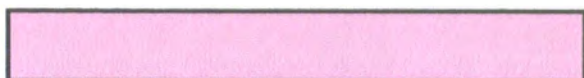
AB010693

AC024128

terminal end but is much longer at the N-terminal end. Two further proteins are homologous to the N terminal end of this protein (Fig. 4.5, see Appendix 1 for sequences). A single published homologue exists which was isolated from a tobacco BY-2 cell culture (Sano *et al.* 1999). The gene was isolated by differential screening of phosphate-starved cells during cell cycle arrest and following readdition of phosphate. The gene is rapidly induced following the addition of phosphate and was named *phosphate induced* (*PHI-1*). Although PHI-1 and EXO share 76% homology at the amino acid level, they are not regulated in the same way, as *PHI-1* is expressed at the same level throughout the cell cycle (Sano *et al.* 1999).

The EST database reveals that homologues of *EXO* are present in a range of Angiosperms, both monocotyledonous and dicotyledonous, and there are two examples from the Gymnosperm *Pinus*. No other groups are represented, although this may in part reflect the fact that lower plants are less well studied than higher ones. The fully sequenced *E.coli* and *S.cerevisiae* genomes reveal that there is no homologue in these organisms and there is no homologue in the human genome, suggesting that *EXO* is exclusive to plants.

4.2.2 Sequence prediction

Various prediction programmes were used to analyse the *EXO* cDNA, its promoter and the predicted protein. Putative cis elements were identified in the promoter and intragenically at the 5' end of the *EXO* gene, notably auxin responsive elements (AREs, sequence: TGTCTC). The positions of these elements are shown in Fig. 4.6A +B. Although such sequences are most commonly considered to be important in directing gene activity when located in the promoter, there is evidence of intragenic sequences being involved in regulating gene expression, for example in the spinach *PsaD* gene (Bolle et al., 1996).

No protein motifs were identified using the SMART program, however PSORT predicts that the EXO protein has a possible cleavage site between positions 21 and 22 (alanine and arginine) and that this represents a cleavable N-terminal signal sequence (Fig. 4.7). The protein may be targeted to the endoplasmic reticulum, both membrane and lumen, or the Golgi apparatus, but it is most likely extracellular.

Fig. 4.6a   Putative cis elements in the *EXO* promoter

Putative TATA boxes are shown in blue, putative auxin responsive elements (AREs) are shown in purple. The sORF is underlined and the T-DNA insertion site is asterisked in pink.

```
   1 ACTTAAAACA AAATTAATGA CAACGTGTAC CGCGCGTGAT AATCACGTGA

  51 CAAATTGATG ACGTGGAAGC GCGTAGTAAC ACGTGCCGAC ATAGTGTCAG

 101 TAAATGCTCT CAAATTACCT ATGGATAGGA ATTGTAGGAT TACTCTCTCA

 151 TCTTCCTCTA TCTACTAACT TGTGCCAGCT GTACACTCTT CCCCATATTT

 201 TCCAGCCTGT CCAAAAAGT CCTCCCAACG TCTCTCAGAA GTTTCACATT

 251 TTTTCTTTAA CTGCCCACGC GCTTTACCCA CAGAAGGAAA AAAAAACACA

 301 GCTTGCATAA GGGATTTACG TCATTAGATC TGTAACTAAG CTGTATATTC

 351 TTCAACACAT TTTTATCGAT ATGGTCAATT TTGAGTCCTC TTTTTAATCA

 401 ATTTTTTATT AGCTGTATGT AACTATTGCT TAATCTGACA CAATTAAATC

 451 TTTATTAATT GTTTTATAGT TTATATAATA CATATATAAA CACTAATTAT

 501 TCCTTAAATA TTTATTTAGA TTACCACTAG CAACTCTTTT TTATTGGTCC

 551 AACTTGATTT CATTTTCTAA TTTGTAGTAT TAAATAAAAA CATCCACAAC

 601 ATGTACAGCT TTTACAACAG GAATCCTATG TCTCATTCTA GCCTCCATGA

 651 GTAGTCTTAT CCAAATCCAA ATATATTCAA AATATTTGTA TATATTTAAA

 701 AATAAAATTA TACTTAGCAT TATATAATAC TCTTTCTGTT TCATCAAGAT

 751 TGATGTTATA G*GTTTTTTTT CTTATTCCAA AAAGTTGATG TTTTAGATCA

 801 ATATTTTTTT TTACTTTTAT TTGTAACTAA TAGATAGAGC AGAGAAGAGA

 851 GAAATAGTGT CTGATATTAT TAAACAAATA AAAAATAAAA AAATAAAAAA

 901 ATTAATTTTT GTGAAAAACC TACAACATCA AACTTTATGG AACGGATGGA

 951 GTATTTATAT GAAGTTAAAC ATAGCATTAT ACATATTTTT ATGAAAACTT

1001 TTGATGAAAA TCATTAAAGA AACTGAGGAA TTTATAATGA CATTTTCACA

1051 TTATAACCAT AACATTGAAA AATAAGAATT TTAAACCAAA GTTGTATGTA

1101 ATGAATAAGT TGTCGGACTC GAAACTGAGT AAATTATAAT GACATTTGAC

1151 TATATGACCA TAACATAATC AAGAAAATTC AAATTTTGAC ATAAACCAAA

1201 AGTTGTGTAG AACTATGGAT CATTCGTTAG CTGGCAACAG TACAATGGTC

1251 CATTACTCCT TTGTATCTTT TCTTTCAATT TCTCTGCACC TTCCCTTTCT

1301 CTTTCGCTTT AAAGCATAAT AATAACATAC CTCTCTCTTT TTCCACCTTT

1351 ATTATTTAAA AATCATATTC AAAATCTAAT TTCCATTTTT AATAAAAATA

1401 AGTCCACTCT GAGAGAGGCA TATATATAGC CCCATCACAC TCCATACATC

1451 TCACAAACCA ATCTAAACAT CAAAAGACAA AAACCAAAGC TCTTTCACTA

1501 TTACACAACC TTCTATTTCA CTTTTCCTCA AAAAAAAGAA AAATG
```

Fig. 4.6b   Putative cis elements in the *EXO* coding region

Putative auxin responsive elements (AREs) are shown in purple.

```
  1 ATGTATTTGT TAGTGTTTAA ACTCTTTCTA TTTTTGTCTC TTCTTCAAAT

 51 CTCTGTCTCT GCTAGAAACC TAGCTTCACA AGAACCAAAC CAGTTTCAAC

101 TACTCAAGTA CCACAAAGGA GCTCTTCTCT CCGGCAAAAT CTCCGTTAAC

151 CTAATCTGGT ACGGCAAATT CAAACCATCT CAAAGAGCAA TCATCTCCGA

201 CTTCATTACC TCACTCACAC ACACTTCTCC TACGTCCAAA ACTCTCCACC

251 AACCATCTGT TGCCACGTGG TGGAAAACCA CAGAAAAATA CTACAAACTC

301 GCAACACCCA GCAAAAACTC ATCACCTCTG TCTCTAACTC TCGGGAAACA

351 AATCATCGAC GAGTCTTGCT CTCTAGGAAA ATCTTTAACC GATAAAAAGA

401 TTCAAACCCT AGCTTCGAAA GGAGACCAAC GCAACGCCAT CAACGTCGTT

451 TTGACTTCAG CTGACGTTAC GGTTACAGGA TTTGGTATGA GTCGTTGCGG

501 GACTCACGGA CACGCTCGTG GTTTAGGTAA ACGTGGCTCC AAGTTTGCTT

551 ACATTTGGGT TGGAAACTCT GAGACACAAT GTCCTGGTCA ATGCGCGTGG

601 CCTTTCCACG CGCCGGTGTA CGGTCCACAG AGTCCACCAC TAGTGGCACC

651 AAACAATGAC GTGGGACTCG ATGGGATGGT TATTAACTTA GCTAGTCTTT

701 TAGCTGGAAC CGCAACGAAC CCTTTTGGTA ATGGTTACTA TCAAGGTCCA

751 CAAAACGCAC CGCTTGAAGC TGCTTCTGCT TGTCCTGGCG TTTATGGTAA

801 AGGAGCTTAT CCTGGTTACG CTGGAGATTT GCTTGTGGAT ACTACAACGG

851 GAGGTAGTTT TAATGCGTAT GGTGCAAACG GCAGGAAGTT TTTGCTTCCT

901 GCTTTGTATG ATCCTACGAC GTCGGCTTGC TCTACTATGG TCTGA
```

4.3 Summary

EXORDIUM appears to be a novel gene and its predicted protein has no homology with any protein of known function. Its expression is associated with cell cycle activity, and is up- and down-regulated by auxin and cytokinin treatment respectively.

Fig. 4.7   Predicted EXO sequence

The potentially membrane-bound N-terminal region is underlined and the putative cleavage site is asterisked

```
  1 MYLLVFKLFL FLSLLQISVS A*RNLASQEPN QFQLLKYHKG ALLSGKISVN

 51 LIWYGKFKPS QRAIISDFIT SLTHTSPTSK TLHQPSVATW WKTTEKYYKL

101 ATPSKNSSPL SLTLGKQIID ESCSLGKSLT DKKIQTLASK GDQRNAINVV

151 LTSADVTVTG FGMSRCGTHG HARGLGKRGS KFAYIWVGNS ETQCPGQCAW

201 PFHAPVYGPQ SPPLVAPNND VGLDGMVINL ASLLAGTATN PFGNGYYQGP

251 QNAPLEAASA CPGVYGKGAY PGYAGDLLVD TTTGGSFNAY GANGRKFLLP

301 ALYDPTTSAC STMV
```

5.0 Transgenic Analysis

Previous chapters describe the expression of GUS in the promoter trap line EM2 and the regulation of *EXORDIUM* in a wild-type background. This chapter details the transgenic approach which was undertaken to answer two questions arising from the aforementioned work, namely: 1) Does the GUS expression pattern seen in the EM2 line resemble that of *EXO*? and 2) Would knocking out or overexpressing *EXO* result in a mutant phenotype?

5.1 Promoter Analysis

5.1.1 Creating promoter-GUS constructs

Study of the transgenic line EM2 reveals a consistent and tightly regulated pattern of GUS expression. It has been shown that the T-DNA in this line is inserted 782bp upstream of the ATG of the *EXO* coding region, and so constructs were designed to test the importance of the 782bp immediately upstream of the gene in its regulation.

1542bp of genomic sequence upstream of the *EXO* ATG had been cloned previously by Dr.I.M.Evans (unpublished). This sequence was considered likely to contain the full length promoter driving *EXO* expression in the wild-type, and the first 761bp of the sequence (up to the position at which the T-DNA is inserted) responsible for the GUS expression pattern observed in EM2. Both the full length (1487bp; *EXO*fp) and truncated (715bp, *EXO*tp) promoter fragments were cloned into vectors containing the β-glucuronidase (GUS) reporter gene and transferred to a binary vector for transformation into plants to allow a comparison of GUS expression driven by the two promoters to be made. Fig. 5.1 outlines the approach taken.

In order to clone the promoters, PCR primers were designed to amplify both the full length (*EXO*fp) and the truncated (*EXO*tp) promoter sequences. Positions of the primers used are shown in Fig. 5.2. The primers were designed to include a restriction enzyme recognition site at each end of the amplified product to facilitate subsequent cloning. Primer sequences are detailed in section 2.1.4.

Fig. 5.1   Approach taken to clone promoter::GUS constructs

The *EXO* promoter was amplified as a PCR product using the primers shown in Fig. 5.2 and cloned into the pGUS plasmid upstream of the GUS gene. The promoter::GUS cassette was then transferred into the pCIRCE binary vector plasmid and transferred into plants via Agrobacterium mediated transfer.

GUS

EXO promoter
(PCR product)

pGUS → Promoter
pGUS

Promoter-GUS

pCIRCE
binary
vector

→

Promoter-
GUS-
pCIRCE

↓

Transformed into
plants via
Agrobacterium
mediated gene
transfer.
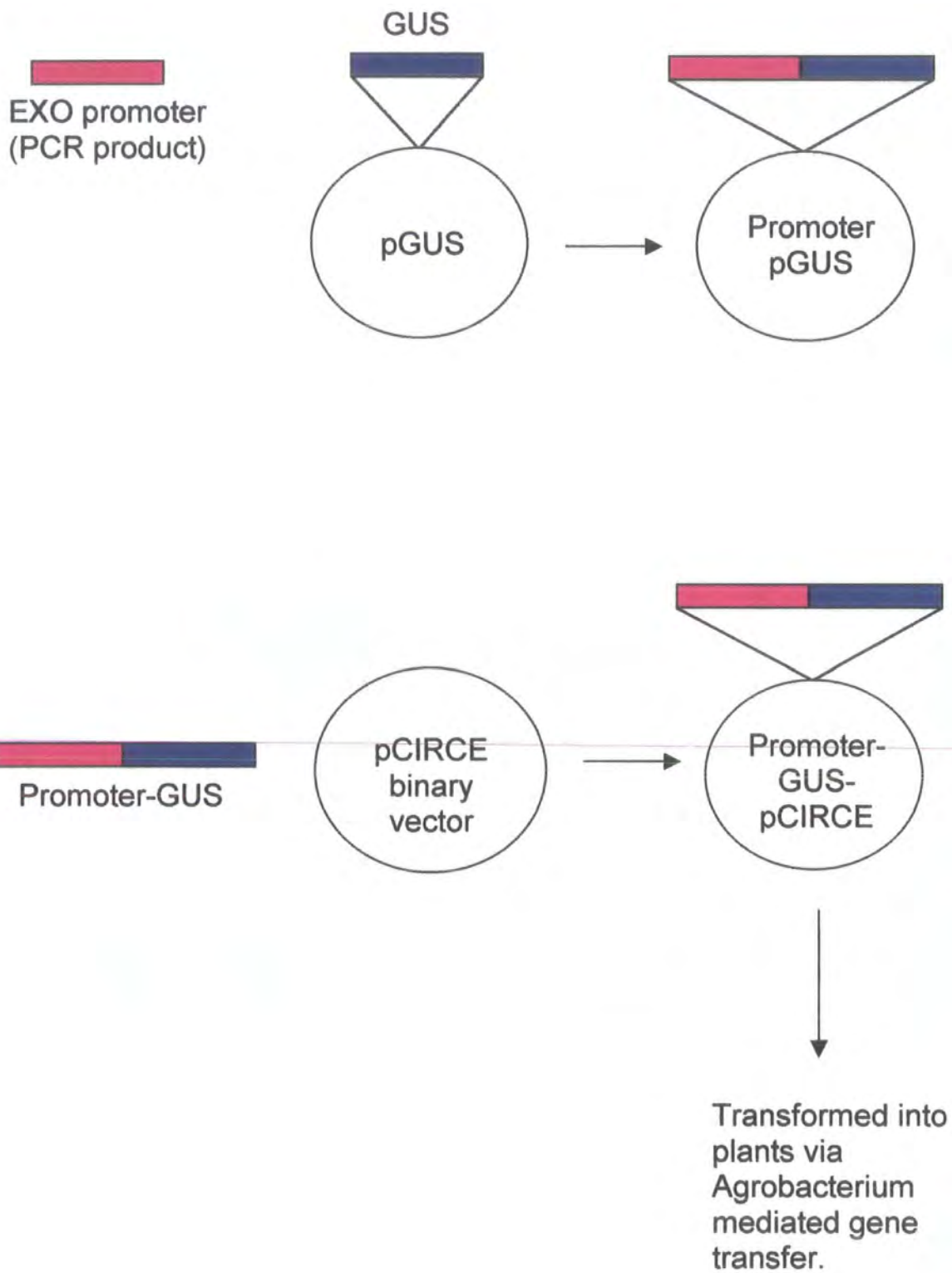
5.1.1.1 PCR for promoter fragments

1) Truncated promoter (*EXO*tp).

A PCR mix containing 1µl of each primer (Promoter start, tp stop), 2µl template DNA (sequence shown in Fig. 5.2, cloned into pGEM), 10µl 10x reaction buffer (including $MgCl_2$), 1µl (3.5units) Expand Taq polymerase (Boehringer Mannheim), 2µl dNTPs and sdH_2O to a final volume of 100µl was split into two tubes. The tubes were heated to 94°C for $2^1/_2$ minutes to denature the DNA template and then the reaction proceeded through 30 cycles of 30s denaturation at 94°C, 1 minute primer annealing at 63°C, and 1 minute product extension at 72°C before a final 10 minutes at 72°C to ensure all products were fully extended. An aliquot of the reaction mix was run out on a gel to confirm the products were of the expected size (Fig. 5.3A). The products were purified using the High Pure PCR kit (Boehringer) as described in section 2.12.2 and quantified by running out an aliquot of the purified product on a gel with DNA markers.

2) Full length promoter (*EXO*fp).

A PCR mix containing 1µl of each primer, 1µl template DNA (sequence shown in Fig. 5.2, cloned into pGEM), 10µl 10x reaction buffer, 4µl $MgCl_2$, 0.5µl taq polymerase (Bioline), 2µl dNTPs and sdH_2O to a final volume of 100µl was split into two tubes and underwent PCR as follows: $2^1/_2$ minutes at 94°C, 30 cycles of 30s at 94°C, 1 minute 55°C, and 2 minutes at 72°C, followed by a final 10 minutes at 72°C. An aliquot of the reaction mix was run out on a gel to confirm the products were of the expected size (Fig. 5.4A) and then cloned directly into TOPO using the TOPO Cloning kit (Invitrogen as described in section 2.12.1. Positive colonies were identified (as described in section 2.12.3, Fig. 5.4B), cultured (as described in section 2.1.6), and the plasmid extracted (as described in section 2.10).

5.1.1.2 Cloning the promoter into the pGUS vector

pGUS is a pUC based plasmid containing the β-glucuronidase gene downstream of a multiple cloning site as shown in Fig. 2.2 (Jefferson, 1987). In order to introduce the promoter into the vector, restriction digestion by endonucleases was performed. The use of two different enzymes allows orientation of the promoter in the vector during subsequent ligation.

Fig. 5.2   Primer sites in the *EXO* promoter

Primers were designed to amplify both the full length promoter and a truncated fragment upstream of the T-DNA insertion site in the EM2 line (asterisked).

Primer sites are shown in pink. 'Promoter Start' (31-51) was used as the foreward promoter for both fragments, 'tp stop' (730-748) was used as the reverse primer for the truncated promoter fragment and 'fp stop' (1504-1521) was used as the reverse primer for the full length promoter. In addition to the sequences shown, primers also carried a restriction endonuclease recognition site to facilitate subsequent cloning, for details see section 2.1.4.

```
   1 ACTTAAAACA AAATTAATGA CAACGTGTAC CGCGCGTGAT AATCACGTGA
  51 CAAATTGATG ACGTGGAAGC GCGTAGTAAC ACGTGCCGAC ATAGTGTCAG
 101 TAAATGCTCT CAAATTACCT ATGGATAGGA ATTGTAGGAT TACTCTCTCA
 151 TCTTCCTCTA TCTACTAACT TGTGCCAGCT GTACACTCTT CCCCATATTT
 201 TCCAGCCTGT CCAAAAAAGT CCTCCCAACG TCTCTCAGAA GTTTCACATT
 251 TTTTCTTTAA CTGCCCACGC GCTTTACCCA CAGAAGGAAA AAAAAACACA
 301 GCTTGCATAA GGGATTTACG TCATTAGATC TGTAACTAAG CTGTATATTC
 351 TTCAACACAT TTTTATCGAT ATGGTCAATT TTGAGTCCTC TTTTTAATCA
 401 ATTTTTTATT AGCTGTATGT AACTATTGCT TAATCTGACA CAATTAAATC
 451 TTTATTAATT GTTTTATAGT TTATATAATA CATATATAAA CACTAATTAT
 501 TCCTTAAATA TTTATTTAGA TTACCACTAG CAACTCTTTT TTATTGGTCC
 551 AACTTGATTT CATTTTCTAA TTTGTAGTAT TAAATAAAAA CATCCACAAC
 601 ATGTACAGCT TTTACAACAG GAATCCTATG TCTCATTCTA GCCTCCATGA
 651 GTAGTCTTAT CCAAATCCAA ATATATTCAA AATATTTGTA TATATTTAAA
 701 AATAAAATTA TACTTAGCAT TATATAATAC TCTTTCTGTT TCATCAAGAT
 751 TGATGTTATA G*GTTTTTTTT CTTATTCCAA AAAGTTGATG TTTTAGATCA
 801 ATATTTTTTT TTACTTTTAT TTGTAACTAA TAGATAGAGC AGAGAAGAGA
 851 GAAATAGTGT CTGATATTAT TAAACAAATA AAAAATAAAA AAATAAAAAA
 901 ATTAATTTTT GTGAAAAACC TACAACATCA AACTTTATGG AACGGATGGA
 951 GTATTTATAT GAAGTTAAAC ATAGCATTAT ACATATTTTT ATGAAAACTT
1001 TTGATGAAAA TCATTAAAGA AACTGAGGAA TTTATAATGA CATTTTCACA
1051 TTATAACCAT AACATTGAAA AATAAGAATT TTAAACCAAA GTTGTATGTA
1101 ATGAATAAGT TGTCGGACTC GAAACTGAGT AAATTATAAT GACATTTGAC
1151 TATATGACCA TAACATAATC AAGAAAATTC AAATTTTGAC ATAAACCAAA
1201 AGTTGTGTAG AACTATGGAT CATTCGTTAG CTGGCAACAG TACAATGGTC
1251 CATTACTCCT TTGTATCTTT TCTTTCAATT TCTCTGCACC TTCCCTTTCT
1301 CTTTCGCTTT AAAGCATAAT AATAACATAC CTCTCTCTTT TTCCACCTTT
1351 ATTATTTAAA AATCATATTC AAAATCTAAT TTCCATTTTT AATAAAAATA
1401 AGTCCACTCT GAGAGAGGCA TATATATAGC CCCATCACAC TCCATACATC
1451 TCACAAACCA ATCTAAACAT CAAAAGACAA AAACCAAAGC TCTTTCACTA
1501 TTACACAACC TTCTATTTCA CTTTTCCTCA AAAAAAAGAA AAATG
```

1) Truncated promoter

Both the purified truncated promoter PCR product and pGUS were digested with the enzymes BamH1 and Xba1 as described in section 2.13.1, the products were run out on a gel to allow purification of the vector and insert DNA. The required bands were excised from the gel under UV light and the DNA purified using the Geneclean kit (Scotlab) as described in section 2.9.2. Following purification an aliquot of the vector and insert DNA was run out on a gel with DNA markers to quantify each product (Fig. 5.3B).

Ligation occurs efficiently at a 3:1 insert:vector molar ratio (Promega Protocols and Applications Guide 1996). Accordingly, a ligation mixture containing GUS vector, purified truncated promoter, T4 ligase, 10x buffer and sdH$_2$O was incubated in a tube for ten minutes at room temperature before transformation by electroporation into XL1blue *E.coli* cells as described in section 2.13.3.

2) Full length promoter

*EXO*fp-TOPO was digested using Xba1 and BamH1 as described in section 2.13.1, and run out on a gel (Fig. 5.4C). The promoter fragment was excised and purified using the Geneclean kit, as described in section 2.9.2. pGUS was digested in two stages as the restriction sites were located very close to one another which resulted in a decreased efficiency of digestion. The plasmid was first digested with Bam HI for 2h at 37°C. The enzyme was then heat inactivated for 10 minutes at 65°C and the linearised plasmid was then digested with Xba1 for 2h at 37°C. The mixture was incubated a second time for ten minutes at 65°C to inactivate the second enzyme. The vector and insert were run out to calculate their relative concentrations, and a 3:1 molar ratio of insert:vector was calculated. Vector and insert were ligated and transformed into *E.coli*.

Positive colonies were identified from both transformations by PCR (Figures 5.3C and 5.4E). Restriction analysis of the plasmids isolated from the positive colonies was performed using Bam H1 and Xba1 to ensure they were not false positives (Figures 5.3D and 5.4F). All but one of the *EXO*tp-pGUS colonies were true positives. DNA from a single positive colony from each transformation was sent for sequencing to

Fig. 5.3 Cloning of the tp::GUS construct

A   The truncated promoter (tp) was amplified by PCR and demonstrated to be of the expected size

B   The truncated promoter fragment and pGUS vector were digested using compatible restriction endonucleases and purified. The vector and insert were then analysed by gel electrophoresis with DNA markers to quantify each product prior to ligation and transformation into *E.coli*.

C   Colony PCR was performed using the tp primers . Five putative positives were identfied.

D   Restriction analysis of the putative positives confirmed that four of the five (asterisked) were true positives containing the truncated promoter.

E   The truncated promoter::pGUS plasmid and the pCIRCE plasmid were digested using compatible restriction endonucleases and analysed with DNA markers to confirm complete digestion had occurred prior to ligation and transformation into *E.coli*.

F   Identification of positives by colony PCR. True positives are asterisked. One of these was subsequently sequenced and transformed into plants via Agrobacterium mediated gene transfer.

750bp

5.5kb

750bp

A

B

C

D

* * * *

>10kb

3kb
2.8kb

E

F

*   *

Fig. 5.4   Cloning of the fp::GUS construct

A   The full length promoter (fp) was amplified by PCR and demonstrated to be of the expected size. The fragment was then cloned directly into the TOPO vector.
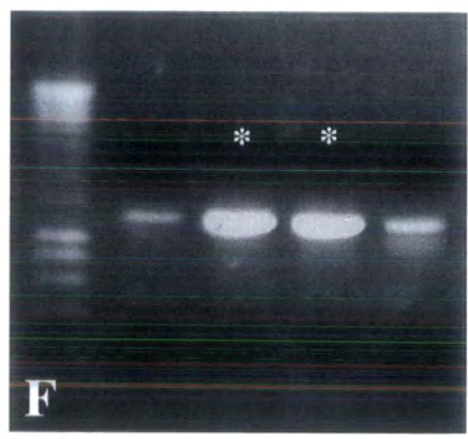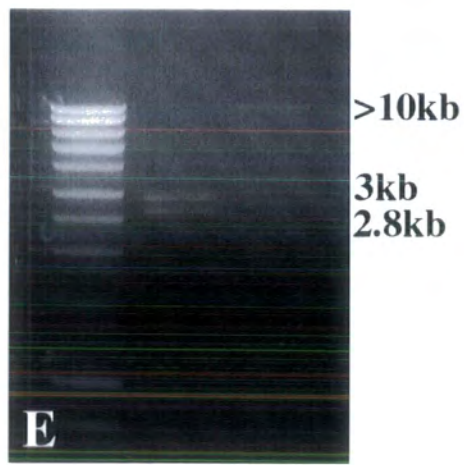
B   Colony PCR using the fp primers was used to identfy colonies containing the promoter fragment.

C   The promoter fragment was released from TOPO using restriction endomucleases. The 1.5kb fragment was then cut from the gel and purified
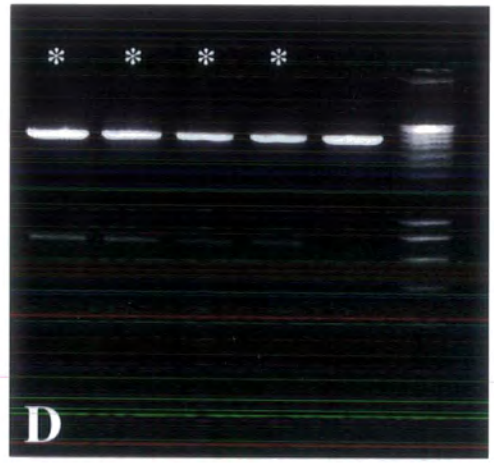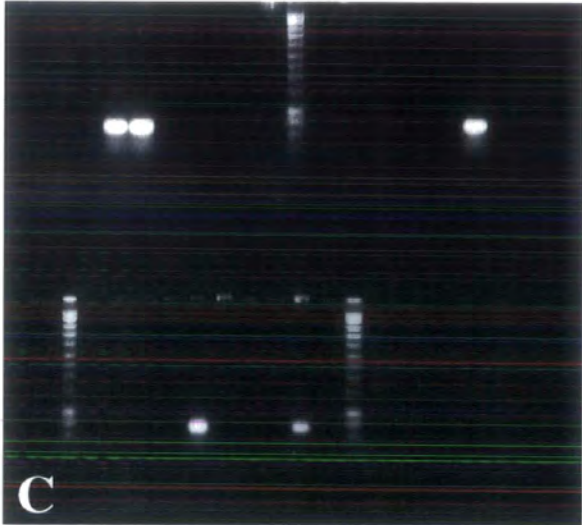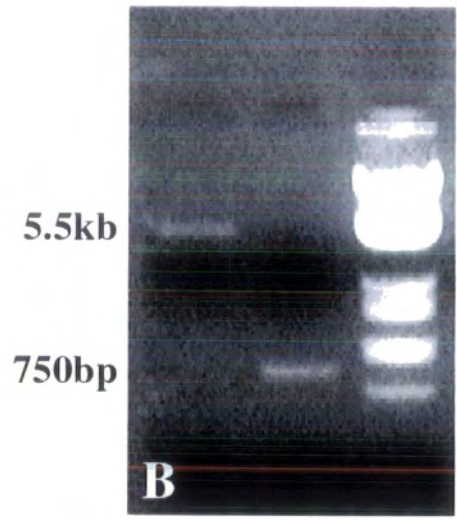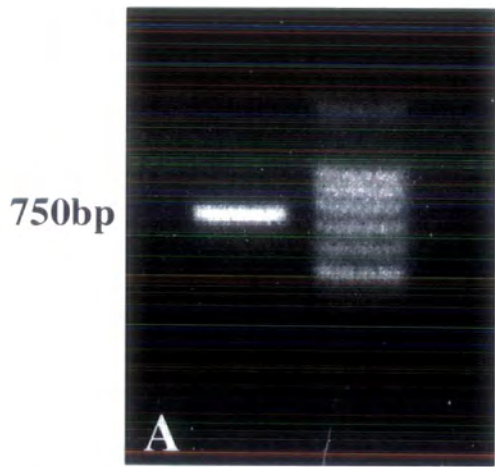
D   The truncated promoter fragment and pGUS vector were digested using compatible restriction endonucleases and purified. The vector and insert were then analysed by gel electrophoresis with DNA markers to quantify each product prior to ligation and transformation into *E.coli*.

E   Colony PCR was performed using the fp primers . Five putative positives were identified.

F   Restriction analysis of the putative positives confirmed that all five colonies were true positives containing the full length promoter.

G   The truncated promoter::pGUS plasmid and the pCIRCE plasmid were digested using compatible restriction endonucleases. The promoter::GUS cassette was cut from a gel, purified and analysed by gel electrophoresis along with the digested pCIRCE vector to establish their relative concentrations prior to ligation and transformation into *E.coli*.

H   Identification of positives by colony PCR.

I   Restriction analysis of the putative positives showing six of the nine were true positives. One of these was subsequently sequenced and transformed into plants via Agrobacterium mediated gene transfer.

1.5kb

A

B

1.5
kb

C

1.5kb

D

E

3.5
2.7
kb

F

~10kb

~4kb

G

H

2.0kb
1.5kb

I
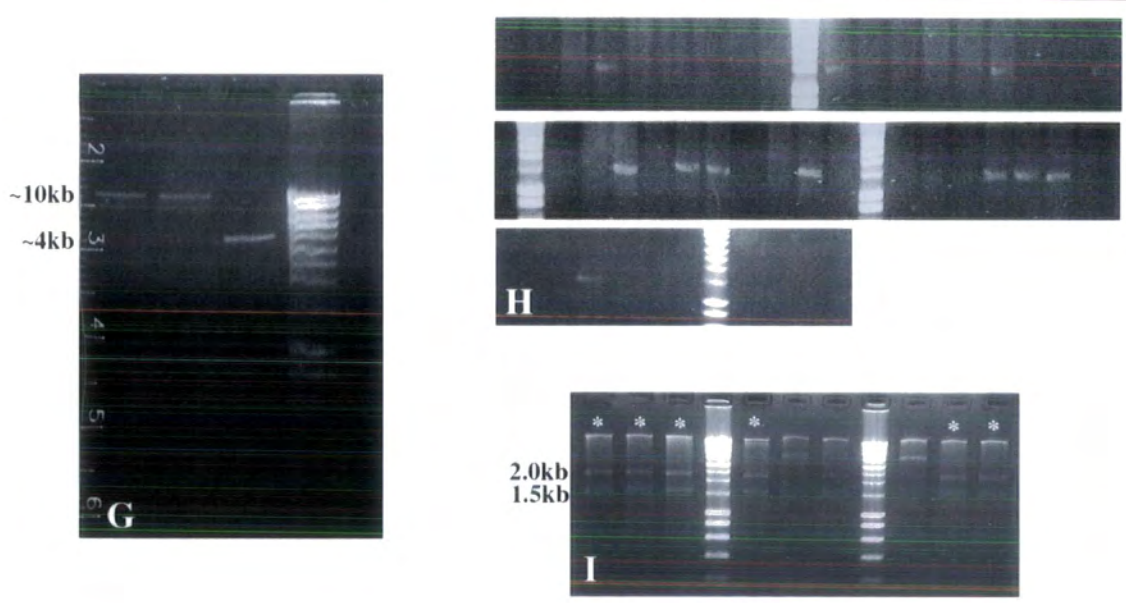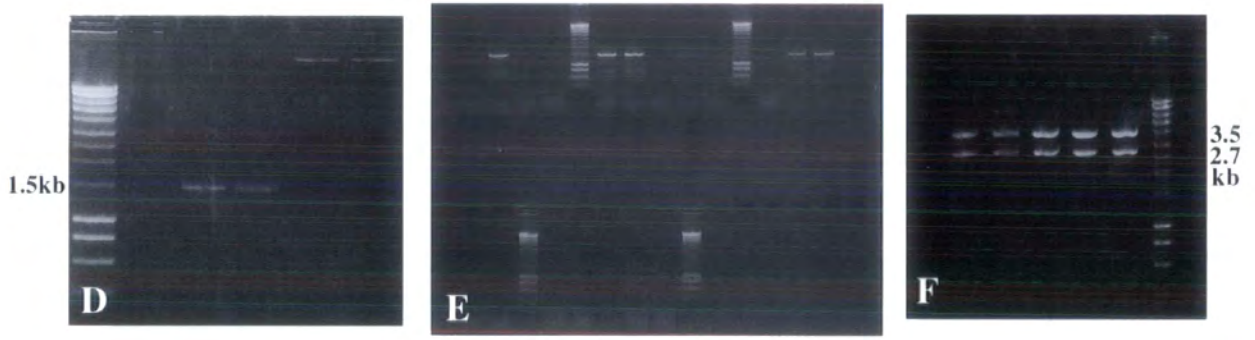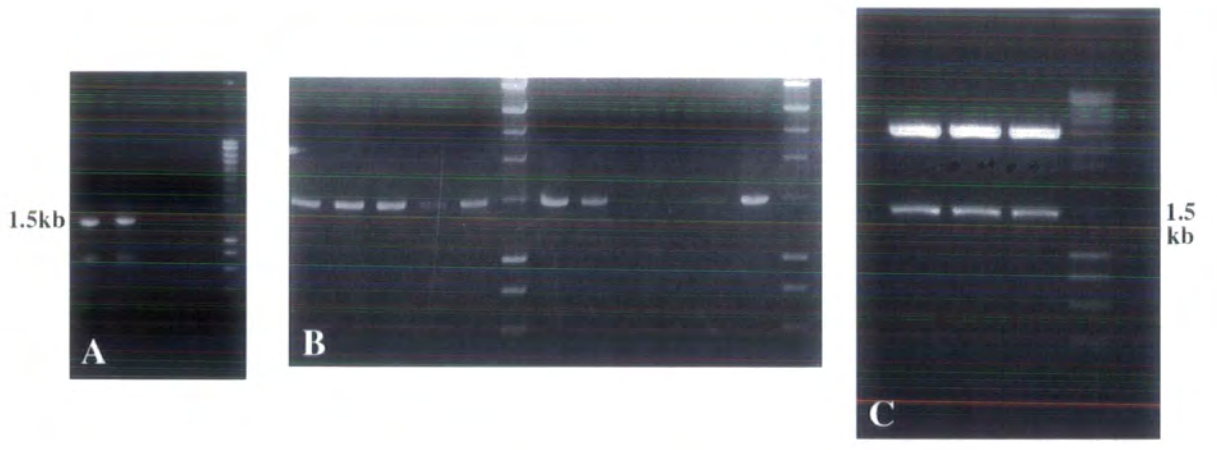
check for any errors prior to subsequent cloning and all fragments used were confirmed as being of correct sequence.

5.1.1.3 Cloning cassette into Binary vector pCIRCE

Both promoter-GUS plasmids and pCIRCE were digested with HindIII and EcoRI and run out on a gel to ensure complete restriction had occurred (Figures 5.3e and 5.4G). The promoter-GUS cassettes were ligated into pCIRCE, transformed into XL1blue cells and positives selected (Figures 5.3f and 5.4h). A single positive colony was selected from each transformation and their DNA sequenced to confirm there were no errors prior to transformation into plants.

5.1.1.4 Introducing the cassette into plants

E.coli colonies containing the constructs were grown up and the constructs were introduced into *Agrobacterium* by either tri-parental mating or electroporation, as described in section 2.15.1. Plants were transformed using the dipping method described in section 2.15.3, before being grown to maturity, and their seed collected. Seed was germinated on 35mg/l kanamycin to select for transformants.

5.1.2 Analysis of GUS expression in transgenic plants

5.1.2.1 Primary analysis of promoter-GUS transformants

Kanamycin selection for transformants generated four resistant plants containing the full length promoter construct. These were named *EXO*FPG1, *EXO*FPG2, *EXO*FPG3 and *EXO*FPG4. No plants containing the truncated promoter construct were successfully obtained. *EXO*FPG1, *EXO*FPG2, *EXO*FPG3, and *EXO*FPG4 were removed from Petri dishes to be analysed for GUS activity and planted in soil to set seed. Portions of root and a mature leaf were removed from each plant and stained histochemically overnight. Although not identical, all four showed GUS activity in a restricted pattern, which is similar in each of the lines. The results are shown in figures 5.5-5.8. Staining in the leaf is patchy in lines *EXO*FPG1, *EXO*FPG2 and *EXO*FPG3 but appears constitutive in the *EXO*FPG4 leaf. Expression in the root is similar in the four lines. GUS expression is observed in the majority of root tips and in the vascular tissue of the root, although not continuously throughout all vascular tissue. At least one root tip in each line is either unstained or shows very little staining with respect to the majority of tips. There is no staining in other parts of the root.

Figures 5.5 – 5.8 show GUS activity in individual transgenic lines generated with the full length EXORDIUM promoter fused to the GUS reporter gene

Fig. 5.5   *EXO*FPG1 GUS activity

A + B Staining in the root tip as in EM2
    Additional staining in the vascular tissue not observed in EM2

Scale bars = 0.1cm

C   Patchy staining in the leaf

Scale bars = 0.3cm

Fig. 5.6   *EXO*FPG2 GUS activity

A   Staining in the root tip as in EM2
       Additional staining in the vascular tissue not observed in EM2

Scale bar = 0.1cm

B   Patchy staining in the leaf

Scale bar = 0.15cm

Fig. 5.5

Fig. 5.6

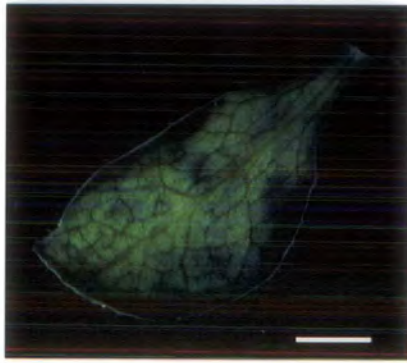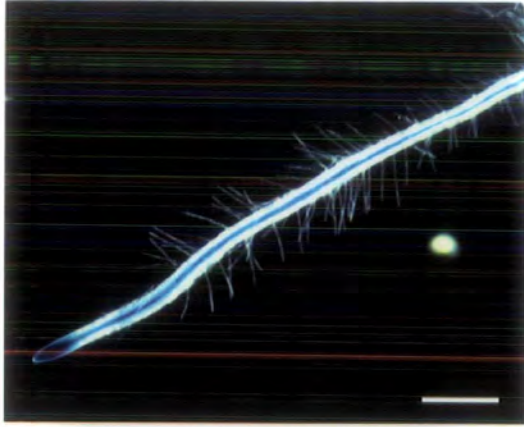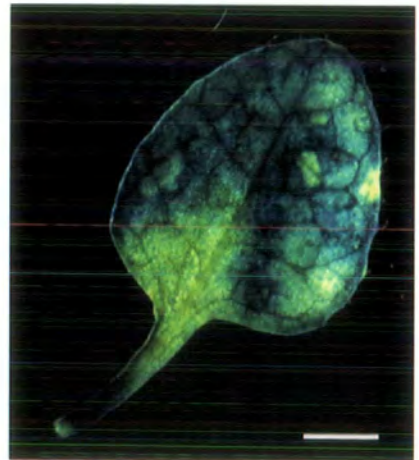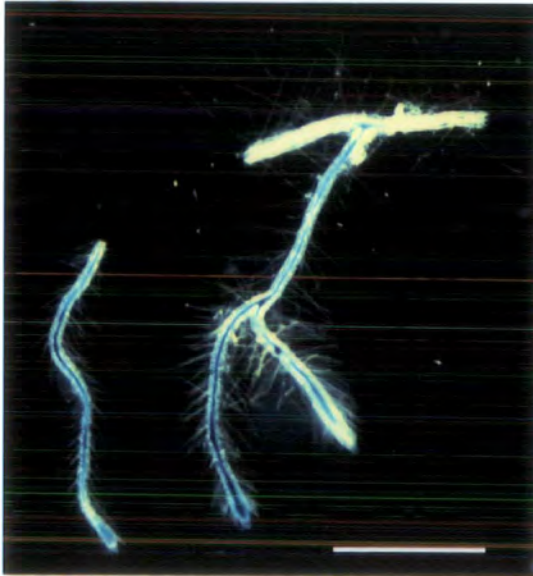## Fig. 5.7 *EXO*FPG3 GUS activity

A   Staining in the root tip as in EM2
       Additional staining in the vascular tissue not observed in EM2

B   Patchy staining in the leaf

Scale bars = 0.2cm

Fig. 5.8 *EXO*FPG4

Staining in the root tip as in EM2
Additional staining in the vascular tissue not observed in EM2

Intense staining throughout the leaf

Scale bar = 0.5cm

Fig. 5.7
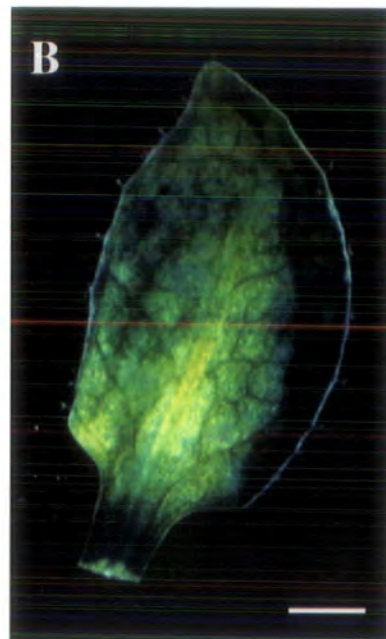
Fig 5.8
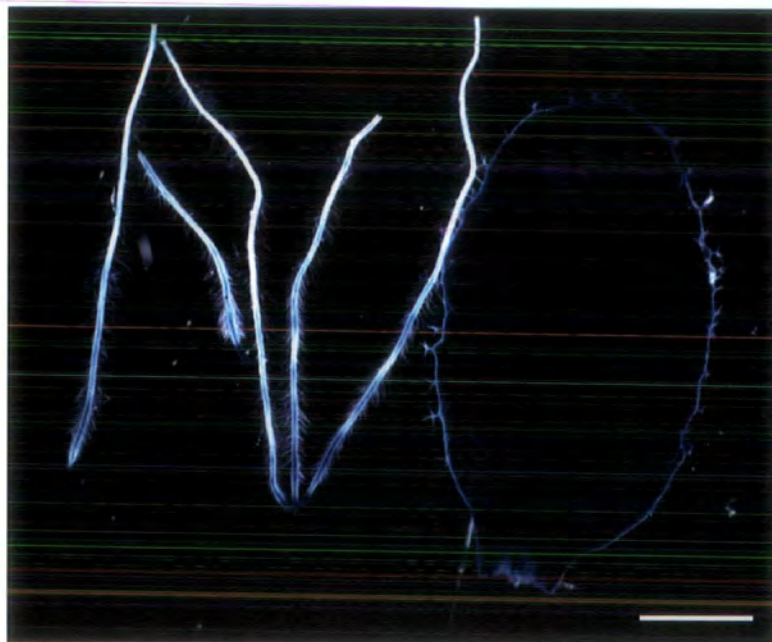
5.1.2.2 Analysis of GUS activity in the embryo

Following growth in soil, siliques of various stages of development were removed from plants *EXO*FPG1, *EXO*FPG2, and *EXO*FPG3 and analysed for GUS expression by histology. *EXO*FPG4 was isolated at a later date and so was not screened for GUS activity in the embryo. In order to stain embryos, siliques were split using a fine pointed needle, and each seed pierced individually to facilitate penetration by the histochemical buffer and substrate. This was achieved by two five minute vacuum infiltrations prior to incubation overnight at 37°C.

*EXO*FPG1, *EXO*FPG2 and *EXO*FPG3 have very similar patterns of GUS activity in the silique as shown in Figures 5.9A, 5.10A and 5.11A respectively. In all three lines GUS activity appears to be constitutive throughout the embryo from the globular stage onwards (Figures 5.9, 5.10, 5.11). No embryos of an earlier developmental stage were screened. In each line staining in young embryos (globular and heart stages) is intense, becoming less so by the torpedo stage and greatly diminished in the mature embryo.

5.1.2.3 Comparison with the EM2 line

Staining in lines *EXO*FPG1, *EXO*CFPG, *EXO*FPG3 and *EXO*FPG4, which contain the full length promoter fused to the GUS gene, show similarity to the staining observed in the EM2 line in which the GUS gene is inserted in the promoter 782bp upstream of the main open reading frame. The EM2 line was isolated from a screen for GUS expression in the early embryo, and the three lines generated which have been analysed for GUS expression in the embryo also show constitutive expression from an early stage. Further analysis is required to ascertain whether expression in the four lines can be observed earlier in embryogenesis. Unlike the EM2 line, *EXO*FPG1, *EXO*FPG2 and *EXO*FPG3 show staining in parts of the silique other than the embryo, including the silique itself, the testa and the endosperm.

GUS activity in the root of the four transformants generated appears in a less restricted pattern than that seen in EM2, with staining observed in the vascular tissues of *EXO*FPG1, *EXO*FPG2, *EXO*FPG3 and *EXO*FPG4. Direct comparison of the four lines is difficult as each line represents an independent transformation, and the

Figures 5.9 – 5.11 show GUS activity in individual transgenic lines generated with the full length EXORDIUM promoter fused to the GUS reporter gene

Fig. 5.9   *EXO*FPG1 GUS activity in the silique

A   Staining in the silique wall, becoming diminished in mature siliques

Scale bar = 0.5cm

B   Intense staining throughout the heart stage embryo (H)
     H = heat stage embryo

C   Intense staining throughout the torpedo stage embryo (T)

D   Intense staining in the globular embryo (G)
     Less intense staining in the cotyledonary stage embryo (top right)

Scale bars = 0.1cm
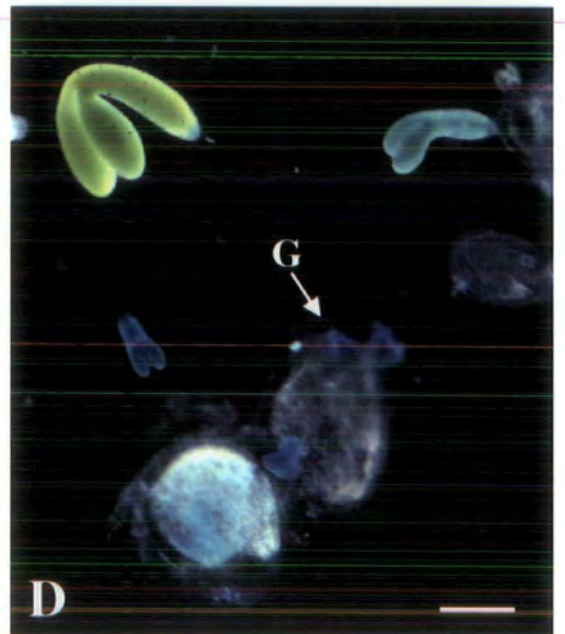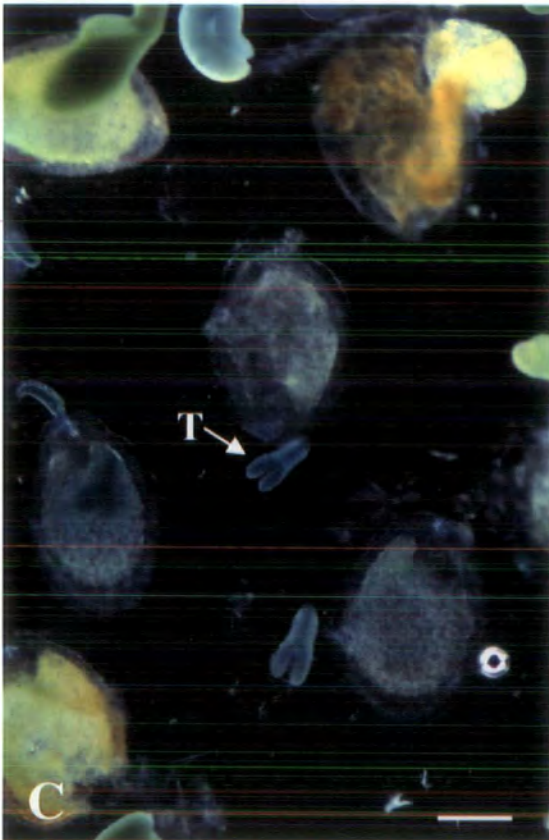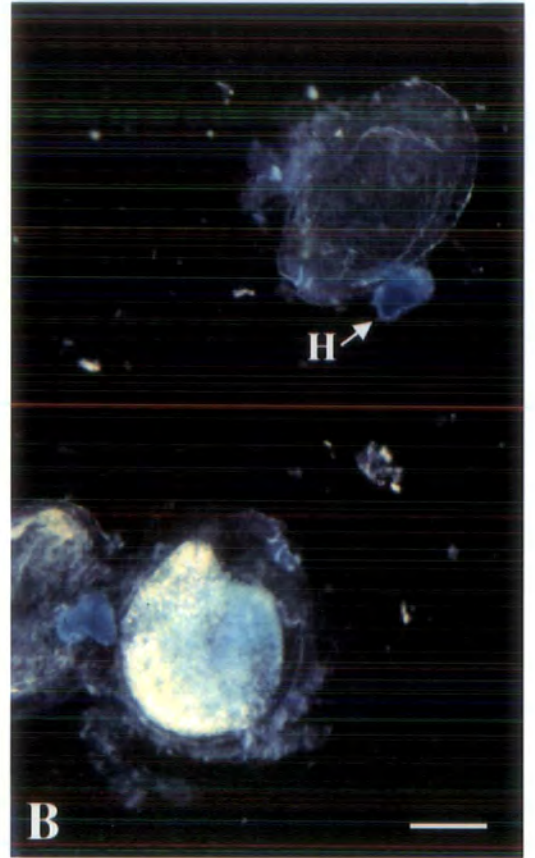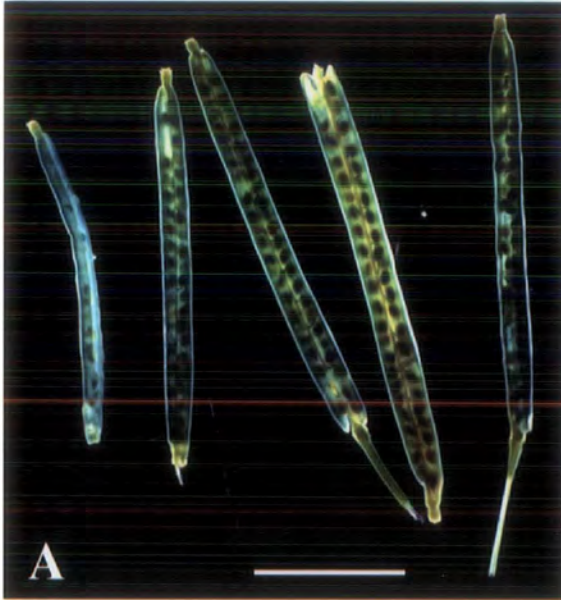
Fig. 5.10 *EXO*FPG2 GUS activity in the silique

A   Staining in the silique wall, becoming diminished in mature siliques

Scale bar = 0.5cm

B   Intense staining throughout the heart stage embryo (H)
        H = heat stage embryo

C   Intense staining throughout the globular embryo (G)

D   Intense staining in the torpedo stage embryo (T)

Scale bars = 0.1cm
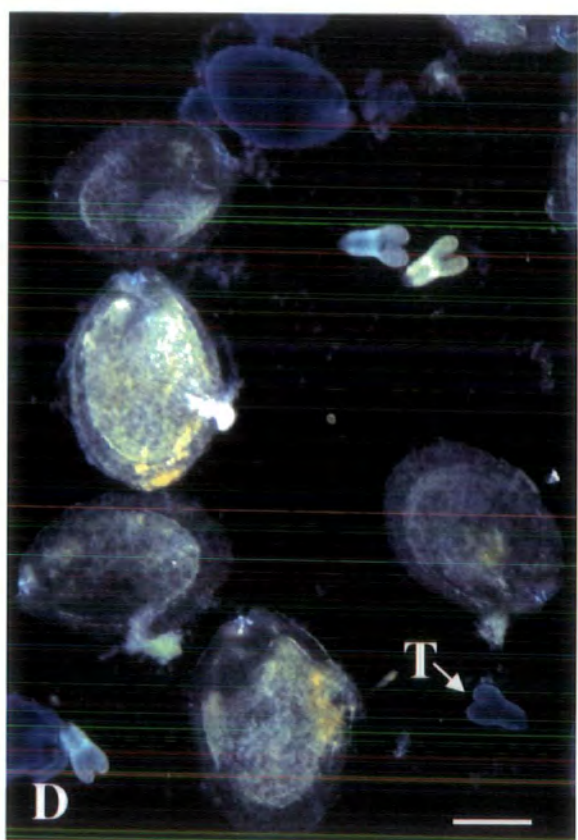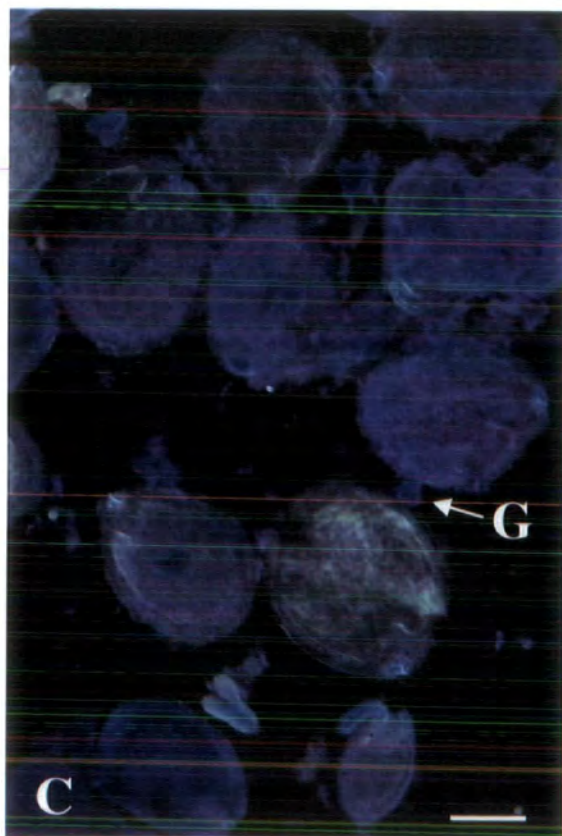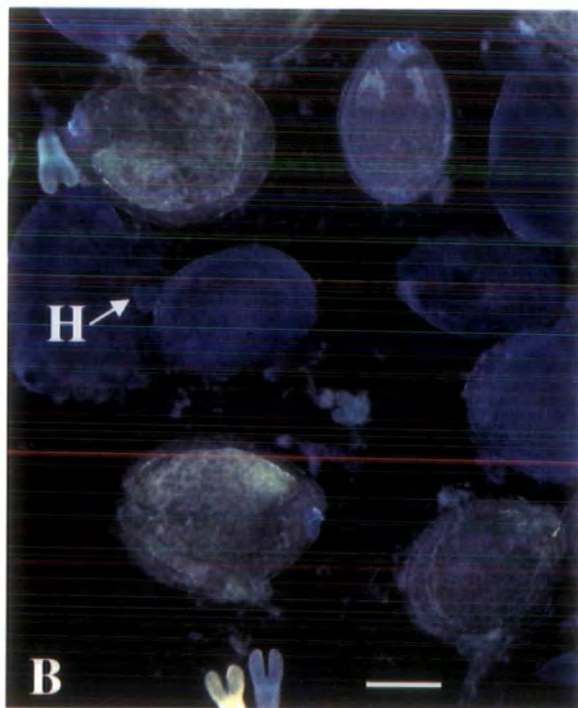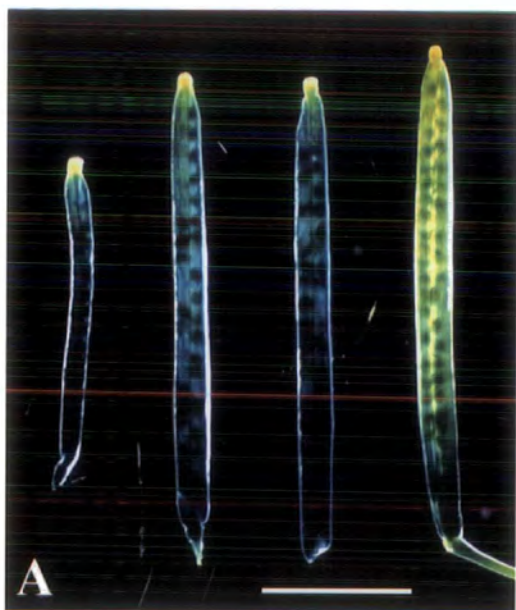
Fig. 5.11   *EXO*FPG3 GUS activity in the silique

A   Staining in the silique wall

Scale bar = 0.5cm

B   Intense staining throughout the globular embryo (G)

Scale bar = 0.1cm

C   Intense staining throughout the heart stage embryo (H)

Scale bar = 0.05cm

D   Intense staining in the torpedo stage embryo (T)
        Less intense staining in the mature cotyledonary stage embryo (M)

Scale bar = 0.1cm
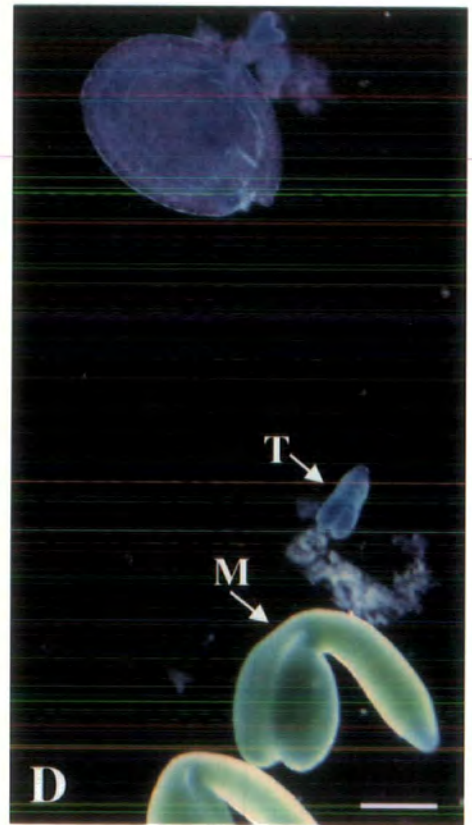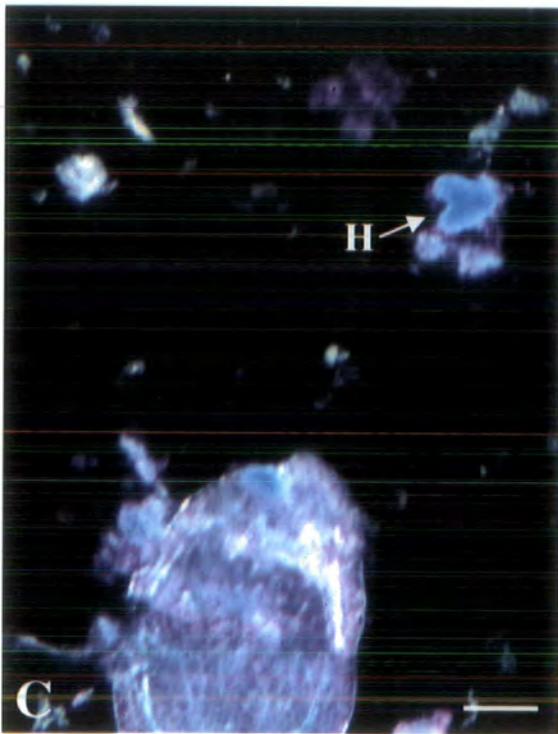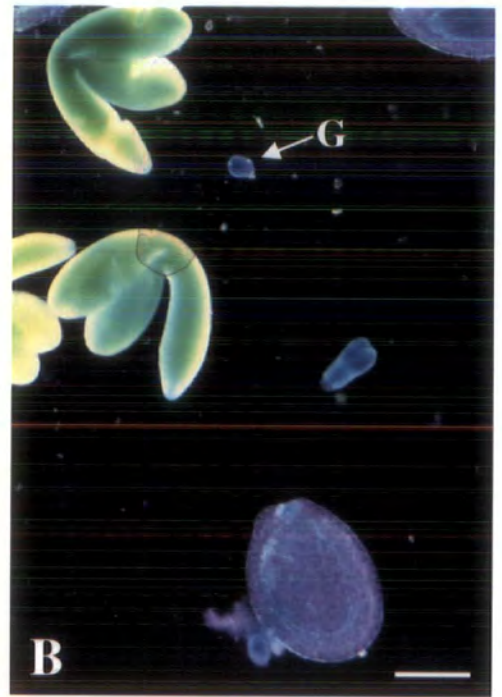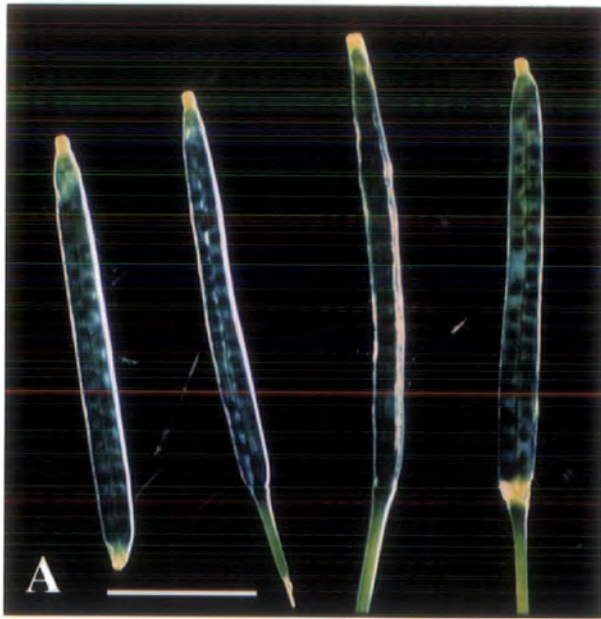
position of the integration event may influence the level of GUS expression. The intense staining observed in the leaf of the *EXO*FPG4 line and the endosperm of the *EXO*FPG2 line may represent an enhanced level of expression in these lines rather than a difference in spatial regulation of expression.

5.2   Antisense and overexpression analysis

Northern analysis of the promoter trap line EM2 reveals that the presence of the T-DNA causes a significant decrease in the mRNA levels with respect to the wild-type (see section 4.1.1). The line does not however represent a complete knockout of *EXORDIUM* as low levels of expression are present in the EM2 line (Fig. 4.2). This low level of expression may be sufficient for gene function and thereby account for the lack of phenotype in the promoter trap line. An alternative hypothesis is that the gene is functionally redundant, and the identification of a small gene family in *Arabidopsis* adds weight to this possibility.

5.2.1   Creating sense and antisense plants

In order to test whether knocking out *EXO* and closely related genes would lead to a mutant phenotype that may elucidate their function, antisense analysis was employed. A constitutive promoter was used instead of the *EXO* promoter in order to knock out any homologues that might be regulated differently. Simultaneously, overexpression analysis, also using the constitutive promoter, was performed to determine whether excess and/or ectopic *EXO* expression would cause an aberrant phenotype.

Constructs were designed using the cDNA sequence cloned previously by Dr.I.M.Evans (unpublished) cloned into the DH51 plasmid, which contains the CaMV 35S constitutive promoter and terminator (Pietrzak *et al.* 1986). These were then transferred to a binary vector for transformation into plants. Figure 5.12 outlines the approach taken.

In order to clone the cDNA, primers were designed to amplify the sequence with added Xba1 endonuclease recognition sequences at each end to facilitate subsequent cloning. Positions of the primers are shown in figure 5.13 and primer sequences are detailed in section 2.1.4.

Fig. 5.12 Approach taken to clone the *EXORDIUM* sense and antisense constructs

The *EXO* cDNA was amplified as a PCR product using the primers shown in Fig. 5.13 and cloned into the pDH51 plasmid downstream of the CaMV35S constitutive promoter. The promoter::*EXO* cassette was then transferred into the pCIRCE binary vector plasmid and transferred into plants via Agrobacterium mediated transfer.

EXO cDNA
(PCR product)

35S promoter

pDH51

→

pDH51-EXO

Sense + antisense

35S-EXO cassette
(sense + antisense)

pCIRCE
binary
vector

→

35S-pCIRCE

Sense + antisense
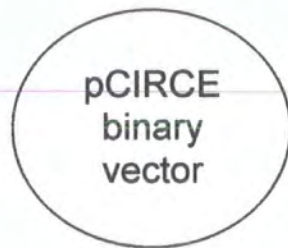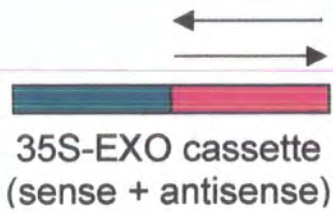
Transformed into
plants via
Agrobacterium
mediated gene
transfer.

5.2.1.1 PCR for *EXO* cDNA

The cDNA sequence was amplified using a PCR mix consisting of 1μl of each primer (Promoter start, fp stop), 2μl template DNA (sequence shown in Fig. 5.13, cloned into pGEM), 2μl dNTPs, 10μl 10x buffer (including $MgCl_2$), 1μl Expand Taq polymerase and sdH_2O to a total volume of 100μl. The mixture was split into two tubes and heated to 94°C for $2^1/_2$ minutes to denature the template DNA. The reaction mix subsequently underwent 30 cycles of denaturation at 94°C for 30s, primer annealing at 60°C for 1 minute, and product extension at 68°C for 1 minute followed by a final extension period of ten minutes at 68°C to ensure all products were fully extended. An aliquot of the reaction products was run out on a gel to ensure all products were of the expected size (Fig. 5.14A), and the product was then cloned into the TOPO TA PCR II vector using the TOPO Cloning kit (Invitrogen) as described in section 2.13.4. Positive colonies were identified by PCR (Fig. 5.14B) and the plasmid isolated and sent to be sequenced. All sequences were confirmed to be correct prior to subsequent cloning.

5.2.1.2 Cloning the cDNA into the pDH51 vector

pDH51 is a pUC18-based plasmid containing the CaMV 35S promoter and terminator with a multiple cloning site between them. In order to introduce the *EXO* coding region into the vector, restriction digestion by endonucleases was performed. A single enzyme was used as it was expected to produce a mixture of plasmids containing *EXO* in both the forward (sense) and reverse (antisense) orientation.

In order to introduce the *EXO* coding region into the pDH51 vector, both *EXO*-TOPO and pDH51 were digested with Xba1 overnight at 37°C as described in section 2.13.1. The insert was excised from a gel (Fig. 5.14C) and purified using the Geneclean kit as described in section 2.9.2. The vector was heated to 70°C for 20 minutes to inactivate the enzyme, and dephosphorylated using shrimp alkaline phosphatase (Sigma) to prevent self religation of the vector, as described in section 2.9.2. An aliquot of the vector and insert were run out on a gel in order for a comparison of concentrations to be made (Fig. 5.14D). A 3:1 (insert:vector) molar ratio was calculated, and the *EXO* coding sequence and pDH51 were ligated as described in section 2.13.2 and transformed into *E.coli* as described in section 2.13.3. A single enzyme was used as it

Fig. 5.13   Promoter sites used to clone the EXO coding region

The *EXO* cDNA is underlined with the ATG shown in bold.
Primer sites are shown in pink. 'EXOf' (12-27) was used as the foreward promoter and 'EXOr' (1094-1107) was used as the reverse primer.

In addition to the sequences shown, primers also carried a restriction endonuclease recognition site to facilitate subsequent cloning, for details see section 2.1.4.

```
   1 TAATAATAAA TCCACTCTGA GAGAGGCATA TATATAGCCC CATCACACTC
  51 TCATATATCT CACAAACCAA TCTAAACATC AAAGACAAAA ACCAAACCTC
 101 TTTCACTATT ACACTTTTCC TCAAAAAAAA GAAAAATGTA TTTGTTAGTG
 151 TTTAAACTCT TTCTATTTTT GTCTCTTCTT CAAATCTCTG TCTCTGCTAG
 201 AAACCTAGCT TCACAAGAAC CAAACCAGTT TCAACTACTC AAGTACCACA
 251 AAGGAGCTCT TCTCTCCGGC AAAATCTCCG TTAACCTAAT CTGGTACGGC
 301 AAATTCAAAC CATCTCAAAG AGCAATCATC TCCGACTTCA TTACCTCACT
 351 CACACACACT TCTCCTACGT CCAAAACTCT CCACCAACCA TCTGTTGCCA
 401 CGTGGTGGAA AACCACAGAA AAATACTACA AACTCGCAAC ACCCAGCAAA
 451 AACTCATCAC CTCTGTCTCT AACTCTCGGG AAACAAATCA TCGACGAGTC
 501 TTGCTCTCTA GGAAAATCTT TAACCGATAA AAAGATTCAA ACCCTAGCTT
 551 CGAAAGGAGA CCAACGCAAC GCCATCAACG TCGTTTTGAC TTCAGCTGAC
 601 GTTACGGTTA CAGGATTTGG TATGAGTCGT TGCGGGACTC ACGGACACGC
 651 TCGTGGTTTA GGTAAACGTG GCTCCAAGTT TGCTTACATT TGGGTTGGAA
 701 ACTCTGAGAC ACAATGTCCT GGTCAATGCG CGTGGCCTTT CCACGCGCCG
 751 GTGTACGGTC CACAGAGTCC ACCACTAGTG GCACCAAACA ATGACGTGGG
 801 ACTCGATGGG ATGGTTATTA ACTTAGCTAG TCTTTTAGCT GGAACCGCAA
 851 CGAACCCTTT TGGTAATGGT TACTATCAAG GTCCACAAAA CGCACCGCTT
 901 GAAGCTGCTT CTGCTTGTCC TGGCGTTTAT GGTAAAGGAG CTTATCCTGG
 951 TTACGCTGGA GATTTGCTTG TGGATACTAC AACGGGAGGT AGTTTTAATG
1001 CGTATGGTGC AAACGGCAGG AAGTTTTTGC TTCCTGCTTT GTATGATCCT
1051 ACGACGTCGG CTTGCTCTAC TATGGTCTGA ATACTAAAAA AAAGTCCAAT
1101 GTTTGGTCTT TGGTAGTAGA AAATAATATT CTAGCGTAAT TCTTTTGTTA
1151 TGTTTGGTTA CAAATTCTAG TATAATTCAA TTTATTCATT AATATTATTC
```

Fig. 5.14   Cloning of the *EXORDIUM* sense and antisense constructs

A   The *EXO* cDNA was amplified by PCR and demonstrated to be of the exected size (1.1kb). The fragment was then cloned into the TOPO vector.

B   Colony PCR using the cDNA primers to identify colonies containing the promoter fragment.

C   The cDNA fragment was isolated from the TOPO vector by restriction nuclease digestion with a single enzyme using the recognition sequences included in the primers. The 1.1kb fragment was cut out and purified.

D   Following digestion of the pDH51 vector, which contains the CaMV35S promoter, with a single restriction endonuclease, both vector and insert were analysed to determine their relative concentrations prior to ligation and transformation into *E.coli*

E   Identification of positives by colony PCR. Five putative positives were identified, numbered 2, 5, 8, 15 and 22.

F   Restriction analysis to determine the orientation of the cDNA in each colony. Colonies 2, 8, and 22 have inserts in the sense orientation and colonies 5 and 15 are antisense.

G   The 35S::sense and 35S::antisense cassettes and the pCIRCE vector were digested with complementary restriction endonucleases and analysed to determine their relative concentrations prior to ligation and transformation into *E.coli*.

H   Colony PCR to identify positives. S: sense positives, A: antisense positives. A single colony was selected for subsequent transformation into plants via Agrobacterium mediated gene transfer.
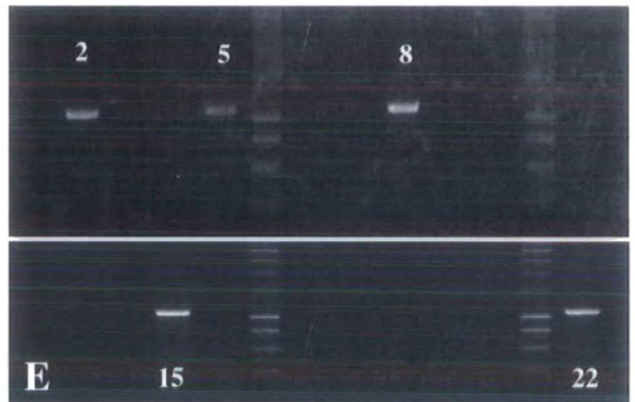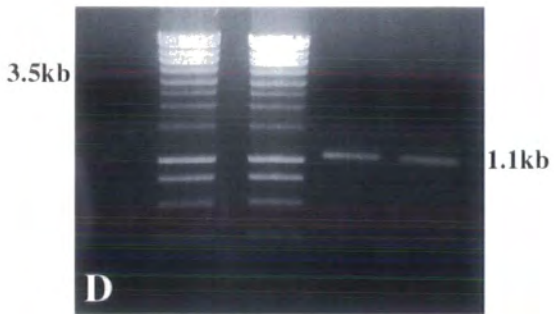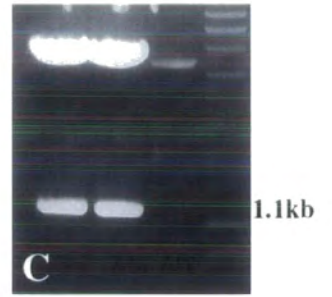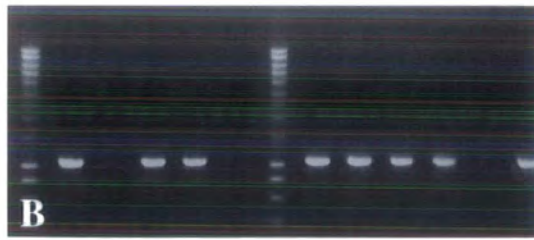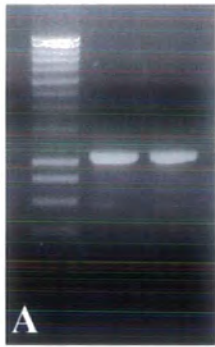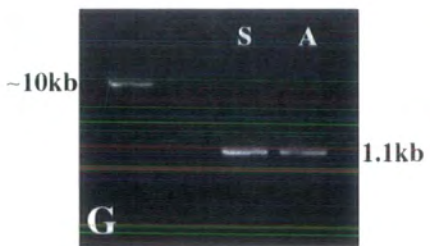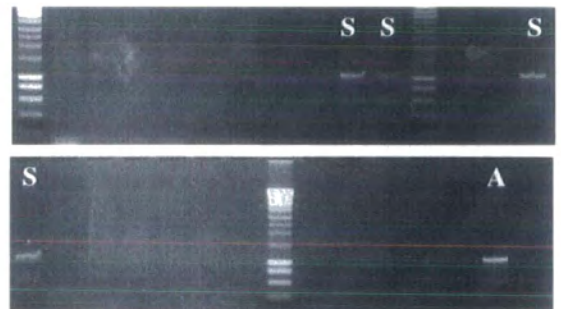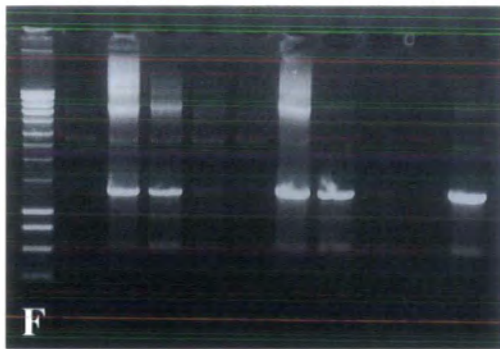
was expected to produce a mixture of plasmids containing the *EXO* insert in both the forward (sense) and reverse (antisense) orientations. Positive colonies were identified by PCR (Fig. 5.14E) and the plasmid isolated and analysed for orientation of the *EXO* insert.

In order to orientate the *EXO* insert, the M13R primer site situated upstream of the 35S promoter in the pDH51 vector was utilised. Two PCR reactions were set up for each positive colony, one containing the M13R primer and the EXOf primer (reaction A) and the other containing M13R and EXOr (reaction B). For each colony, only one *EXO* primer should produce a PCR product with the M13R primer, and the orientation of the insert may be deduced by observing which primer produces a product. If the *EXO* insert is in the sense orientation, reaction B should produce a product and if the insert is in the antisense orientation, reaction A should produce a product. Three colonies were identified as containing the *EXO* fragment in the sense orientation and two contained *EXO* in the antisense orientatation (Fig. 5.14F). A single colony was selected for each orientation and the isolated plasmid sent for sequence analysis to ensure there were no errors prior to subsequent cloning.

5.2.1.3 Cloning the sense and antisense cassettes into pCIRCE

Both sense and antisense *EXO*pDH51 and pCIRCE were digested using EcoR1. The inserts were excised from a gel using the Geneclean kit and the vector DNA was heated to 65°C for 10 minutes to inactivate the restriction enzymes. The vector was dephosphorylated using shrimp alkaline phosphatase as described in section 2.9.2 to prevent self-ligation. An aliquot of the sense and antisense constructs and the dephosphorylated pCIRCE were run out on a gel to estimate their relative concentrations (Fig. 5.14G). The constructs were ligated into pCIRCE and transformed into *E.coli*. Positive colonies were identified by PCR as shown in Fig. 14h. A single colony was selected for both sense and antisense constructs, and their plasmid DNA was isolated and sent to be sequenced to ensure the sequence was correct.

5.2.1.4 Introducing the sense and antisense cassettes into plants

Constructs were grown up and the constructs were introduced into *Agrobacterium* by electroporation, as described in section 2.15.1. Plants were transformed using the

dipping method described in section 2.15.3, before being grown to maturity, and their seed collected. Seed was germinated on 35mg/l kanamycin to select for transformants.

5.2.2 Analysis of sense and antisense plants

5.2.2.1 Primary analysis of sense and antisense plants

Kanamycin selection of the transformed seed generated 11 plants containing the sense construct (named *EXO*S1-11) and 15 plants containing the antisense construct (named *EXO*A1-15). Figure 5.15A shows a single sense plant and 5.15B shows 8 putative antisense plants which were transferred from Petri dishes to soil to be analysed and to set seed. The antisense plants appear to be slower growing than the sense which was grown on identical media.

5.2.2.2 Analysis of a putative antisense phenotype

Of the 15 antisense transformants, one plant (*EXO*A1) demonstrated a distinctive abnormal phenotype. The plant was dwarfed with respect to the wild-type, its leaves were small and its siliques were small and undeveloped (Fig. 5.16A). Further analysis revealed several architectural deviations from the wild-type. Irregular branching occurred in several positions with organs appearing to be fused together (Fig. 5.16B), ectopic organogenesis was exemplified by the observation of a silique emerging from the branch of two stems (Fig. 5.16C) and some inflorescences were deformed in a manner reminiscent of the pin1 mutant (Fig. 5.17A). The majority of flowers appeared to be male sterile as no pollen was visible and the siliques were small and undeveloped (Fig. 5.17B). Presence of the T-DNA in this line was confirmed by PCR.

5.3 Summary

The results in this chapter suggest that the T-DNA insertion in the promoter trap line EM2 has occurred downstream of regulatory elements directing *EXO* expression as *EXO*FPG lines generated show a similar pattern of GUS expression to that in the EM2 line. The pattern of expression in the *EXO*FPG lines is not identical to that in EM2 however, suggesting that other important regulatory elements such as those directing expression in the root vascular tissue and the silique are situated downstream of the T-DNA insertion site. A putative antisense plant has been generated which displays a phenotype suggestive of abnormal meristematic or cell division activity.

Fig. 5.15   Putative sense and antisense transformants recovering on $^1/_2$MS10 following kanamycin selection (approximately three weeks post germination)

A   A single putative sense plant amongst untransformed seedlings

B   Putative antisense plants showing retarded growth with respect to the sense plant

Approximately lifesize
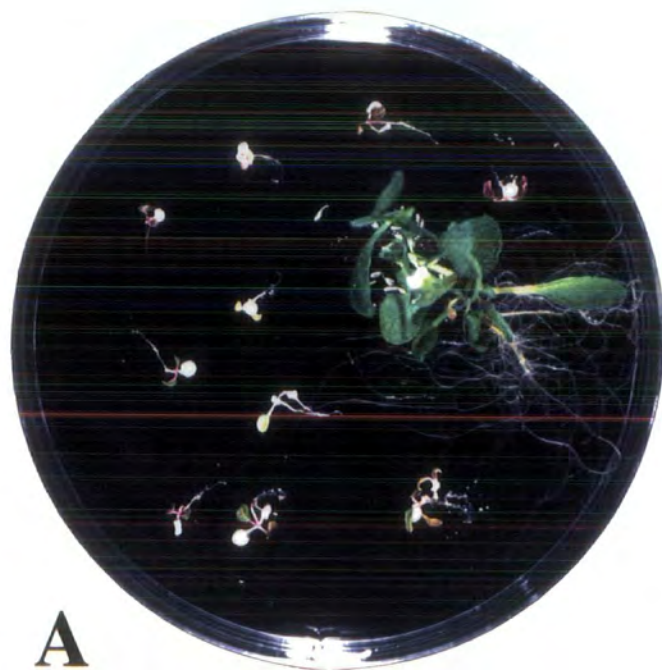
A
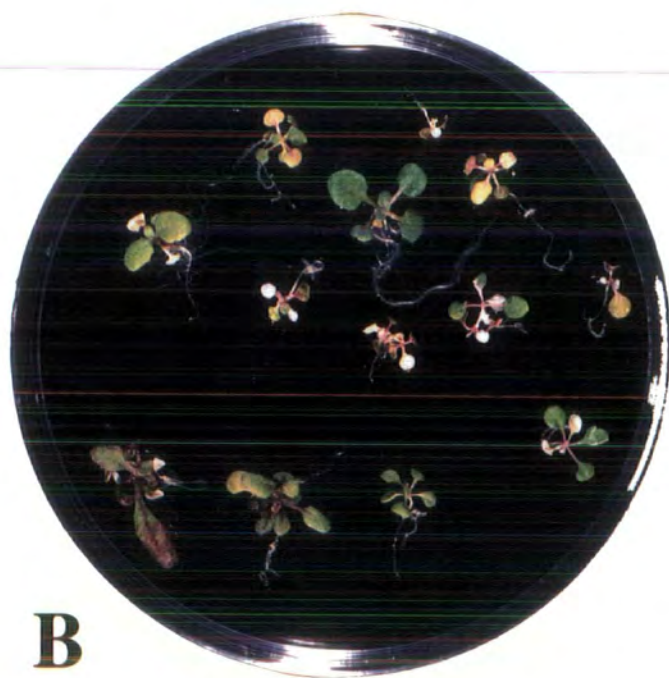
B

Fig. 5.16 Putative *EXORDIUM* antisense phenotype

A   Antisense plant (i) with wild-type control (ii)
     Antisense plant shows reduced apical dominance, small leaves and
     undeveloped siliques

Scale bar = 4cm

B   The antisense plant shows abnormal architecture including fused
     organs

C   Ectopic organ formation in the antisense plant, a silique has formed
     in place of an indeterminate inflorescence (centre) and two floral
     axes have developed opposite one another instead of alternately as
     occurs in the wild-type

Scale bars = 0.5cm
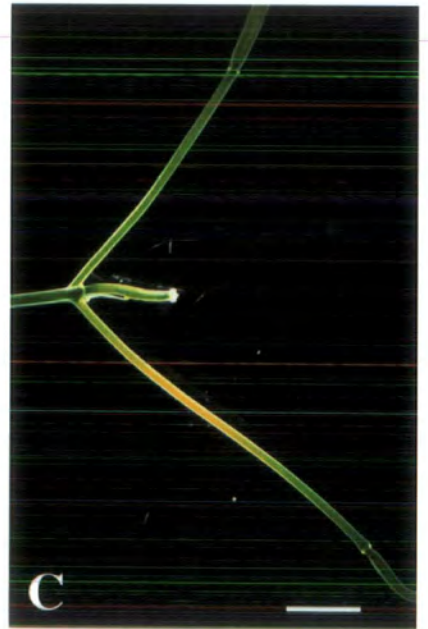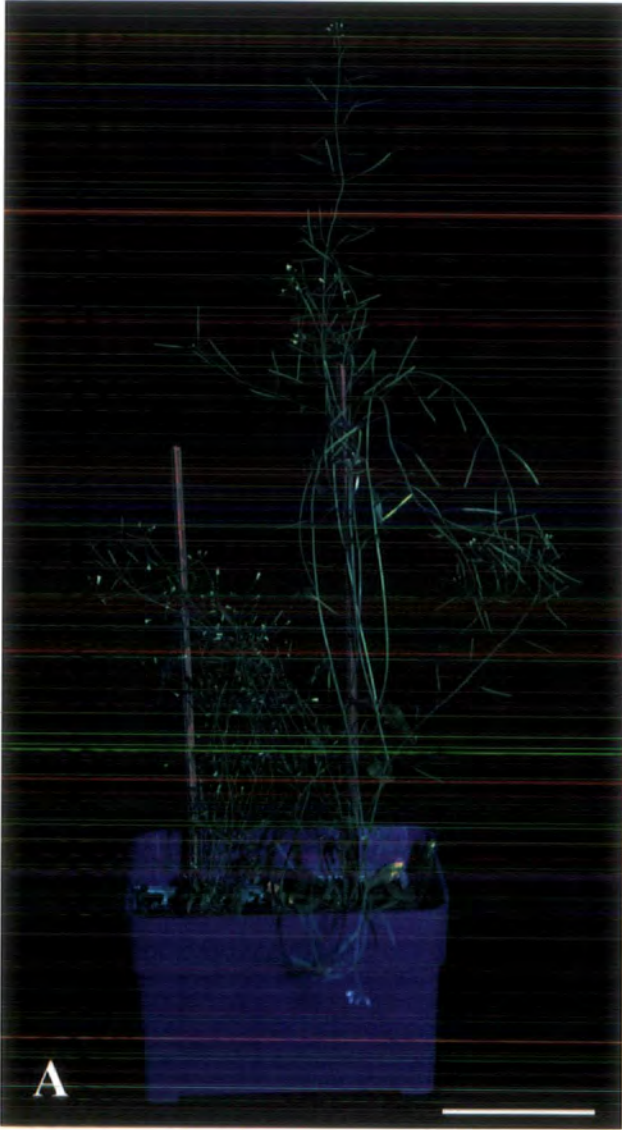
Fig. 5.17 Putative *EXORDIUM* antisense phenotype in the flower

A   Defective floral organ flormation. 'Flower' showing fused and abnormally formed organs

Scale bar = 0.5cm

B   Phenotypically normal male sterile flower containing no pollen.

Scale bar = 0.25cm
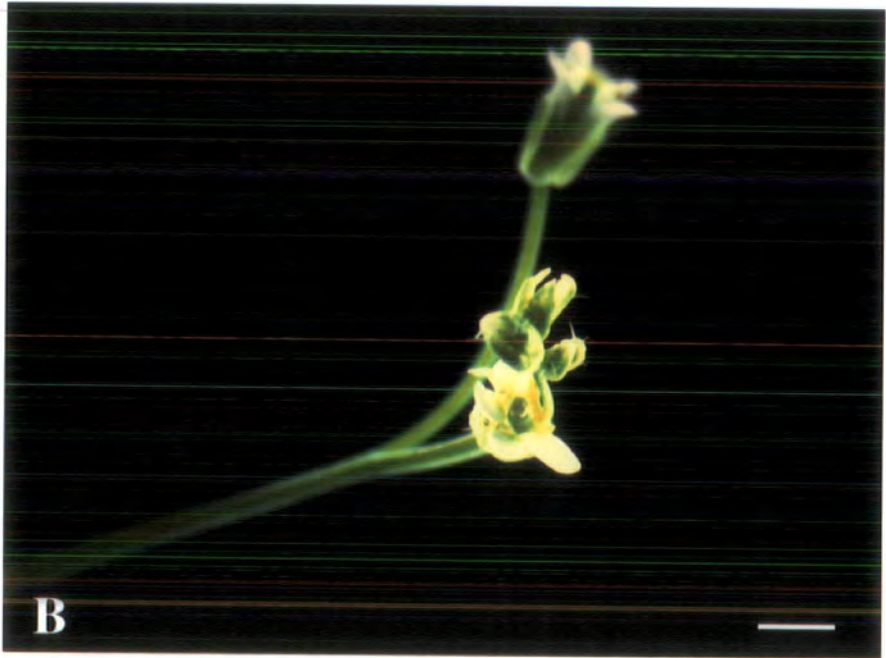
DISCUSSION

6.0 Discussion

The aim of this thesis was to characterise the promoter trap line EM2. To date, few genes tagged by promoter trapping have been cloned. One gene that has been cloned using this technique is a nucleic acid helicase that was isolated following analysis of the *Arabidopsis* promoter trap line HVT-1. This line shows GUS activity in the vascular tissue and tapetum (Wei *et al.*, 1997). The EM2 line shows GUS activity in the early embryo. The native gene was named *EXORDIUM*, and has been cloned previously by I.M.Evans (unpublished).

Regulation of GUS expression in the EM2 line has been analysed under different conditions and in different mutant backgrounds, and compared with the regulation of the native gene *EXORDIUM*. Promoter-GUS transgenic plants were created in order to compare the GUS expression pattern in the EM2 line with expression driven by the full length promoter. It has been demonstrated that the T-DNA in the promoter trap line has tagged certain promoter elements responsible for the regulation of *EXO* gene expression, but that other important elements such as those directing expression in the vascular tissue and siliques are situated downstream of the insertion site. The predicted EXO protein shares no homology with any protein of known function, and no aberrant phenotype was detected in the promoter trap line. Sense and antisense plants were created in order to study gene function. The GUS expression in the EM2 line is associated with meristem acivity and the native gene has been demonstrated to be regulated in a cell cycle-dependent manner.

6.1 Expression analysis

6.1.1 GUS expression in the EM2 line

GUS expression in the EM2 line occurs in a regular pattern, indicating that it is spatially regulated. The pattern of expression remains consistent under a wide range of environmental, nutritional and hormonal conditions (see Chapter 3.0). GUS expression in the promoter trap line corresponds with areas of cell cycle activity and appears to be under hormonal control, being upregulated by auxin (Figures 3.6-3.8) and downregulated by cytokinin (Figures 3.10 &3.11).

Previous experiments crossing EM2 into seedling mutant backgrounds confirm that meristematic activity is required for EM2-GUS expression (Topping & Lindsey, 1997). The *hydra* mutant is disrupted in cell shape and morphology but shows correct pattern formation and has both a shoot and a root meristem (Topping *et al.*, 1997). In this background, EM2-GUS expression is observed throughout the embryo, and in the seedling is restricted to root tips and young leaves and cotyledons as it is in the EM2 line. When EM2 is crossed into the *gnom* mutant (Mayer *et al.*, 1993, Shevell *et al.*, 1994), which has no root meristem, GUS expression is not constitutive throughout the embryo but is restricted to the apical region. In the seedling, there is no GUS activity in the basal part of the plant, indicating that expression is dependent on meristematic organization or activity. This is in direct contrast to another promoter trap line (EM101, Topping *et al.*, 1994), which shows a similar pattern of GUS expression to EM2 in the seedling root. EM1-GUS expression is restricted to the basal part of the *hydra* embryo and is present in the basal (meristemless) part of the *gnom* seedling. The tagged gene is considered to be a positional marker and has been named *POLARIS* (*PLS*, Topping & Lindsey, 1997). This result indicates that the *EXO* and *PLS* genes are regulated by independent pathways, *EXO* being part of a *GNOM*-dependent pathway. *GNOM* encodes an ARF GEF protein required for correct vesicle trafficking and is necessary for polar auxin transport and meristem construction (Steinmann *et al.*, 1999, Shevell *et al.*, 1994).

Phytohormones have a profound effect on root development, with auxin and cytokinin acting antagonistically to promote and inhibit lateral root initiation respectively (Topping & Lindsey, 1997, Su & Howell, 1992, Laskowski *et al.*, 1995). Exogenous auxin application is suggested to cause lateral roots to develop in sections of mature roots from cells that had previously been arrested in the cell cycle. In contrast, uninduced lateral roots form sequentially from pericycle cells that are actively dividing and expanding (Lasowski, et al., 1995). Sabatini *et al.* (1999) constructed a synthetic auxin-induced promoter-GUS construct named DR5::GUS in order to assess auxin localisation in *Arabidopsis*. DR5::GUS plants show staining of all cells of the root upon treatment with 2,4-dichlorophenoxy acetic acid (2,4-D), whereas in untreated plants GUS activity is restricted to the root tips. This pattern of expression is reminiscent of that in the EM2 line (Fig. 3.6, 3.7) and the GUS expression pattern observed in the line may be a marker of some aspect of meristematic or cell division

activity which is induced by auxin. This idea is supported by the fact that EM2 plants grown on NPA, an inhibitor of polar auxin transport, show a dramatic upregulation of GUS activity at the root tip. NPA treatment also causes increased GUS activity at the root tip of DR5::GUS plants, consistent with the idea that NPA treatment causes pooling of auxin at the root tip. This idea is also consistent with the fact that EXO expression is defective in the *gnom* mutant, which is also defective in PIN protein localisation and polar auxin transport (Topping & Lindsey, 1997, Steinmann *et al.*, 1999). Additionally, when crossed into auxin-resistant backgrounds, little or no EM2-GUS activity is observed at the root tips, indicating that auxin perception is required for expression. EM2-GUS expression was unaffected in the root tips of the *pin-1* mutant (Okada *et al.*, 1991), which exhibits reduced polar auxin transport and shows a dramatic aerial phenotype with the inflorescences being reduced to a simple pin-like structure. The roots in this line however are morphologically normal (unlike NPA-treated roots), confirming that the inhibition of polar auxin transport is incomplete in this line, with the mutation mainly causing inhibition of polar auxin transport in the inflorescence axis (Okada *et al.*, 1991). Together these data suggest that EXO expression is dependent on functional meristem activity, which is in part regulated by polar auxin transport.

EM2 was crossed into a further two mutant backgrounds: *rooty*, which has elevated auxin levels (King *et al.*, 1995) and *amp-1* which has elevated cytokinin levels (Chaudhury *et al.*, 1993), but the results of these experiments had not been obtained at the time of writing. It is expected that GUS expression will be elevated in the *rooty* mutant and diminished in the *amp-1* background.

Surprisingly, ectopic GUS expression was observed in the hypocotyls of etiolated EM2 seedlings (Fig. 3.2). The elongation of Arabidopsis hypocotyls occurs without cell division (Kurata &Yamamoto, 1998). Auxin transport has been demonstrated to be required for light-mediated hypocotyl elongation but not for etiolation (Jensen et al., 1998). GUS expression is not observed in light grown EM2 hypocotyls but is expressed in those that are etiolated (Fig. 3.2). This result may indicate that there is an accumulation of auxin in etiolated hypocotyls; there may be an alternative role for *EXO* in these conditions; or it may be that there is some cell cycle activity in the etiolated hypocotyls. A further possibility is that cytokinin levels may be reduced in

the etiolated hypocotyls, causing an increase in the auxin:cytokinin (aux:CK) ratio to activate GUS expression. In support of this idea, it has been demonstrated that exogenous cytokinin treatment causes inhibition of hypocotyl elongation, and this effect may be mediated through ethylene production (Carey et al., 1995). In order to test the hypothesis that GUS expression in the etiolated hypocotyls of the EM2 line is due to an increased aux:CK ratio, plants should be grown on exogenous kinetin (a synthetic cytokinin), ACC (the immediate precursor of ethylene), and $AgNO_3$ (which blocks ethylene perception). It is predicted that the kinetin and ACC treatments should cause both inhibition of hypocotyl elongation and reduction of GUS expression in this tissue. The $AgNO_3$ treatment is predicted to have the opposite effect, causing extra elongation of the hypocotyls in relation to untreated dark-grown plants, and a corresponding upregulation of GUS activity.

Interestingly van der Zaal et al., (1991) observed root tip-specific GUS expression when the reporter gene was fused to the promoter of an auxin-responsive gene (*GNT1*) isolated from tobacco cells. They attributed the specificity to a high auxin concentration or a high auxin sensitivity at the root tips and suggested that the lack of GUS activity in other tissues may be due to the fact that only meristematic cells are competent to react quickly with cell division in response to auxin.

6.1.2 Regulation of *EXO* expression

Northern analysis revealed that *EXO* steady state mRNA levels are increased with auxin treatment and decreased with cytokinin treatment (Fig. 4.2), indicating that at least some elements responsible for *EXO* regulation in the wild-type have been tagged by the T-DNA, despite it having inserted 782bp upstream of the coding region. The discovery of putative Auxin Responsive Elements (AREs) in the promoter and intragenically (Fig. 4.7) provide a possible mechanism by which such regulation may be mediated. The TGTCTC sequence present in *EXO* was identified within two auxin-inducible elements of the soybean *GH3* promoter (Liu et al., 1994) and soybean small auxin-upregulated (SAUR) gene promoters (Li et al., 1994). The TGTCTC sequences may not be functional in the *EXO* promoter as they are not associated with other sequences demonstrated to be present in the *GH3* and SAUR promoters, and the TGTCTC element has been demonstrated to have no activity when fused to a minimal CaMV 35S promoter (Li et al., 1994). Alone, the TGTCTC sequence may be required

but not sufficient to confer auxin-responsiveness to a promoter. However, Sabatini *et al.* (1999) used multiple copies of this element to create the synthetic auxin regulated DR5 promoter, which consists of 7 tandem repeats of the TGTCTC sequence fused to a minimal CaMV promoter. Promoter deletion studies should be undertaken in order to test whether the element is important in mediating the auxin responsiveness of *EXO*. It is possible that *EXO* has an alternative, as yet unidentified, mechanism by which auxin response is mediated and it is possible that the intragenic elements are involved in the regulation of gene expression as well as those upstream of the coding region. Intron sequences of the spinach *PsaD* gene are required for gene expression regulation. For example, Sieburth &Meyerowitz (1997) demonstrated that intragenic *cis* elements are responsible for the spatial regulation of the *AGAMOUS* gene. In order to test the importance of any intragenic regulatory elements, transgenic plants should be created containing a GUS-*EXO* gene fusion. Attachment of GUS to the 3' end of the *EXO* gene should reveal the pattern of EXO protein localisation following expression directed by all relevant regulatory elements.

Analysis of the full length promoter-GUS transgenic lines revealed that the pattern of GUS expression is similar to that in the EM2 line, indicating that sequences important for directing *EXO* expression are located upstream of the T-DNA insertion site. FP::GUS expression is observed constitutively in the embryo from the globular stage onwards, and in the root tips, root vascular tissue and leaves of adult plants. The pattern of GUS expression is less restricted than that in the EM2 line, suggesting that sequences directing expression in root vascular tissue and leaves may be located downstream of the T-DNA insertion site. Further analysis of FP::GUS expression is required to characterise the expression pattern at different stages of development, and to determine whether the expression is regulated in a manner similar to EM2. Further screening of the transformed truncated promoter-GUS seed is required to isolate TP::GUS lines for further comparison with EM2 and FP::GUS lines.

The relationship between *EXO* expression and the cell cycle was confirmed by the demonstration that there is an accumulation of *EXO* steady state mRNA levels in response to HU treatment which blocks the cell cycle at the G1/S transition (Hemerly *et al.*, 1993). This accumulation (shown in Fig. 4.3a) suggests that there is usually a turnover of the *EXO* transcript at a point beyond the HU inhibition, *i.e.* during S

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phase. This hypothesis was subsequently confirmed and *EXO* mRNA levels were revealed to accumulate to a maximum at a timepoint calculated to correspond to M phase and decrease to a minimum twelve hours later (Fig. 4.3b). Further analysis is required to ascertain whether the EXO protein is turned over in a cell-cycle-dependent manner.

Manipulation of the cell cycle is predicted to have profound effects on plant growth and development. Plants often mediate responses to environmental changes through control of the cell cycle, and so an improved understanding of the mechanisms by which such changes may be instigated represents a major goal for the manipulation of plant form for crop improvement (den Boer & Murray, 2000a). Plant growth rate is largely regulated by the proportion of cells that are actively cycling at any given time and the length of the cycle. Both elevated CO_2 (Kinsman et al., 1997) and temperature (Francis & Barlow, 1988) have been demonstrated to cause faster growth, through an increase in the number of dividing cells in the meristem, and a decrease in the length of the G1 phase respectively. Cell cycle progression is driven by periodic peaks in cyclin (cyc) and cyclin dependent kinase (CDK) gene expression (den Boer & Murray, 2000b) and elements of this pathway have been shown to be conserved between mammals and plants. Root growth in *Arabidopsis* was accelerated by expression of *CycB1* under the control of the weak but widespread *cdc2a* promoter (Doerner *et al.*, 1996), demonstrating that these cyclins and the associated kinases represent excellent targets for the manipulation of plant growth. The D-type cyclins, which regulate CDK activity during G1 phase, are sensitive to extracellular signals and so represent a link between the environment and cell cycle control.

6.2 Phenotypic analysis

6.2.1 No conditional phenotype was discovered in the EM2 line

No aberrant phenotype is observed in the promoter trap line EM2. There are at least three possible reasons for this:

- There is a phenotype that is observed only under certain conditions, *ie.* the mutant phenotype is conditional.

- The T-DNA insertion in the EM2 line has not disrupted gene function.

- *EXO* is redundant, its function being fulfilled by a homologue or an alternative pathway in its absence.

In order to address the first of these possibilities, EM2 and wild-type plants were grown under various environmental and hormonal conditions in an attempt to reveal a conditional phenotype in the transgenic line. The divergent signalling pathways by which phytochrome A (PHY A) mediates its responses were elucidated by the characterisation of a conditional mutant (*fhy3*, Yanovsky *et al.*, 2000). Etiolated seedlings of both wildtype and mutant plants exposed to hourly pulses of far-red light show similar inhibition of hypocotyl growth and greening upon transfer to white light, but while continuous far-red light treatment is significantly more effective in the wild-type, for the *fhy3* mutant, hourly pulses are equally effective.

Many of the experiments performed resulted in drastic alteration of plant morphology (see chapter 3), but in no case did EM2 respond in a manner different from the wildtype. It is possible that there is a phenotype in EM2, but either it is very subtle or specific and was not observed, or it is a response to a condition not tested.

6.2.2 *EXO* is expressed at low levels in the EM2 line

Another possible reason for the lack of aberrant phenotype in the EM2 line is that the T-DNA insert has not significantly disrupted gene function. Cloning and sequencing of the gene locus (I.M.Evans, unpublished) demonstrated that the T-DNA is inserted not within the gene, but 782bp upstream, presumably within the promoter (Fig.4.1). Northern analysis revealed that steady state mRNA levels of *EXO* mRNA are much reduced in the EM2 line with respect to those in the wildtype. Some expression is however detectable (Fig.4.2). It is possible that there is sufficient *EXO* expression in the EM2 line to perform gene function and this may explain the lack of mutant phenotype in the line.

6.2.3 *EXO* may be functionally redundant

The third possible reason that no aberrant phenotype is detected in the promoter trap line is that the gene may be functionally redundant. *EXO* has been discovered to be a member of a small gene family in *Arabidopsis*. Database searches reveal two predicted proteins homologous to, and of similar length to, *EXO* and a third predicted

protein has homology with *EXO* at its C terminal end but is much longer at the N terminal end (Fig.4.5). Any one of these three predicted proteins may serve a similar function to *EXO*. In order to test whether *EXO* is functionally redundant, homologues should be knocked out using either antisense or RNAi (Chuang & Meyerowitz, 2000). Alternatively mutations in the homologues could be screened for from commercially available mutant populations.

The *cup-shaped cotyledon* (*cuc*) mutant of *Arabidopsis* is caused by the double mutation of two unlinked loci (*CUC1* and *CUC2*), neither of which cause the full *cuc* phenotype as single mutations (Aida *et al.* 1997) and so are considered to be functionally redundant. Another example of functional redundancy is the *cauliflower* (*cal*) mutant of *Arabidopsis* which is phenotypically normal as a single mutant but in the *apetala* (*ap1*) background displays a mutant phenotype (Bowman *et al.*, 1993). The two proteins, *AP1* and *CAL*, show a high level of similarity to one another and both encode MADS domain proteins (Mandel *et al.*, 1992, Kempin *et al.*, 1995).

EM2 plants were crossed with different mutants in order that any double mutant phenotype might be observed. Unfortunately no double mutant phenotype was observed in any of the EM2 mutant crosses performed, and so no indication of EXO function was gained from these experiments. It can however be determined that EXO is not functioning in parallel with any of the proteins of the mutants tested (AUX1-7, AXR1-12, PIN1). It would be desirable to obtain knockout lines for *EXO* and each of the *EXO* homologues and to cross them together to create double and triple mutants. These may display a mutant phenotype that might suggest a role for the function of the genes.

A single antisense plant was generated which displays a mutant phenotype (Fig. 5.16a). The physical abnormalities observed - short stature, small leaves, irregular branching (Fig. 5.16b), ectopic organ formation (Fig. 5.16c) and deformed floral parts (Fig. 5.17a) - might be explained by aberrant meristematic function caused by abnormal cell cycle activity, as plant morphology is determined by the rate and number of cell divisions, the plane in which thay occur and the direction of cell growth. Such a disruption to cell cycle activity could also be expected to account for the male sterility observed; flowers do not contain pollen and siliques remain

undeveloped. Quantitative analysis is required to determine whether *EXO* mRNA levels are reduced in this line. In the other 14 antisense lines generated, no obvious mutant phenotype was observed. A number of genes have been identified that are required for wild-type *Arabidopsis* architecture. For example, mutants of the *AXR1* and *AXR3* genes, reqired for auxin signalling, are defective in axillary shoot formation and branching pattern (Hobbie, 1998). It is possible that a knockout of *EXO* expression interferes with auxin-mediated apical dominance to produce the observed phenotype in the putative *EXO* antisense transgenic line.

The lack of mutant phenotype in the majority of the antisense lines may be due to any of the following reasons. The 35S promoter is not constitutively active in plants. Wilkinson *et al.* (1997) demonstrated that there is variability between lines transformed with a 35S-GUS construct, both in the patterning and level of expression. It may be that there is sufficient *EXO* expression in the relevant tissues to perform gene function in these lines. Correspondingly, the lack of obvious aberrant phenotype may be due to the fact that a range of phenotypes, from severe (depending on the function of the gene) to wildtype, is expected in antisense studies. This is due to position effects of the genomic DNA; some insertion events may occur at sites where enhancers are active, while others may occur in areas of little transcriptional activity. These lines may represent the least dramatic examples in a range of growth habits and that given a larger population of *EXO*-antisense plants, multiple individuals displaying similar mutant phenotypes of varying severity might be observed. Sense and antisense studies are further complicated by gene silencing, in which the expression of multiple copies of homologous DNA sequences is repressed by the plant (Matzke & Matzke, 1995). Quantitative analyses of *EXO* expression levels, by either RT PCR or Northern analysis, are required to assess whether putative antisense mutant phenotypes correspond to decreased *EXO* expression with respect to the wild-type. A third possibility is that the abnormal phenotype in the *EXOA1* plant is not due to an antisense effect but to an insertional mutagenesis of a second gene. It is important that co-segregation analyses are carried out to correlate antisense T-DNA with the abnormal phenotype.

An antisense phenotype may be due to down-regulation of expression of the *EXO* gene or it may be due to the knockout of *EXO* and its homologues. Quantitative

analysis of homologue expression in the wildtype and antisense lines may indicate whether *EXO* is functionally redundant.

No mutant phenotype was observed in any of the sense plants generated, suggesting that an excess of the protein might be tolerated by the plant with no adverse effects on development. Alternatively, as for the antisense lines, a larger population of sense plants may reveal a range of phenotypes, with the phenotypically wildtype lines representing those in which there is little protein overexpression.

6.3 *EXORDIUM* gene sequence

Analysis of the predicted EXO protein reveals that it is a novel gene. A single published homologue (*PHI1*) exists, which was isolated from tobacco (Sano *et al.*, 1999). Cell suspension cultures starved of phosphate enter a static state and upon re-addition of phosphate, cells re-enter the cell cycle semi-synchronously. *PHI1* was isolated by differential screening of phosphate-starved BY-2 cells before and after the re-addition of phosphate (Sano *et al.*, 1999). Several genes involved in phosphate metabolism are induced by phosphate starvation in *Saccharomyces cerevisiae* as nutrients are often scarce and so efficient usage of available resources is important. In limiting conditions the expression of a high-affinity phosphate transporter and phosphatases are increased and prolonged periods of phosphate starvation induce arrest of the cell cycle (Lenburg &O'Shea, 1996). In contrast, *PHI1* was found to be rapidly induced (within 20 minutes) following re-addition of phosphate to arrested cells, reaching a maximum by 1h. Despite sequence similarity (see Appendix 1), *PHI1* is not regulated in a cell cycle dependent manner and so is unlikely to function in the same way as *EXO*. *PHI1* is suggested to play a role in causing phosphate-starved cells to re-enter the cell cycle (Sano *et al.* 1999). Following similar experiments using auxin-starvation to cause cell-cycle arrest, Sano *et al.* (1999) suggest that two different mechanisms exist for cell cycle arrest, and that *PHI1* is specific to phosphate-induced cell cycle activity. Despite no sequence homology with any gene of known function, the N-terminal region was noted to have some homology with plasma membrane H+-ATPases of fungi and plants, including a conserved sequence (Lys-Gly_Ala) suggested to be involved in ATP-binding (Sano *et al.*, 1999). This sequence is also present in the predicted EXO protein, but it is unknown whether

EXO has any ATP binding properties. It is interesting to note that *PHI1* has some role in cell cycle activity, and although its function is unlikely to be the same as that of *EXO*, the two genes may be involved in similar processes. Both may have some involvement in sensing environmental conditions in relation to the competence of the cell to cycle. In the case of *EXO*, its function may be as part of auxin-induced cell cycle activation at the G2-M transition.

Analysis of the *EXO* promoter revealed several putative cis elements. Without performing promoter-deletion experiments it is impossible to determine whether any of these are involved in *EXO* expression. The presence of putative Auxin Regulatory Elements upstream of the T-DNA insertion site provides a possible mechanism for the auxin-mediated up-regulation of both GUS expression in the EM2 line and *EXO* expression in the wild-type line. Further comparison of the FP::GUS line and EM2 is required to reveal the role of elements situated downstream of the T-DNA insertion site.

6.4   Studying plant development

Recently much work in the area of plant development has focused on cell fate and the role of positional cues. Scheres *et al.* (1994) have studied the relationship between the embryo and the seedling and have generated a 'fate map' depicting the origin of seedling tissue types in the heart stage embryo. Elegant laser ablation studies have demonstrated that cell position rather than lineage determines cell identity (van den Berg *et al.*, 1995, Berger *et al.*, 1998), indicating that positional information and signalling are of great importance during plant development (Scheres, 1996, 1997). Mutant studies have also been successfully employed to elucidate the mechanisms underlying development (Berleth *et al.*, 1996, Scheres *et al.*, 1995, Schiefelbein & Benfey, 1991). Characterisation of the *EXO* gene suggests that its role is not associated with cell position or signalling, but with cell activity.

It is important to consider that the majority of mutant studies are based on single gene mutations as these are relatively easy to identify. Many genes involved in important aspects of plant growth and function may well be redundant, with function being fulfilled by another gene or pathway in their absence. Such multiple gene effects may

be more difficult to elucidate but are likely to constitute a large proportion of the mechanism by which the plant functions. *EXO* is likely to belong to this second category and is probably redundant in *Arabidopsis*. Promoter trapping is an excellent method for isolating such genes, as it does not depend on the observation of a mutant phenotype. In addition to the advantage of having a T-DNA insertion to facilitate subsequent cloning, some idea of the gene expression pattern can be determined without further cloning. Comparison of the GUS expression in the promoter trap line EM2 and the promoter-GUS transgenic plants generated in this study confirm that this method is viable.

6.5   Final summary and future work

In addition to the specific experiments outlined in the previous pages, there are several broad directions in which this project should be continued.

Further GUS expression analysis should be performed using the FP::GUS lines generated in this study. In addition to the 2h staining time used in this study, a 24h staining period should also be employed to detect downregulation of GUS expression in the cotyledons and leaves. It is predicted that treatments causing cotyledons to remain unexpanded (etiolation, ABA) will result in reduced GUS expression in those organs. FP::GUS plants should also be crossed into mutant backgrounds and the GUS expression pattern analysed. Morphological mutants of interest include: *hobbit*, which has no functional root meristem (Willemsen *et al.*), *short root*, which shows determinate root growth, *cobra* and *lions tail*, which have abnormal cell expansion (Benfey *et al.* 1993) and *fass*, which has abnormal morphology but correct patterning (Torres-Ruiz & Jurgens, 1994). It is predicted that expression will be absent in roots with no meristem activity and present in incorrectly expanded cells. Expression in the *short root* background is predicted to be particularly interesting as root tip expression is expected to persist only as long as the roots continue growing.

In situ localisation should be performed in wild-type plants using the EXO cDNA as a probe to confirm GUS expression in the FP::GUS line represents the true expression pattern of *EXO*. Intragenic sequences may be important in the regulation of the gene in the wild-type plant and these will not be active in the promoter-GUS line.

Comparison of FP::GUS expression with both DR5::GUS (which marks auxin localisation, Sabatini *et al.*, 1999), CYCAT1:CDB:GUS expression (which marks mitosis, Hauser & Bauer, 1999) and *CycB*1;1::*uid*A (which also marks mitosis, Colón-Carmona et al., 1999) would be interesting as *EXO* is suggested to play a role in cell cycle regulation via auxin signalling.

Analysis of the expression pattern of the *EXO* homologues would also be of interest as a similar pattern of expression may suggest an overlap of gene function and explain the possible redundancy of the *EXO* gene. Obtaining knockout lines for these genes and crossing them together to create double and triple mutants might produce a mutant phenotype and identify the homolgues as being functionally redundant with *EXO*.

In order to discover second site regulators of *EXO* expression, secondary mutagenesis could be employed on either the FP::GUS line or an antisense line. Alteration of GUS expression in mutagenised FP::GUS lines could identify genes responsible for upstream regulation of *EXO* gene expression, while altered phenotypes in mutagenised antisense lines would allow the identification of enhancer or suppressor mutants. In this way a regulatory pathway involving *EXO* may be elucidated. A yeast two-hybrid system could be used in order to identify any proteins which interact with EXO (Allen *et al.*, 1995).

Localisation of the protein is important in ascertaining its function. The simplest method that might be employed to visualise EXO location in the cell is that of a GFP-EXO fusion. Alternatively, overexpression of the protein should be performed in order to raise antibodies to EXO for immunolocalisation studies.

To summarise, the work described in this thesis describes the characterisation of a novel gene in *Arabidopsis thaliana*, identified by promoter trapping. Comparison of GUS gene expression in the promoter trap line with native *EXO* expression demonstrates the benefits of promoter trapping over insertional mutagenesis. Not only has the *EXO* gene been cloned in the absence of a mutant phenotype, but the pattern of GUS expression indicates that important regulatory elements regulating *EXO* gene expression have been tagged. The gene is regulated in a cell-cycle dependent manner,

with auxin and cytokinin causing up- and down-regulation of expression in the root respectively. A putative antisense plant has been generated which is small in stature, has unexpanded leaves, shows irregular branching and organ formation and is male sterile. These results indicate that the *EXO* gene may play a role in an auxin-mediated pathway of cell division control in *Arabidopsis*.

Note added in proof:

A second putative antisense mutant was identified subsequent to the submission of this thesis. The plant was greatly reduced in stature with abnormal cell files and organ development, and pin-like structures on the inflorescence meristem.

Following completion of the sequencing of the *Arabidopsis* genome, additional homologues were identified, all of unknown function. The complete putative gene family in *Arabidopsis* is as follows:

    AF12839/AL161513 (EXO)
    AB018441 (PHI1: tobacco)
    AC079605
    AB008268
    AF324998
    AL391712
    AF325723
    AC018363
    AB018109
    AP002542
    AC007127
    AC004667

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APPENDIX

C24 root (mm)		C24 seedling (mm)		EM2 root (mm)		EM2 seedling (mm)	
5		12		7		18	
13		22		10		20	
5		18		11		21	
10		21		7		19	
9		17		7		16	
7		15		4		15	
8		17		7		19	
6		19		8		18	
7		20		9		17	
8		19		9		18	
6		19		5		15	
10		21		10		22	
8		17		5		17	
7		17		9		20	
6		17		4		16	
7		16		4		15	
7		19		8		19	
9		18		6		18	
10		17		9		23	
9		18		7		20	
12		20		4		16	
Sum	169	Sum	379	Sum	150	Sum	382
Count	21	Count	21	Count	21	Count	21
Mean	8.047619	Mean	18.04762	Mean	7.142857	Mean	18.19048
S.D.	2.132515	S.D.	2.246691	S.D.	2.197401	S.D.	2.293884

>dbj|BAA33810.1| (AB018441) phi-1 [Nicotiana tabacum]
         Length = 313

 Score = 453 bits (1152), Expect = e-126
 Identities = 226/314 (71%), Positives = 260/314 (81%), Gaps = 11/314
 (3%)

Query: 5   VFKLFLFLSLLQISVSARNLAS--QEPNQFQLLKYHKGALLSGKISVNLIWYGKFKPSQR
62
            + KLFL +S   + ++R L +   QEP   QLL+YHKGALL GKISVNLIWYGKFKPSQR
Sbjct: 7   ILKLFLVISFCNVCFASRKLTALVQEPEN-QLLQYHKGALLFGKISVNLIWYGKFKPSQR
65

Query: 63   AIISDFITSLTHTSPTSKTLHPSVATWWKTTEKYYKLATPSKNSSPLSLTLGKQIIDES
122
            AI+SDFITSL+ ++P SKT   PSVA WWKTTEKYY LA   K+   LSL LGKQ++ E+
Sbjct: 66   AIVSDFITSLSSSTP-SKT--DPSVAKWWKTTEKYYHLANSKKS---LSLYLGKQVLVEN
119

Query: 123 CSLGKSLTDKKIQTLASKGDQRNAINVVLTSADVTVTGFGMSRCGTHGHARGLGKRGS--
180
             SLGKSLT K+I LASKG+Q++AIN+VLT++DV V GF ++RCGTHG ++G   RG
Sbjct: 120 YSLGKSLTQKQIVQLASKGEQKDAINIVLTASDVAVDGFCVNRCGTHGSSKGAIIRGKTY
179

Query: 181 KFAYIWVGNSETQCPGQCAWPFHAPVYGPQSPPLVAPNNDVGLDGMVINLASLLAGTATN
240
            KFAYIWVGNSETQC G CAWPFH P+YGPQSPPLVAPNNDVG+DGMVINLASLLAGTATN
Sbjct: 180 KFAYIWVGNSETQCAGYCAWPFHQPIYGPQSPPLVAPNNDVGVDGMVINLASLLAGTATN
239

Query: 241 PFGNGYYQGPQNAPLEAASACPGVYGKGAYPGYAGDLLVDTTTGGSFNAYGANGRKFLLP
300
            PFGNGYYQG +APLEAASACPGVY KGAYPGYAGDLLVD TTG S+NA+G NGRK+LLP
Sbjct: 240 PFGNGYYQGEADAPLEAASACPGVYAKGAYPGYAGDLLVDKTTGASYNAHGTNGRKYLLP
299

Query: 301 ALYDPTTSACSTMV 314
            ALYDP+TS CST+V
Sbjct: 300 ALYDPSTSTCSTLV 313

>dbj|BAB09857.1| (AB008268) phi-1-like protein [Arabidopsis thaliana]
         Length = 305

 Score = 351 bits (890), Expect = 8e-96
 Identities = 175/312 (56%), Positives = 220/312 (70%), Gaps = 16/312
 (5%)

```
Sbjct:  64  VDFIHSL-NSKDVASSAAVPSVASWWKTTEKY--    ---KGGSSTLVVGKQLLLENYPL
            114

Query: 126  GKSLTDKKIQTLASK--GDQRNAINVVLTSADVTVTGFGMSRCGTHGHARGLGKRGSK-F
            182
            GKSL +   ++ L++K   G  R+ I VVLT+ DVTV  F MSRCGTHG +     +R +
Sbjct: 115  GKSLKNPYLRALSTKLNGGLRS-ITVVLTAKDVTVERFCMSRCGTHGSSGSNPRRAANGA
            173

Query: 183  AYIWVGNSETQCPGQCAWPFHAPVYGPQSPPLVAPNNDVGLDGMVINLASLLAGTATNPF
            242
            AY+WVGNSETQCPG CAWPFH P+YGPQ+PPLVAPN DVG+DGM+INLA+LLA T TNPF
Sbjct: 174  AYVWVGNSETQCPGYCAWPFHQPIYGPQTPPLVAPNGDVGVDGMIINLATLLANTVTNPF
            233

Query: 243  GNGYYQGPQNAPLEAASACPGVYGKGAYPGYAGDLLVDTTTGGSFNAYGANGRKFLLPAL
            302
             NGYYQGP  APLEA SACPG++G G+YPGYAG +LVD TTG S+NA G  GRK+LLPA+
Sbjct: 234  NNGYYQGPPTAPLEAVSACPGIFGSGSYPGYAGRVLVDKTTGSSYNARGLAGRKYLLPAM
            293

Query: 303  YDPTTSACSTMV 314
            +DP +S C T+V
Sbjct: 294  WDPQSSTCKTLV 305
```

>emb|CAC05470.1| (AL391712) putative protein [Arabidopsis thaliana]
       Length = 278

 Score = 293 bits (742), Expect = 2e-78
 Identities = 152/274 (55%), Positives = 186/274 (67%), Gaps = 29/274 (10%)

```
Query:  43  LSGKISVNLIWYGKFKPSQRAIISDFTTSLTHTSPTSKTLHQPSVATWWKTTEKYYKLAT
            102
            L+G I++NLIWYGKF P QR+II DFI S++  +        PSVA+WWKTTEKY
Sbjct:  32  LNGNITLNLIWYGKFTPIQRSIIVDFIRSISSVTAAKG----PSVASWWKTTEKY-----
            82

Query: 103  PSKNSSPLSLTLGKQIIDESCSLGKSLTDKKIQTLASKGDQRNA--INVVLTSADVTVTG
            160
               +   +L +GKQ++ E+  LGKSL    ++ L+SK +   A  I VVLT+ DVTV G
Sbjct:  83  ---KTGVSTLVVGKQLLLENYPLGKSLKSPYLRALSSKLNAGGARSITVVLTAKDVTVEG
            139

Query: 161  FGMSRCGTHGHARGLGKRGSKFAYIWVGNSETQCPGQCAWPFHAPVYGPQSPPLVAPNND
            220
            M+RCGTHG        G   AY+WVGNSETQCPG CAWPFH P+YGPQSPPLVAPN D
Sbjct: 140  LCMNRCGTHGSKSSSVNSG---AYVWVGNSETQCPGYCAWPFHQPIYGPQSPPLVAPNGD
            196
```

```
Sbjct: 245  KTSGASYNALGLACRKYLLPALWDPQTSTCKTMV 278
```

>gb|AAF26962.1|AC018363_7 (AC018363) phi-1-like protein [Arabidopsis thaliana]
            Length = 332

 Score = 168 bits (422), Expect = 6e-41
 Identities = 109/337 (32%), Positives = 169/337 (49%), Gaps = 55/337 (16%)

```
Query: 8    LFLFLSLLQISVSARNLASQEPNQFQLLKYHKGALLSGKISVNLIWYGKFKPSQRAIISD
67
            +FL L+ L +S   R   SQ PN                 G + ++L+WYG+F P+Q+  + D
Sbjct: 17   IFLLLAPLCLS---REPPSQIPN--------------GTLDLSLLWYGQFTPTQKERVHD
59


Query: 68   FITSLTHTSPTSKTLHQPSVATWWKTTEKY---------YKLATPSKNSSP-LSLTLGKQ
117
            FI SL   +  K    P V+ WWK  E Y          Y+      ++   +P + + +
Sbjct: 60   FIESLNFDA---KEGLDPKVSAWWKVVESYQERFEVKDIYRQKKSNRTVAPRIKVKVVRS
116


Query: 118  IIDESCSLGKSLT----DKKIQTLASKGDQRNAINVVLTSADVTVTGFGM--SRCGTHGH
171
            +DE    GK LT    +K ++T   G+      + VVL SA V   G G     C  +
Sbjct: 117  YVDEKMKYGKELTMGNGEKLVETAI--GNMSKVVPVVLLSAQVRAHGVGFCDGTCQHNAL
174


Query: 172  ARGLGKRGSKFAYIWVGNSETQCPGQCAWPFHAPVYGPQSPPLVAPNNDVGLDGMVINLA
231
            A+  G++  +  YI V N E +CPG+CAWPFH      GP+         + +VG D +VI LA
Sbjct: 175  AKIKGQKEPR-RYIMVSNPEVECPGECAWPFHTADKGPRGMTYQPASGEVGADALVIQLA
233


Query: 232  SLLAGTATNP-------------FGNGYYQGPQNAPL---EAASACPGVYGKGAYPGYAG
275
            + LA  ATNP              + +   + +++ +    + A+ C  V+G GA+PG+ G
Sbjct: 234  TGLADLATNPDLTKSLFKSETTPYNDDVKKNHESSSMYIVDPATKCTRVFGSGAFPGFTG
293


Query: 276  DLLVDTTTGGSFNAYGANGRKFLLPALYDPTTSACST 312
            + VD  TGG+FN++G N  KFL+P+++DP T +C T
Sbjct: 294  RIRVDPITGGAFNSHGINHLKFLIPSIWDPKTKSCWT 330
```

>dbj|BAB08671.1| (AB018109) gb|AAD25141.1~gene_id:K17N15.10~similar to unknown
            protein [Arabidopsis thaliana]
            Length = 337

 Score = 168 bits (420), Expect = 1e-40

```
Query:  95  EKYYKLATPSKNSSPLSLTLGKQIIDESCSLGKSLTDKKIQTLASKG-----------DQR
144
            + Y       + -+   ++ LG++  D   S GKSLT   IQ++             + +
Sbjct: 110  QLY---TDQTGSNITGTVRLGEEKNDRFYSHGKSLTRLSIQSVIKSAVTSRSRPLPVNPK
166


Query: 145  NAINVVLTSADVTVTGFGMSRCGTHGHARGLGKRGSKFAYIWVGNSETQCPGQCAWPFHA
204
            + + ++LT+ DV V  F    CG H +          G   Y WVGNS   CPG CA+PF
Sbjct: 167  SGLYLLLTADDVYVQDFCGQVCGFH-YFTFPSIVGFTLPYAWVGNSAKLCPGVCAYPFAV
225


Query: 205  PVYGPQSPPLVAPNNDVGLDGMVINLASLLAGTATNPFGNGYYQGPQN-APLEAASACPG
263
            P + P    P+ +PN DVG+DGM+  +A  +A ATNP  N +Y GP   AP+E A   C G
Sbjct: 226  PAFIPGLKPVKSPNGDVGVDGMISVIAHEIAELATNPLVNAWYAGPDPVAPVEIADLCEG
285


Query: 264  VYGKGAYPGYAGDLLVDTTTGGSFNAYGANGRKFLLPALYDPTTSACS 311
            +YG G   Y G +L D  +G ++N  G   R++L+   L+    S C+
Sbjct: 286  IYGTGGGGSYTGQMLND-HSGATYNVNGIR-RRYLIQWLWSHVVSYCT 331
```

>gb|AAD25141.1|AC007127_7 (AC007127) unknown protein [Arabidopsis thaliana]
          Length = 363

 Score = 159 bits (399), Expect = 3e-38
 Identities = 100/289 (34%), Positives = 144/289 (49%), Gaps = 22/289 (7%)


```
Query:  35  LKYHKGALLSGK-ISVNLIWYGKFKPSQRAIISDFITSLTHTSPTSKTLHQPSVATWWKT
93
            L+YH G +LS    I++ +IWYG++    +++I DF+ S++      S     PSV+ WW+T
Sbjct:  79  LRYHMGPVLSSSPINIYVIWYGQWSRPHKSLIRDFLNSISDAKAPS-----PSVSEWWRT
133


Query:  94  TEKYYKLATPSKNSSPLSLTLGKQIIDESCSLGKSLTDKKIQTLASKG--------DQRN
145
                    Y       + ++   S+ +   +  D   S G+ LT   IQ + +        D +N
Sbjct: 134  ASLY---TDQTGSNVSRSVLIAGEYSDSKYSHGQHLTRLTIQEVIASAARSASFPVDHKN
190


Query: 146  AINVVLTSADVTVTGFGMSRCGTHGHARGLGKRGSKFAYIWVGNSETQCPGQCAWPFHAP
205
            + +VLTS DVT+  F  + CG H +          G   Y WVG S  QCP  CA+PF  P
Sbjct: 191  GMYLVLTSHDVTMQDFCRAVCGFH-YFTFPSMVGYTMPYAWVGQSGKQCPEVCAYPFALP
249
```

>gb|AAC61821.1| (AC004667) unknown protein [Arabidopsis thaliana]
        Length = 899

 Score = 159 bits (397), Expect = 5e-38
 Identities = 105/290 (36%), Positives = 151/290 (51%), Gaps = 23/290
(7%)

Query: 35   LKYHKGALLSGKI-SVNLIWYGKFKPSQRAIISDFITSLTHTSPTSKTLHQPSVATWWKT
93

            L+YH G ++S + S+ +IWYG++ P+ ++II DF+ S++   +P   PSV+ WWKT
Sbjct: 612 LQYHLGPVISSPVTSLYIIWYGRWNPTHQSIIRDFLYSVSAPAPAQ----YPSVSNWWKT
667

Query: 94   TEKYYKLATPSKNSSPLSLTLGKQIIDESCSLGKSLTDKKIQ----TLASKGDQRNAIN-
148

            + Y+ T S + +L L + D + S G LT   +Q   T +   NA+N
Sbjct: 668 V-RLYRDQTGSNITD---TLVLSGEFHDSTYSHGSHLTRFSVQSVIRTALTSKLPLNAVNG
724

Query: 149 --VVLTSADVTVTGFGMSRCGTHGHARGLGKRGSKFAYIWVGNSETQCPGQCAWPFHAPV
206

            +VLTS DV + F + CG H +   G+ Y WVGNSE QCP CA+PF P
Sbjct: 725 LYLVLTSDDVEMQEFCRAICGFH-YFTFPSVVGATVPYAWVGNSERQCPEMCAYPFAQPK
783

Query: 207 YGPQS-----PPLVAPNNDVGLDGMVINLASLLAGTATNPFGNGYYQGPQ-NAPLEAASA
260

             P S   + PN +VG+DGM+ +A LA ++NP NG+Y G AP E A
Sbjct: 784 PFPGSGFVAREKMKPPNGEVGIDGMISVIAHELAEVSSNPMLNGWYGGEDATAPTEIADL
843

Query: 261 CPGVYGKGAYPGYAGDLLVDTTTGGSFNAYGANGRKFLLPALYDPTTSAC 310
            C GVYG G GY G + D   +N G GRK+L+ ++D   + C
Sbjct: 844 CLGVYGSGGGGGYMGSVYKDRWR-NVYNVKGVKGRKYLIQWVWDLNRNRC 892