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An *in vitro* and *in vivo* study of the mechanical stress-controlling region of the *extA* extensin gene promoter from *Brassica napus*

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A thesis submitted to the University of Wales Bangor in candidature for the degree of Doctor of Philosophy

School of Biological Sciences, University of Wales Bangor

2003

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Abstract

Extensins are hydroxyproline-rich glycoproteins, which are abundant in the cell wall of most plant species. It has been found that expression of extensin genes is induced in areas of the plant that are subjected to mechanical stresses. The promoter of the *extA* extensin gene from oilseed rape (*Brassica napus*) has been previously characterised and a negative regulatory region (NRR), which repressed expression in non-phloem cell types, was located between -664 and -433bp relative to the transcription start site. This repression was shown to be overcome in regions of the plant subjected to mechanical stresses, resulting in high expression levels.

In the current work, the NRR from the *extA* gene promoter was dissected further using a series of PCR-generated 5′ promoter truncations fused to the β-glucuronidase (*gus*) reporter gene. These truncations terminated at -636bp, -619bp, -586bp, -569bp, -547bp, -524bp, -505bp, -481bp and -452bp, relative to the transcription start site. Transgenic tobacco plants containing these truncations were regenerated and *extA*-driven *gus* expression was analysed.

Histochemical analysis of mature stem cross sections from transgenics containing the -636bp, -619bp, -586bp, -569bp truncations revealed that GUS activity was located in the phloem. Expression became non-specific in transgenics containing the -547bp, -524bp, -505bp, -481bp, and -452bp truncations, suggesting that a negative regulatory element, repressing expression in non-phloem cell types, exists between -569 and -547bp.

The ability of the -569 to -547bp region to bind a putative repressor protein was investigated in electrophoretic mobility shift assays (EMSAs) using specific oligonucleotides. A MYB core sequence (CTGTTA), which is disrupted in the -547bp truncation construct, was located and EMSAs revealed that an oligonucleotide containing the MYB core and 44bp of flanking sequence bound rape leaf nuclear proteins and formed two specific complexes of reduced mobility. These specific complexes were designated B1 and B2.

Northern analysis identified developmental stages when *extA* was expressed; the *extA* transcript is absent in mature laminae and present to high levels in mature petiole. B1 and B2 complexes were only formed with nuclear protein extracts from

mature *B. napus* lamina and not with extracts from mature petiole, implying that the complexes consist of repressor proteins, preventing *extA* expression in the lamina.

Different regions of the NRR formed additional complexes with nuclear proteins extracted from rape leaves, indicating that the NRR is functionally composite.

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List of abbreviations used in text

- Standard chemical formulae and SI units of measurement have been used throughout this work.
- Standard DNA IUPAC codes are used and DNA sizes are measured in either kilobases (kb) or base pairs (bp).
- Standard three letter abbreviations were used to represent amino acids.

BSA	bovine serum albumin
CHO	'cold' homologous oligonucleotide
CN-HO	'cold' non-homologous oligonucleotide
Cps	counts per second
Da	Daltons
Dicots	dicotyledonous (plants)
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EMSA	electrophoretic mobility shift assay
EDTA	ethylenediaminetetraaceticacid
GUS	β-glucuronidase or *uidA* gene
HRGP	hydroxyproline rich glycoprotein
IAA	isoamyl alcohol
λ	lambda
LB	Luria broth
mRNA	messenger RNA
MS	Murashige and Skoog (medium)
NRE	negative regulatory element
NRR	negative regulatory region
Oligo	oligonucleotide
OD_{600}	optical density (absorbance) at 600nm
pH	hydrogen ion potential

PCR	polymerase chain reaction
PMSF	phenyl methyl sulphonyl fluoride
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	saline sodium citrate
TCS	transcription start site
T-DNA	transfer DNA (*Agrobacterium tumefaciens*)
TEMED	N, N, N', N'- tetramethylenediamine
Tris	tris(hydroxymethyl) aminomethane
UV	ultraviolet
V	Volts
v/v	volume for volume ratio
W	Watts
w/v	weight for volume ratio
w/w	weight for weight ratio
xg	centrifugal force expressed relative to gravity
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

1. Introduction

1.1. The plant response to mechanical stress

The earliest documentation of the mechanical stress phenomenon was by Charles Darwin in his 1881 book "The Power of Movement in Plants." Darwin noticed that tactile stimulation of pea roots changed the direction and amount of their growth. It has long been observed by loggers that trees within a dense stand readily blow over when exposed to wind for the first time. Interior trees are tall, un-tapered, weak-wooded and shallow rooted (Osawa, 1993), whereas solitary trees are shorter, tapered and strong, the types traditionally selected by shipbuilders for masts (Neel and Harris, 1971). In addition, the shortest stalks of maize in a field are found on the windward edge of the stand.

Natural mechanical stress vectors are not confined to wind and include rain, hail and animal movements. For example, trails through woodland are bordered by stunted undergrowth and plants on greenhouse benches bordering heavily trafficked aisles develop a gradient of increasing height away from the aisle (Mitchell and Myers, 1995). Agricultural practices provide mechanical stress in the form of pruning, pinching and tying. Even urban environments create their own mechanical stresses for plants when prevailing winds are deflected by high-rise buildings, creating air turbulence and strong downdrafts. The inability of plants to move from an adverse environment has led to the evolution of adaptive mechanisms enabling them to respond to physical and chemical stimuli in order to survive.

1.1.1. Physiological and morphological effects of mechanical stress

Mechanical perturbation can have many effects on plant growth and fitness in the field, partly through direct damage caused by desiccation, leaf tearing, lodging and wind throw (Telewski, 1995; Ennos; 1997) and partly through morphological and developmental changes to the plant, which occur over the long term. Mechanical stress has been shown to delay development, reduce height and/or reduce flowering and fruiting, leading ultimately to a reduction in crop yield (Latimer *et al.*, 1986; Giridhar and Jaffe, 1988; Latimer, 1990; Jones and Mitchell, 1992; Garner and Bjorkman, 1996).

A major difficulty with understanding the effects of wind on plants is that it doesn't act as a single stimulus but has two different effects: it increases the air flow past the leaves and it mechanically stimulates the plant, in particular by flexing the stem. It has been shown that wind stress reduces extension growth at a whole plant level (Coutland and Moulia, 2000) but stimulates local secondary thickening (Goodman and Ennos, 1998). In a unique study by Smith and Ennos (2003), it was found that the two aspects of wind stimulation, air-flow and flexing, had opposite effects on sunflower plants. Airflow increased stem height, while reducing the strength and rigidity of the stem whereas stem flexure reduced height, while increasing the strength and rigidity of the stem. There was a clear trade-off between hydraulic and mechanical capability. A plant's reaction to wind must therefore be a complex response to at least two different stimuli, explaining why the effect of wind stress varies with species and environment. Plants in the Smith and Ennos experiment (2003) subjected to both air flow and stem flexure tended to have shorter stems with lower rigidity than those subjected to neither, although these differences would have depended on the exact conditions.

Such a trade-off has also been observed with the root response to touch. Touch has been shown to modulate gravity sensing and to regulate growth of primary roots of *Arabidopsis*, enabling roots to avoid obstacles in the soil while maintaining downward growth (Massa and Gilroy, 2003). The root tip and the peripheral cells of the cap exhibit touch sensitivity and touch-induced Ca^{2+} increases have been shown in these areas (Legué *et al.*, 1997) (see section 1.1.3). Upon encountering an object (continuous touch stimulation), roots respond by compromising between the gravitropic and thigmotropic (touch) response. Massa and Gilroy (2003) proposed that touch stimulus reduces the gravitropic signalling events generated by the cap cells leading to a tip angle of 136° in *Arabidopsis*, representing a compromise between vertical gravitropic growth and direct sideways growth to avoid the obstruction.

Over 80% of plant species tested display altered development as a result of mechanical stimulation (Jaffe, 1973). This response is distinct from that of specialised plants such as Venus fly trap (*Dionaea muscipula*) and the sensitive plant

(*Mimosa pudica*), in that touch-induced growth changes take place gradually and are not readily recognisable (Braam and Davis, 1990).

Plant growth and developmental responses to shaking, vibration and wind have been described as 'seismomorphism' and the process as 'seismomorphogenesis' (Mitchell *et al.*, 1975). The term 'thigmomorphogenesis' describes morphological changes that occur when plants are lightly touched (Jaffe, 1973). Plant responses to the two kinds of mechanical stress are similar but not always identical (Heuchert *et al.*, 1983). Characteristics of thigmomorphism are internode compression and lateral enlargement of stems whereas the effect of shaking on plants is a general retardation of internode elongation and inhibition of leaf expansion, which dwarfs plant size and mass, depending on the extent of the stress delivered (Mitchell and Myers, 1995).

Growth inhibition resulting from mechanical stress probably contributes substantially to loss of yield by field crops under windy conditions. In studies in which soil moisture was not limiting, crop yield increased when tomato and bean plants (Bagley, 1964; Rosenberg *et al.*, 1966) and soybean (Radke and Burrows, 1970; Radke and Hagstrom, 1973; Frank *et al.*, 1974; Agbuehi and Brandle, 1981) were protected from prevailing winds. Managing the extent of seismomorphogenesis is an important factor contributing to yield enhancement and is a promising candidate for sustainable environmentally friendly cropping systems.

Although wind-exposed plants generally develop fewer and smaller leaves, they contain a higher proportion of mechanical tissues (Martin and Clements, 1935; Grace and Russell, 1977; Niklas, 1996). According to the constant-strain hypothesis for developing wood in tree stems, the distribution of new wood is regulated by the degree of strain in various regions along the stem (Mattheck, 1991). Increased allocation of plant resources to mechanical strength-enhancing proteins and/or their substrates e.g. phenolic monomers for lignin (DeJaegher *et al.*, 1985) may account for the observation by Cipollini (1999) that relative increases in stem structural integrity with increasing exposure to mechanical perturbation are inversely related to plant development and flowering. This is likely to be the result of intrinsic resource allocation trade-offs (Cipollini and Schultz, 1999). Proteins with structural functions whose expression is known to be significantly enhanced in plants exposed to mechanical perturbation include annexins (Thonat *et al.*, 1997), xyloglucan endotransglycosylases (Braam *et al.*, 1996), peroxidases (DeJaegher *et al.*, 1985;

Cipollini, 1997, 1998), and finally the extensins (Tiré *et al.*, 1994; Shirsat *et al.*, 1996a). It has long been accepted that extensins are found in tissues that require mechanical strengthening (see section 1.3.4.1).

1.1.2. Mechanically stressed plants are at an adaptive advantage

Such changes in the morphology and mechanics of aerial tissues are structurally adaptive when the plant is subjected to constant mechanical stress, although effects vary between species. Mechanical perturbations have been shown to stimulate radial growth, decrease flexibility and increase numbers of tracheids (and therefore lignin) in seedlings of pine (Telewski and Jaffe, 1986a) and fir (Telewski and Jaffe, 1986b). Conversely, angiosperm trees accumulate an elevated amount of highly crystalline cellulose with a concomitant decrease in lignin in the cell walls of tension-stressed tissues (Wu *et al.*, 2000). When shaken, xylem tissue cylinder mass was increased in stems of genetically modified tobacco plants which had been engineered to produce secondary xylem of a reduced tensile stiffness (Hepworth and Vincent, 1999).

Mechanical perturbation of the shoot can also induce changes in root architecture and composition that appear to increase anchorage and strength (Gartner, 1994; Crook and Ennos, 1996; Goodman and Ennos, 1996). Thigmic stress of potted potato (*Solanum tuberosum*) caused by stroking the stem tips or seismic stress from shaking the entire shoot led to a decrease in the size and mass, but not the number of tubers formed during treatment (Mitchell, 1992). This suggests that shoots are able to communicate mechanical stress signals to subterranean plant parts.

These cellular and physical changes are part of the natural 'hardening' process that plants undergo when transferred from protected environments to the field. In addition to the direct adaptive benefits that thigmomorphogenesis can have on the prevention of mechanical damage to plants, hardening by mechanical perturbation is also known to enhance plant establishment (Latimer, 1990), chilling tolerance (Keller and Steffen, 1995), pest resistance (Latimer and Oetting, 1994; Cipollini, 1997) and drought tolerance (Suge, 1980). For example, in a study by Jaffe and Biro (1979), rubbed kidney beans survived and recovered from severe drought stress, whereas undisturbed controls did not.

1.1.3. The role of calcium and calmodulin in the mechanical stress response

Calcium is a ubiquitous second messenger in plants, playing a vital role during plant growth and development. Intracellular Ca^{2+} concentrations are modulated in response to various signals including hormones, light, abiotic stress and pathogen elicitors (Reddy, 2001; Rudd and Franklin-Tong, 2001). The information encoded in transient Ca^{2+} signals is deciphered by various intracellular Ca^{2+} sensors such as sensor responders and sensor relays that convert the Ca^{2+} signals into a wide variety of biological responses (Luan *et al.*, 2002; Sanders *et al.*, 2002). There is much evidence to suggest that transduction signals may involve rapid increases in intracellular calcium ion concentration in response to mechanical stress. For example, aequorin, a calcium sensitive bioluminescent protein that occurs naturally in jellyfish emits blue light in the presence of calcium and coelenterazine, a luminophore, and can be used to measure quantitative cytosolic calcium changes in transformed plant cells (Knight *et al.*, 1991). In an experiment by Knight *et al.* (1992) *Nicotiana tabacum* seedlings genetically transformed to express aequorin were placed in a cuvette of a luminometer and when exposed to puffs of air from a syringe, gave off a pulse of blue light proportional to the force received.

The identification of genes responsive to mechanical stress provides further evidence that the calcium flux is involved in early mechanical perception/transduction mechanisms. For example, Braam and Davis (1990) identified five touch (TCH) genes that were up-regulated by mechanical stress in *Arabidopsis thaliana*, which is readily stunted by thigmic treatments. Ten to 30 minutes following treatment, mRNA levels for these TCH genes increased up to 100 fold above control levels, although by 2-3 hours post-stress, most of these transcripts had disappeared. Three of the touch genes were almost identical to or had large regions of homology with genes in other species coding for calmodulin. Ito *et al.* (1995) isolated five genes for calmodulin (AtCAL1, 2, 3, 5, 6) and one gene for a calmodulin-related protein (AtCAL4) from an *Arabidopsis* genomic library. Touch stimulus of *Arabidopsis* plants induced the acculmulation of mRNA transcribed from two of these genes, AtCAL4 and AtCAL5. Depège *et al.* (1997) reported that mechanical stimulation leads to a dramatic accumulation of calmodulin mRNA and a significant increase in calmodulin proteins in tomato plants. Other authors have also implicated signal transduction pathways involving Ca^{2+} or calmodulin in changes in

nuclear gene expression in response to mechanical perturbation (Perera and Zielinski, 1991; Galaud *et al.*, 1993; Botella and Arteca, 1994; Oh *et al.*, 1996).

Calmodulin is the most widely distributed member of a specialised group of eukaryotic cellular proteins involved in transducing Ca^{2+} signals from external stimuli, integrating their effects with those of other signalling pathways and maintaining a homeostatic balance of Ca^{2+} to mininise cytotoxic effects. Calmodulin is a small 16kDa, heat stable, acidic Ca^{2+} binding protein. The primary structures of calmodulin proteins are highly conserved across all lines of eukaryotic phylogeny (reviewed by Kawasaki and Kretsinger, 1994), although numerous isoforms of calmodulin may occur within a single plant species.

Calmodulin's mode of action involves transducing second messenger Ca^{2+} signals by binding to short peptide sequences within target proteins and inducing structural changes, causing them to have altered activities (Zielinski, 1998; Snedden *et al.*, 2001; Reddy *et al.*, 2002; Sanders *et al.*, 2002). Molecular recognition and biochemical regulation of calmodulin are dictated by its interaction with short peptide sequences in target proteins. Calmodulin binds with high affinity to peptides ranging from 17 to 25 amino acids in length, having a basic amphiphilic α-helical structure (O'Neil *et al.*, 1990). Binding is accomplished by a change in conformation of the central α-helix of calmodulin, permitting the globular lobes of the protein to engulf the target peptide (reviewed by Crivici and Ikura, 1995). Calmodulin activation can also involve other conformational changes such as multimerisation (Baum *et al.*, 1996; Hellermann and Solomonson, 1997).

Ca^{2+} mediated signalling through calmodulin is an ordered process in that Ca^{2+} enters the cytosol in response to a signal, is bound by calmodulin and the Ca^{2+}-calmodulin complex binds and activates a collection of target proteins leading to a physiological response. The Ca^{2+}-calmodulin complex has been shown to interact with a large number of enzymes such as phosphodiesterase, adenylate cyclase, Ca^{2+}-ATPase and several kinases, the most characterised of which are the calcium dependent protein kinases (Harmon *et al.*, 2001; Cheng *et al.*, 2002 and reviewed by Zhang and Lu, 2003). Many of these enzyme substrates are key regulators of a variety of cellular processes (Cheung, 1980). Activation of calmodulin through the binding of second messenger Ca^{2+} can therefore result in a cascade of regulatory events. Although calmodulin is commonly viewed as a cytosolic protein, it is also

found in the nucleus where it specifically associates with certain transcription factors (Corneliussen *et al.*, 1994; Szymanski *et al.*, 1996), in addition to an NTPase (Chen *et al.*, 1987) and histone H1 (Rasmussen and Garen, 1993).

Genes encoding calmodulin have been isolated from several plant species, including apple (Watillon *et al.*, 1992), *Arabidopsis* (Perera and Zielinski, 1991; Chandra *et al.*, 1994; Ito *et al.*, 1995), potato (Takezawa *et al.*, 1995), rice (Choi *et al.*, 1996) and wheat (Yang *et al.*, 1996). It is likely that multiple calmodulin genes encoding one protein are needed to fine-tune expression to meet the specific needs of different organs and tissues.

The complexity of the response to Ca^{2+} levels is due in part to the large number of calmodulin substrates and the fact that there are four Ca^{2+} binding sites in calmodulin (Cheung, 1980). Certain enzymes, such as phosphodiesterase, prefer calmodulin with all four Ca^{2+} domains filled for activation (Wolff *et al.*, 1977; Crouch and Klee, 1980; Stull *et al.*, 1980). The Ca^{2+} and calmodulin signal transduction pathway in plants may therefore involve changes in the concentration of both the messenger and the mediator and the cell may regulate the ratio of these components to elicit a particular response. In addition, signalling pathways have distinct and characteristic responses to second messenger Ca^{2+} because calmodulin-binding domains are very different, with no consensus calmodulin-binding domain identified from sequence comparisons (O'Neil and DeGrado, 1990; Crivici and Ikura, 1995).

In an attempt to identify the origin of the Ca^{2+} increase following mechanical stress treatments, Knight *et al.* (1992) performed experiments with ion channel blockers. Wind-induced increases in cytosolic Ca^{2+} were unable to be blocked by the plasma membrane channel blocker lanthanum and the stretch channel blocker gadolinium whereas ruthenium red, a blocker of organelle and endomembrane calcium channels completely abolished the wind-induced cytosolic Ca^{2+} increase, leading to the conclusion that wind-induced elevation of cytosolic calcium is of intracellular origin (Knight *et al.*, 1992). According to current models (Guharay and Sachs, 1984), membranes become distorted when force is applied to them and the inelastic linkers connecting various stretch activated ion channels in the membranes tug at their points of attachment to channel proteins. Tugging opens channels briefly (Edwards and Pickard, 1987) and allows specific ions like Ca^{2+} to flow from

mitochondria, endoplasmic reticulum or other internal membranes into the cytoplasm where they activate calmodulin. However, since initial perception of mechanical stress occurs outside the plant cell, the force must be transmitted through the extracellular matrix, across the plasma membrane and signalled along to stretch activated Ca^{2+} channels in the plasmalemma (Pont-Lezica *et al.*, 1993; Wyatt and Carpita, 1993; Trewavas and Knight, 1994). Physical stimuli outside the cell can be transmitted to stretch channels inside by special proteins called 'integrins.' These are physically connected to cell wall proteins on the outside of the plasma membrane and have domains extending into the cytosol where they can bind to actin-like filaments and other elements of the cytoskeleton (Fleurat-Lessard *et al.*, 1988). As these transmembrane extracellular matrix receptors physically interconnect the outside and inside of the cell, they are probably the mechanoreceptors that sense and transmit pressure on the cell wall to endomembranes, causing specific Ca^{2+} channels to open (Wang *et al.*, 1993; Wang *et al.*, 2001). Mechanosensitive gating of stretch channels would release Ca^{2+} into the cytosol, where it would begin its second messenger activity.

To date, there is no direct evidence that the products of touch genes such as calmodulin are involved in the thigmomorphogenic responses of plants. However, there is plenty of correlative evidence. For example, 10- to 100-fold increases in the abundance of mRNAs for calmodulin and calmodulin-like proteins follow mechanical stimulation (Braam and Davis, 1990; Galaud *et al.*, 1993; Sistrunk *et al.*, 1994), suggesting an up-regulation of this calcium-sensing pathway. In addition, another calmodulin-dependent enzyme, glutamate decarboxylase (Ling *et al.*, 1994; Baum *et al.*, 1996) appears to be upregulated in response to mechanical stimulation, leading to the accumulation of γ-aminobutyric acid in plant tissues (Wallace *et al.*, 1984). Further evidence is provided by the kinetics of induction of expression of the *Arabidopsis* TCH genes, which corresponds to the time when there is a complete inhibition and decreased growth rate of stimulated bean plants (Jaffe, 1976a, 1976b). In addition, induction of calmodulin gene expression and the accumulation of calmodulin protein was correlated with the period when growth inhibition was still effective in rubbed tomato plants (Depège *et al.*, 1997). Work by Jones and Mitchell (1989) implied that calmodulin function is at least partially required for touch-induced growth inhibition as partial inhibition of the thigmomorphogenic response in

soybeans was the result of treatment with calmodulin inhibitors. Furthermore, reorientation of the cytoskeleton by microtubules and microfibrils is thought to be required for the expansion of cell diameter and inhibition of cell elongation following ethylene treatment (reviewed by Eisingr, 1983). Calmodulins are known to enhance the Ca^{2+}-induced dissociation of microtubules (Marcum *et al.*, 1978; Keith *et al.*, 1983). Cytoplasmic Ca^{2+} concentration fluxes known to modulate ion channels in plant cells (Hedrich and Neher, 1987) are consistent with the electrical resistance charges (Jaffe, 1976b) and action potentials (Pickard, 1971) that have been observed following mechanical stimulation.

Other studies have provided support for calcium and calmodulin signalling in environmental and mechanical responses. Harding *et al.* (1997) have demonstrated that transgenic tobacco expressing a foreign calmodulin gene show an enhanced production of active oxygen species. Part of plant defence responses to pathogen and environmental stresses is an oxidative burst reaction that generates active oxygen species (AOS) such as H_2O_2 and superoxide (reviewed by Low and Merida, 1996). AOS production is thought to be involved in several responses to these stimuli such as cell wall lignification (Halliwell, 1978), cross-linking of cell wall proteins (Bradley *et al.*, 1992; Brisson *et al.*, 1994) and the expression of defence response genes (reviewed by Lamb, 1994) and has been proposed to be a key signal co-ordinating these various responses (Chen *et al.*, 1993; Levine *et al.*, 1994; Price *et al.*, 1994).

Not all genes up-regulated in response to mechanical stress code for calmodulin or related proteins. TCH-4 codes for xyloglucan endotransglycosylase which modifies cell wall extensibility properties (Xu *et al.*, 1995). This implies that changes at the cell wall are part of the mechanical stress response.

1.1.4. The role of hormones in the mechanical stress response

Hormones are strongly implicated in the mechanical stress response by the fact that developmental responses of such stressed plants are dose dependent (Jaffe, 1976a), saturable (Beyl and Mitchell, 1977) and one or more morphogenic stimuli are transmissible from plant parts that have been physically disturbed to those that have not (Erner *et al.*, 1980) and vice versa (Beyl and Mitchell, 1983). Furthermore, many

symptoms of seismo- and thigmomorphogenesis can be mimicked or antagonised by chemical agents (Erner and Jaffe, 1982: Boyer *et al.*, 1983; Biro and Jaffe, 1984).

Plant hormone levels play an integral role in resource allocation in plants in response to environmental stresses (Trewavas, 1986). One of the strongest correlations between mechanical stress and phytohormones involves the gibberellins (Suge, 1978; Beyl and Mitchell, 1983). High gibberellic acid levels are associated with increased stem elongation and early flowering in many rosette producing species, including *Brassica* (Rood *et al.*, 1990); therefore reductions in gibberellic acid levels in mechanically stressed *B. napus* plants may partially mediate the observed height reductions and delays in flowering (Cipollini, 1999). When gibberellic acid was applied exogenously to rubbed bean stems, the usual stunting of growth was prevented (Jaffe and Biro, 1979). Furthermore, many chemical growth retardants are gibberellic acid biosynthesis inhibitors and the effects of growth retardants often resemble the effects of mechanical stress (Mitchell, 1996).

Enhanced ethylene produced by mechanically stressed plants is also thought to lead to the retarded stem growth responses of bean and *Bryonica dioica* (Biro and Jaffe, 1984; Jaffe *et al.*, 1985; Jaffe and Forbes, 1993). There is a close relationship between touch and ethylene as growth responses are similar and touch results in ethylene production (Goeschl *et al.*, 1966, Biro and Jaffe, 1984; Telewski and Jaffe, 1986b). However, Braam and Davis (1990) reported that the effects of ethylene treatment on TCH gene expression were not identical to those of touch treatment. The kinetics of stress ethylene production are too slow for it to be the main cause of initial signal transduction as mechanical stress has been shown to inhibit stem growth in seconds to minutes in dark and light grown plants respectively, following the onset of stress (Jaffe, 1973). It is therefore likely that ethylene production is a related response that occurs as a result of the regulation of TCH gene expression and may be important in later developmental events. Evidence for this is provided by the fact that the Ca^{2+} influx has been implicated in the stimulation (Lau and Yang, 1974) and maintenance (Lieberman and Wang, 1982) of ethylene production. Rising ethylene can, in turn, lead to cross-linking of cell wall polymers and/or down regulate the activity of cell wall loosening enzymes (Fry *et al.*, 1992). The origin of ethylene may also come from the deposition of callose in the cell wall space just outside the plasma membrane as a result of increased Ca^{2+} concentration. Callose deposition

occurs within seconds after the onset of stress (Jaffe, 1984) and since this area is thought to be the site of ethylene synthesis (Mattoo *et al.*, 1982), wound callose formation may stimulate the ethylene forming complex. On the other hand, soluble, translocatable oligosaccharides arise in the wall space of rubbed plants, either as breakdown products of wall polymers or as their precursors and they may act as elicitors of ethylene formation and the mechanical stress response (Takahashi and Jaffe, 1982).

Auxin, abscisic acid (Erner and Jaffe, 1982) and cytokinins (Beyl and Mitchell, 1983) are also thought to play a role in the mechanical stress response, although there are conflicting reports as to the effects of these phytohormones (reviewed by Mitchell and Myers, 1995).

1.2. Control of plant gene expression

Perception of a stress by a plant triggers rapid defence responses via a number of signal transduction pathways, as in section 1.1.3. A major target of signal transduction is the cell nucleus where the terminal signals lead to the transcriptional activation of a battery of genes as part of the plant defence/stress response.

1.2.1. An overview of the control of plant gene expression

The term 'gene expression' refers to the entire process whereby the information encoded in a particular gene is decoded into a particular protein. Regulation at any one of the various steps in the pathway from DNA to RNA to protein can lead to altered gene expression (figure 1.1), although direct measurements of the transcription rates of multiple genes in different cell types have shown that regulation of transcription initiation is the most widespread form of gene control (Derman *et al.*, 1981). Analysis of an *Arabidopsis* genomic sequence revealed that about 15% of the genes with predicted or known functions were involved in transcription (Bevan *et al.*, 1998).

The control of eukaryotic gene expression depends on an array of interacting regulatory elements that turn genes on and off in the right place and at the right time. Some of these elements are specific DNA sequences in the vicinity of the gene to be regulated, others are DNA binding proteins encoded by separate genes, whereas others are proteins that bind to and modify the activity of DNA binding proteins.

Cis-acting elements of a gene are DNA sequences that serve as attachment sites for the DNA-binding proteins that regulate the initiation of transcription. Enhancers are *cis*-acting sequences able to bind proteins, leading to the augmentation or repression of basal levels of transcription. They are usually orientation independent and can be located far upstream of the proximal promoter. *Trans*-acting elements are genes located elsewhere in the genome, i.e. somewhere other than the target gene. They encode proteins that are able to interact with the target gene's *cis*-acting elements to activate or repress expression of the target gene. These *trans*-acting factors are generically known as transcription factors.

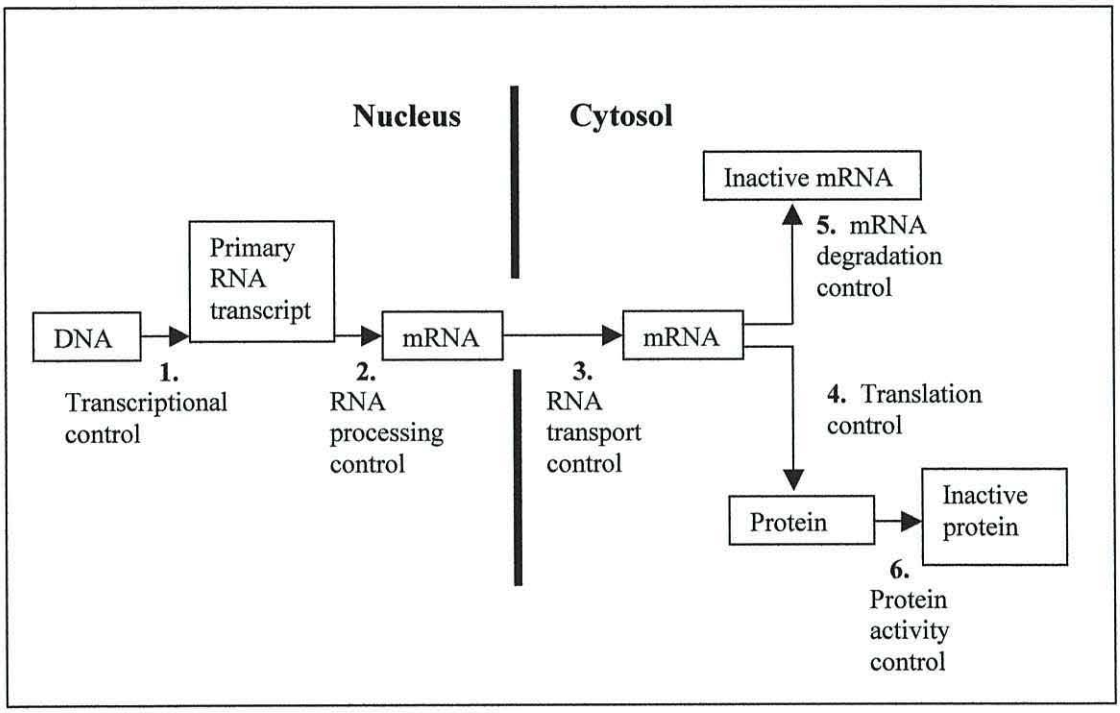


Figure 1.1. The six steps at which eukaryotic gene expression can be controlled from DNA to RNA to protein.

During eukaryotic gene transcription, RNA polymerase makes a single stranded RNA molecule known as the primary transcript that is complimentary to a gene's DNA template strand. Many *cis*-acting DNA elements and *trans*-acting regulatory proteins help to initiate or prevent this first step of gene expression. Activation of transcription is a two-stage process. In the first stage, chromatin must be de-condensed, since most genes exist at some time in an inert state, tightly packaged with histones into chromatin. The second stage involves the interaction of

transcription factors with specific DNA sequences. These DNA binding factors are often a final element in the multi-component signal transduction pathways.

1.2.2. Gene promoters and the role of regulatory elements in transcription

The transcription of each gene is controlled by the promoter, a regulatory region of DNA near the site where transcription begins. However, the term "promoter" is often used to describe the whole 5′ upstream regulatory region of a gene, including sequences far from the transcription start site that are able to modify basal transcription levels. Plant promoters are therefore large, complex arrangements of short DNA sequence elements (*cis*-sequences, boxes or motifs), which provide binding sites for proteins of the transcriptional machinery.

The regulatory sequences in the promoters of most genes can be divided into two categories: the proximal sequences, responsible for the correct initiation of transcription and the distal sequences, which regulate and modulate gene expression.

1.2.2.1. Promoter-proximal elements

These core promoter elements are control regions lying within 100-200 base pairs upstream of the transcription start site. Certain sequence alterations within the promoter proximal elements can greatly reduce transcription rates (Maniatis *et al.*, 1987). Up to two conserved regions may be present in promoter proximal elements:

1. The TATA box, a sequence, approximately 30bp 5′ to the transcription start site (TCS) (Buratowski, 1994), which directs RNA polymerase II to the correct start site for transcription. The presence of the TATA box is necessary for accurate initiation of transcription. The consensus plant TATA box sequence is 5′–CCTATAAATTA-3′.
2. A further control region referred to as the 'CAAT' or 'AGGA' box is often present within 75bp upstream of the TCS and is thought to regulate the frequency of transcript initiation in some genes.

Transcription of genes in plants occurs by similar mechanisms to the process in other eukaryotes. Different classes of genes in eukaryotic cells are transcribed by different RNA polymerases. RNA polymerase I is responsible for the transcription

of rRNA, RNA polymerase III transcribes the tRNAs and other small RNAs whereas RNA polymerase II (pol II) transcribes all protein-encoding genes. The transcription complex contains RNA polymerase and a series of transcription factors designated TFIIA, B, D, E, and F for RNA polymerase II (figure 1.2). These basal factors assist the binding of RNA polymerase II to the promoter and drive the initiation of low levels of transcription (basal transcription).

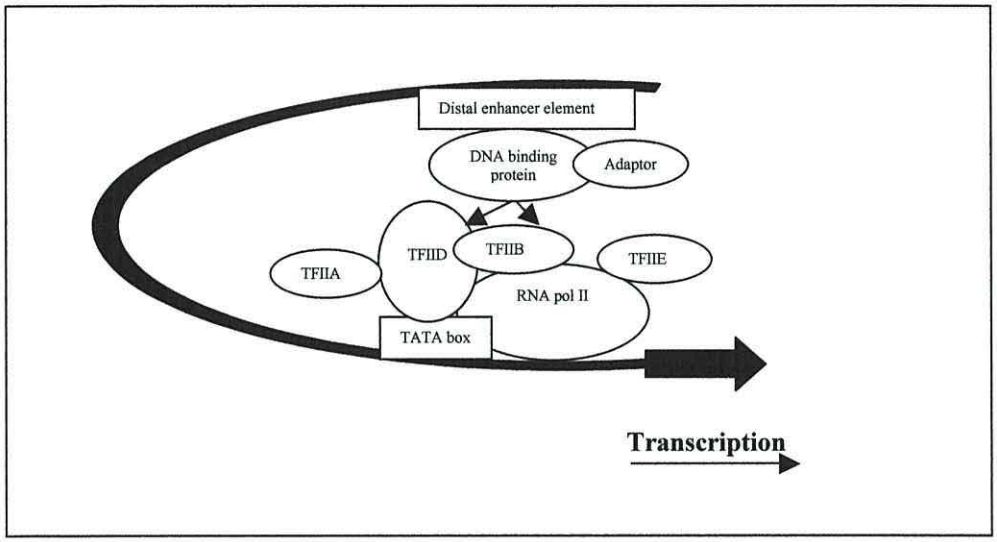


Figure 1.2. Simplified model of the interactions of *trans*-acting DNA binding proteins with the transcription complex containing RNA polymerase II and the transcription factors TFIIA to E.

A stepwise model for the assembly of the transcription initiation complex, starting with the binding of TFIID to the TATA box on the immediate 5′ flanking sequence of the gene, has been proposed (reviewed by Roeder, 1996). However, some of the Pol II in cells has been shown to exist in large protein complexes called the holoenzyme (reviewed by Greenblatt, 1997), suggesting that the transcription initiation complex may already be formed in the absence of DNA.

Most of the primary transcripts produced by RNA polymerase II undergo further processing to generate mRNAs. Under most circumstances, the more mRNA of a gene that accumulates in a cell, the greater the production of that gene's protein product.

1.2.2.2. Distal regulatory elements

Although similar sets of basal factors bind to all the promoters in the eukaryotic genome, different genes can express widely varying amounts of mRNA. This wide range of gene regulation is accomplished through the binding of different transcription factors to different enhancer elements associated with different genes. Enhancer sequences can be located at considerable distances from the TCS, act in a position- and orientation-independent manner to elevate gene transcription, and can stimulate the activity of any gene (Kustu *et al.*, 1991). The principal model for the way in which transcription factors bound to distal *cis*-acting elements influence the rate of transcription involves the intervening DNA being looped out (Martin *et al.*, 1990). Transcription factors bound to distal elements are then brought into contact with the RNA pol II complex at the TATA box, as in figure 1.2. The contact may occur directly or via bridging molecules (Martin, 1991).

Distal regulatory elements enable genes to respond to factors whose presence and/or activity can be regulated by developmental and environmental conditions.

1.2.3. Trans-acting factors (transcription factors)

Transcriptional regulation of gene expression is largely mediated through sequence-specific DNA-binding proteins that recognise *cis*-acting elements located in the promoter regions of the corresponding genes. The binding of these transcription factors to the relevant *cis*-element either facilitates or inhibits the transcription machinery to initiate mRNA synthesis (Meshi and Iwabuchi, 1995).

Transcription factors can be divided into a number of functional classes, with some belonging to more than one class:

1. Activators and repressors bind to specific enhancer DNA sequences and are important in gene-specific regulation.
2. Co-activators and co-repressors mediate the transcriptional effects of specific activators/repressors, in some cases by remodelling chromatin. The factors are not able to bind DNA on their own but can still be promoter-specific as a result of protein-protein interactions with specific activators and repressors.
3. The general transcription factors are important components of the Pol II transcription-initiation complex (see section 1.2.2.1).

Some *trans*-acting factors have a non-specific effect on expression, while others regulate the specific control of expression in relation to development or environmental stimuli.

Whether a transcription factor acts as an activator or repressor, or has no effect, depends on the cell type in which it is expressed and on the gene it is regulating. This is one reason why *cis*-acting elements that bind either activators or repressors are all referred to as enhancers, even though some may repress transcription when associated with the appropriate protein. A regulatory region may contain many enhancer elements, each with the ability to bind different activators and repressors with varying affinities. At any moment there may be dozens of transcription factors in a cell whose affinities for DNA or other polypeptides are being modulated by binding to hormones or other molecules. The transcription factors present in a cell type and their levels of expression within that cell determine which genes or sets of genes are transcribed. The ratio between transcription factors is often crucial, especially if competing positive and negative regulatory factors are involved (Brindle *et al.*, 1990; Grierson *et al.*, 1994). Additional factors other than those that directly bind DNA may be involved, such as protein cofactors (Martin, 1991; Sakurai *et al.*, 1994) and accessory molecules such as metal ions (Berg, 1990) (figure 1.2). Multiple *trans*-acting factors have been shown to bind to the flanking regions of many genes. Such flexibility allows for a precise level of transcriptional repression or activation, depending on the biochemical information being received. Slight changes in a cell's environment can dispatch signal molecules that cause changes in the balance of transcription factors or in their relative affinities for DNA or for each other. These changes lead to the assembly of an altered transcription factor binding complex, which recalibrates gene activity.

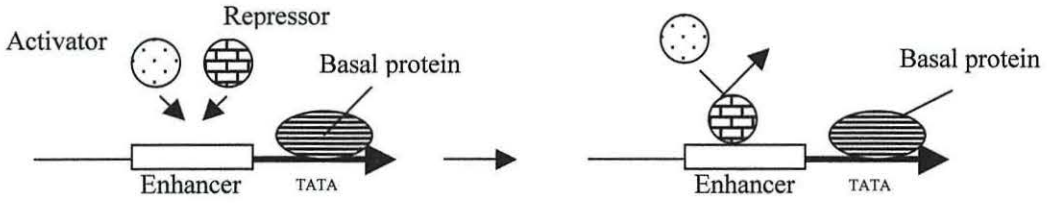
1.2.3.1. Activator proteins

Activator proteins bind to enhancer elements in a sequence specific manner and can interact directly or indirectly with basal factors at the promoter to cause an increase in transcription initiation. Two structural domains within the activator protein, the DNA binding domain and the transcription activator domain mediate these two biochemical functions.

1.2.3.2. Repressor proteins

Some transcription factors suppress the activation of transcription caused by activator proteins. Any transcription factor that has this effect is considered a repressor. In contrast to activators, repressor proteins generally contain a single, small repression domain (reviewed by Hanna-Rose and Hansen, 1996). DNA binding transcriptional repressors act by a variety of mechanisms (reviewed by Hanna-Rose and Hansen, 1996). Some compete with activators for binding to the same enhancer by blocking the activator's access to the same sequence (figure 1.3a), whereas others act without binding DNA and bind to a specific activator, termed quenching (figure 1.3b). In one kind of quenching, a repressor binds to and blocks the activation domain of an activator. These blocked activators still bind to their enhancers but once bound they are unable to carry out their activation. In another kind of quenching, an activator protein is able to bind to the enhancer sequence but the repressor protein binds to its activation domain, preventing interaction with the basal proteins. The repression resulting from both activator-repressor competition and quenching reduces activation but has no effect on basal transcription. However, some eukaryotic repressors act directly on the promoter to eliminate almost all transcriptional activity. Repressors can act by directly blocking the access of RNA polymerase to the promoter or by binding sequences further upstream and by a process known as DNA bending, reach over and contact the basal factor complex, causing the DNA between the enhancer and the promoter to loop out and allow contact between the repressor and the basal factor complex (figure 1.3c). This second mechanism also denies RNA polymerase access to the promoter and reduces transcription below the basal level. Negative regulators of transcription have been shown to displace TFIID from the promoter or to block interactions between TFIID and other basal transcription factors, thus preventing active pre-initiation complex formation (Auble and Hahn, 1993; Auble *et al.*, 1994).

(a). Competition for binding between repressor and activator proteins



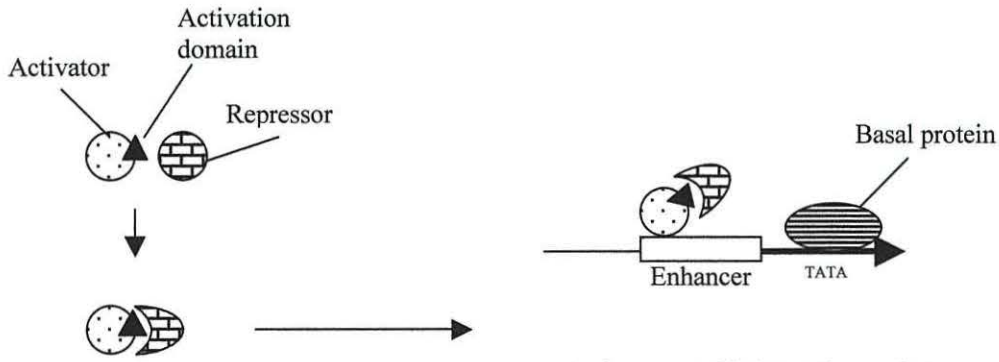
Binding of repressor to enhancer blocks binding of activator

(b). Quenching

Type I: A repressor binds to and blocks the DNA binding domain of an activator

DNA binding domain is blocked. Activator cannot bind to enhancer

Type II: A repressor binds to and blocks the activation domain of an activator



Activator can bind to enhancer but cannot carry out activation

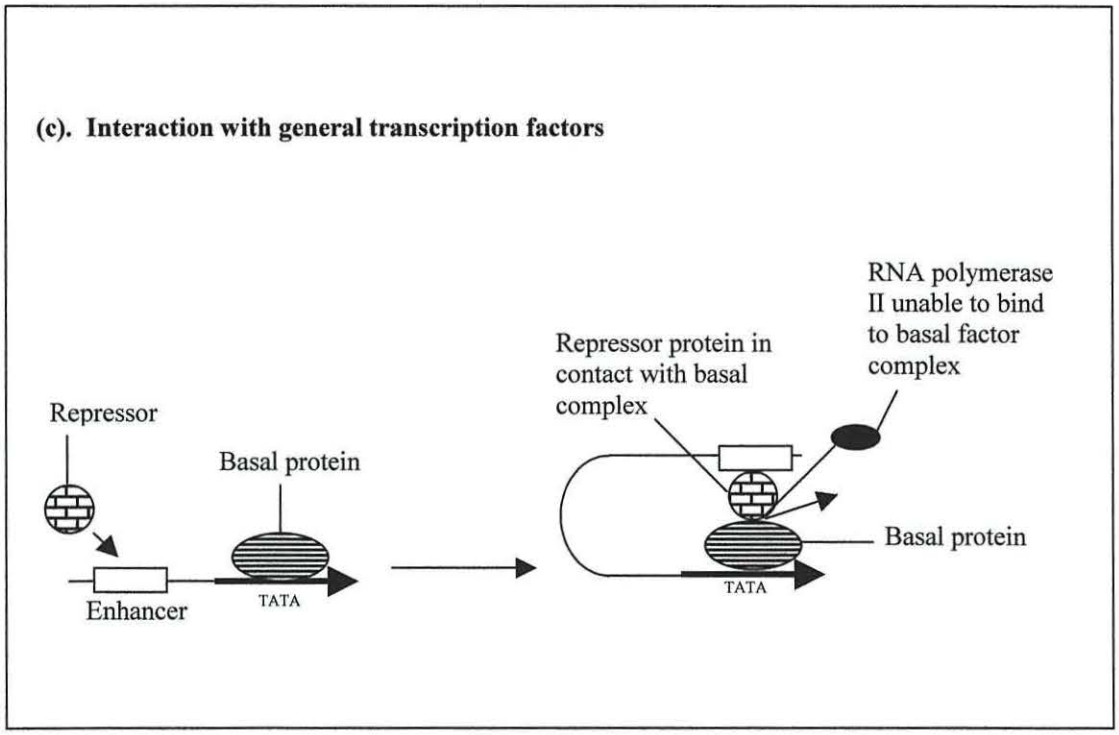


Figure 1.3. Mechanisms of eukaryotic repressor protein action. (a). Some repressor proteins act by competing for the same enhancer elements as activator proteins. However, repressor proteins have no activation domain so when bound to enhancers, no activation of transcription can occur. **(b).** A second class of repressors act by binding directly to the activator proteins themselves to quench activation in one of two ways. Type I quenching occurs when the repressor prevents the activator from reaching the enhancer. Type II quenching is achieved when the activator can bind to the enhancer but the repressor prevents the activation domain from binding to basal proteins. **(c).** Certain repressor proteins are able to prevent RNA polymerase II from initiating transcription by direct interaction with the basal transcription factor complex.

1.2.4. Transcription factor domains

Transcription factors have been described as modular proteins, containing a variety of domains for DNA binding, activation, binding of signalling molecules and interaction with other proteins (Schwechheimer *et al.*, 1998). Modularity permits the combinations of different domains to form transcription factors with discrete functions from a relatively small number of components.

Many eukaryotic transcription factors are homomers (multimeric proteins composed of identical subunits) or heteromers (multimeric proteins composed of nonidentical subunits). Most eukaryotic transcription factors must form dimers (multimers composed of two subunits) to function. The ability to form heterodimers greatly increases the number of potential transcription factors a cell can assemble from a set number of gene products. Different bZIP proteins are able to form homo- and/or heterodimers promiscuously or in a selective way (Armstrong, 1992; Schindler *et al.*, 1992; Guiltinan and Miller, 1994). Regulation of transcription factors through heterodimerisation (Lamb and McKnight, 1991) or modifications by protein kinases has been shown to be important in transcription factor activation or inactivation and in determining their DNA-binding specificity (Hunter and Karin, 1991; Karin and Hunter, 1995).

Transcription factor modularity can therefore explain the ability of a plant to respond to diverse environmental stimuli through similar types of transcription factors. These factors can possess conserved DNA binding and dimerisation interfaces, but still exhibit significant sequence divergence in their activation domains. The variability of activation domains explains how a set of transcription factors with highly conserved DNA binding domains can function in very diverse promoters, be responsive to different sets of signals and be able to interact with or be recognised by different combinations of transcription factors.

Eukaryotic transcription factors contain a variety of structural motifs that interact with specific DNA sequences. It is commonly found that different functions such as DNA binding, dimerisation and transcriptional activation are contained on discrete domains (Keegan *et al.*, 1986). Transcription factors are often classified according to the type of DNA-binding domain they contain. Often similarity in the DNA binding domains predicts similar DNA-binding specificity (reviewed by Mitchell and Tijian, 1989; Gehring *et al.*, 1994; Menkens *et al.*, 1995; Meshi and

Iwabuchi, 1995; Martin and Paz-Ares, 1997; Yanagisawa, 1998). Among the most common structural motifs exhibited by DNA binding domains in eukaryotic transcription factors are the homeodomain, basic zipper/leucine zipper (bZIP), helix-loop-helix and several types of zinc finger (Harrison, 1991).

Transcription factors with novel sequence specificity have been designed by combining known DNA binding domains, as the function of individual domains is often preserved (Ptashne, 1992). For example, a fusion protein that contained a binding domain constructed from zinc fingers and a homeodomain was found to bind optimally to sequences that contained adjacent homeodomain (TAATTA) and zinc finger (NGGGNG) binding sites. When an activator domain was fused onto this binding domain, the resulting fusion protein regulated promoter activity in a sequence specific manner (Pomerantz *et al.*, 1995).

1.2.5. Plant transcription factors

Plants devote a large proportion of their active genome to the control of transcription, with the *Arabidopsis* genome encoding in excess of 1500 transcription factors (Riechmann *et al.*, 2000). Five families of transcription factors have been shown to be particularly involved in plant stress responses. These are ethylene responsive-element-binding factors (ERFs), basic-domain leucine-zipper (bZIP) proteins, WRKY proteins (reviewed by Singh *et al.*, 2002), MYB proteins (Rushton and Somssich, 1998; Jin and Martin, 1999; Hemm *et al.*, 2001; Stracke *et al.*, 2001) and DOF (DNA binding with one finger) proteins, which interact with and stimulate the DNA binding of stress-responsive bZIP proteins (Yanagisawa and Schmidt, 1999; Kang and Singh, 2000).

Plant transcription factors contain a variety of structural motifs that allow for binding to specific DNA sequences. For bZIP transcription factors, DNA binding and dimerisation are mediated by the bZIP motif which is required for dimerisation of the protein binding to the DNA, whereas a basic region contacts the DNA recognition site (reviewed by Hurst, 1995). Some plant transcription factors have DNA binding domains that appear to be unique to plants, for example the AP2/EREBP family found in higher plants (Okamuro *et al.*, 1997), the maize VP1 protein (McCarty *et al.*, 1991) and the *Arabidopsis* ARF1 and Monopteros proteins (Ulmasov *et al.*, 1997a; Hardtke and Berleth, 1998).

Plant transcription factors typically have only a single DNA-binding domain (Singh, 1998), although there are examples in which there are two related DNA binding domains (Ni *et al.*, 1996; Okamuro *et al.*, 1997). The different DNA-binding domains in the GT-2 rice DNA-binding protein have been shown to discriminate between closely related GT-box sequences (Ni *et al.*, 1996). The ability of some transcription factors to form specific heterodimers is a form of combinatorial control (see section 1.2.6.5) and can expand the number of DNA target sequences that can be recognised, in addition to allowing different combinations of activation domains to be recruited to a promoter element (Singh, 1998). Electrophoretic mobility shift assays (EMSAs) (section 3.1.1) have been used to study the ability of DNA-binding proteins to form homo or heterodimers such as the *Arabidopsis* GBF1, 2 and 3 bZIP proteins (Schindler *et al.*, 1992). The addition of one GBF to the binding reaction mixture resulted in a single band representing the homodimer whereas the addition of a second GBF led to the formation of two or three bands, representing homo or heterodimeric complexes.

In *Arabidopsis*, a number of families of transcription factors, each containing a distinct type of DNA binding domain have been implicated in plant stress responses because their expression is induced or repressed under different stress conditions (Shinozaki and Yamaguchi-Shinozaki, 2000). Approximately 5.9% of the total number of estimated genes in *Arabidopsis* encodes transcription factors (Riechmann *et al.*, 2000). Among these, approximately 800 genes encode AP2/EREBP, bZIP, MYB proteins, zinc finger proteins, HD-ZIP and AUX/IAA types of transcription factors (Chen *et al.*, 2002). Recent microarray analysis of *Arabidopsis* transcription factor genes demonstrated that a number of transcription factor genes characterised previously as being activated by abiotic stress were also activated by pathogen infection (Chen *et al.*, 2002). Different stress signalling pathways may therefore overlap or converge at specific points (Ingram and Bartles, 1996), with certain transcription factors representing those points of convergence.

1.2.6. Factors affecting transcription factor binding

1.2.6.1. Chromatin.

Basal factors and RNA polymerase readily bind to promoters on naked DNA *in vitro* to initiate high levels of transcription in the absence of activator proteins. A major

function of chromatin is the reduction of basal transcription from all genes to a very low level. The general suppression of gene activity is achieved by the structure of chromatin which consists of a repeating unit called the nucleosome, made up of an octomer of histone proteins around which 146bp of DNA is wrapped (Kornberg, 1974). Chromatin affects the transcription process at many levels; nucleosomes can prevent the binding of certain transcriptional regulatory proteins and initiation factors and can also impede elongation by RNA polymerase II (Workman and Buchman, 1992). In order for transcription to occur, repressive chromatin must be remodelled by special multiprotein 'chromatin remodelling machines' or CRMs (reviewed by Cairns, 1998) to allow access for transcription factors and the recruitment of RNA polymerase II (Pol II) transcription initiation complex. Chromatin can also affect transcription when organised into active and silent regions.

1.2.6.2. Phosphorylation

There are several examples where phosphorylation of transcription factors has been shown to regulate plant defence gene activation. G/HBF-1 is a bZIP transcription factor, which binds to the G box, and an adjacent H box in the *chs15* promoter (Dröge-Laser *et al.*, 1997). During pathogen-induced activation of the *chs15* gene, the levels of G/HBF-1 did not change. Instead, it was phophorylated rapidly by a cytosolic protein serine-kinase which enhanced its binding to the *chs15* promoter. KAP-1 and KAP-2 are H box binding proteins, which have been isolated from bean cells (Yu *et al.*, 1993). Dephosphorylation of these proteins alters the *in vitro* mobility of the KAP-DNA complexes in gel retardation assays, suggesting that phosphorylation affects the formation of such complexes.

Phosphorylation of the DNA-binding protein PBF-1 increases its binding affinity for the elicitor-inducible *cis*-element of the potato *PR-10a* gene (Despres *et al.*, 1995). The binding kinetics match the transcriptional induction of the gene in response to elicitors. Phosphorylation might therefore activate PBF-1, which in turn activates transcription of the *PR-10a* gene. Finally, pre-treatment of tobacco nuclear extracts with alkaline phosphatase abolished activity of the transcription factor TDBA12, suggesting that protein phosphorylation was important for the high DNA binding activity (Yang *et al.*, 1999).

1.2.6.3. Sequences flanking the core.

There are numerous accounts of the importance of sequences flanking the core *cis*-element in relation to transcription factor binding (Williams *et al.*, 1992; Izawa *et al.*, 1993; Urao *et al.*, 1993; Solano *et al.*, 1995; Ringli and Keller, 1998; Yanagisawa and Schmidt, 1999). For example, nearly all plant bZIP proteins bind to the core sequence ACGT. However, bZIP proteins also require different nucleotide sequences in the flanking region for precise recognition (Tabata *et al.*, 1991; Schindler *et al.*, 1992; Izawa *et al.*, 1993). The common core in the light and ABA response elements (CACGTG) is recognised by all members of the bZIP-P1 family (i.e., EmBP, GBF and CPRF) (Lamb and Dixon, 1994). However, each member, and any possible heterodimer between members, has a preferred binding site that recognises a distinct sequence that borders the core, thereby specifying a response element subclass to enable specific gene activation by a particular signal to be triggered.

1.2.6.4. AT-rich sequences

AT-rich DNA sequences have been found to bind numerous nuclear proteins non-specifically (Laursen *et al.*, 1994; Yamauchi, 1997). This implies that the structural features of the AT-rich DNA sequences are more important than a particular nucleotide sequence for protein binding. DNA bending at AT-rich regions has been well documented (Harrington and Winicov, 1994). AT-element binding proteins (HMGs) binding to AT-elements located in the 5′ flanking sequences of genes may facilitate the integration of transcription factors into the pre-initiation complex of the TATA proximal domain (Gurley *et al.*, 1993). However, in general, AT-elements of plants seem to boost the amplitude of transcription induction, but do not control induction itself.

1.2.6.5. Co-operativity between transcription factors and combinatorial control.

Plants can achieve developmental diversity while maintaining cell specificity and responding dynamically to their environment by employing a limited number of activators to minimise the complexity necessary to link related signalling pathways and to integrate different regulatory cues. It is thought that the cell accomplishes this by combinatorial control where small combinations of ubiquitous signal- and tissue-

specific activators can be used to regulate a much larger number of different responses (Singh, 1998). Co-operativity not only facilitates binding of activators and repressors to the DNA, but also positions them to create a stereo-specific interface for docking with and recruitment of the transcriptional machinery (Carey, 1998).

Combinatorial control is a major mechanism underlying eukaryotic transcriptional regulation. Many genes are regulated by multiple transcription factors by having a specific set of protein binding sites in their promoters. At any given time a distinct set of transcription factors binds to these different sites to produce complexes called enhanseosomes (reviewed by Carey, 1998). The composition of an enhanceosome assembled at a promoter may change in response to cues such as developmental and environmental signals. Therefore, through combinatorial interactions, a given transcription factor can play multiple roles and help regulate different genes whose expression is induced by distinct signals. However, although the interplay of multiple proteins is important in eukaryotic gene regulation, the absence or inactivity of a single factor does not necessarily lead to dissolution of a transcription complex. Genes can be programmed to a state of "competence" awaiting only a single regulatory cue to activate transcription (Burch and Weintraub, 1983).

Examples of combinatorial interactions in plants include the regulation of pigment production in maize by developmental and environmental signals (reviewed by Mol *et al.*, 1996 and 1998) and the response of plants to the hormone abscisic acid (Singh, 1998). Dof proteins also show co-operative binding. Dof proteins are a new class of plant transcription factors that contain a single zinc finger DNA binding domain that is highly conserved in plants (reviewed by Yanagisawa, 1996) and have been shown to interact specifically with bZIP proteins, resulting in stimulation of bZIP binding to DNA target sequences in plant promoters (Chen *et al.*, 1996).

To summarise, eukaryotic gene regulatory proteins are not simple. For example, in several cases, purified proteins have exhibited the capacity to bind, with equal avidity, to sites on DNA that share only minimal nucleotide sequence similarity. In addition, a given *cis*-regulatory sequence can frequently be the target of more than one DNA binding protein and many eukaryotic DNA-binding activities require the co-operation of two or more polypeptide chains (Johnson and McKnight,

1989). Interactions between individual *cis*-elements are also complex and motifs may function independently, co-operatively or competitively (Donald and Cashmore, 1990). However, the combination of multiple *trans*-acting factors acting on *cis* sequences and the interaction of various *cis*-sequences allows for precise and flexible control of gene expression.

1.2. The extensins

1.3.1. The plant cell wall

The cell walls of higher plants form a multicomponent and dynamic interface between plant cells and their environment. Changes in the environment are perceived here first, after which a cascade of signalling events follows, culminating in the transcription of defence genes and ultimately in their expression, as discussed in the previous section.

The plant primary cell wall is composed of polysaccharides, polypeptides and mineral ions such as calcium and borate (Varner and Lin, 1989; Bonilla *et al.*, 1997). The polysaccharide fraction comprises celluloses and hemicelluloses such as xyloglucans (Carpita and Gibeaut, 1993), in a pectin matrix (Jarvis, 1984). The polypeptide fraction consists of structural proteins and enzymes. There are three classes of structural proteins, the hydroxyproline rich glycoproteins (HRGPs), proline rich proteins (PRPs) and the glycine rich proteins (GRPs) (Showalter, 1993; Cassab, 1998). GRPs are characterised by a high content of Gly (50-70%) organised in short repeats of the (gly-X) motif where X = Gly, Ala or Ser. PRPs are characterised by Pro-Pro-X-Y-Lys motifs where X, Y = Val, Tyr, His or Glu.

The most important of the cell wall structural proteins are the HRGPs, characterised by the presence of Ser-$(Hyp)_n$ (n = > 3 motifs) separated by Tyr-, Lys-, His-, and Val-rich regions. Numerous enzymes are found in the cell wall. These include the peroxidases, which may play a role in the formation of networks of cross-linked structural proteins (Schnabelrauch *et al.*, 1996). Other important enzymes are endo-1,4-β-D-glucanases (EGases), which hydrolyse 1, 4-β linkages adjacent to non-substituted glucose residues (Nicol & Höfte, 1998) and xyloglucan endo-transglycosylases (XET), which cleave xyloglucans and join the newly generated reducing end to other xyloglucan chains (Fry *et al.*, 1992). Expansins, whose

enzymatic activity is not fully understood, are able to promote cell wall enlargement by weakening the non-covalent bonding between cellulose microfibrils and hemicelluloses (Cosgrove, 1996).

Carpita and Gibeaut (1993) proposed a model to explain the organisation and role of these components in the primary cell wall (figure 1.4). They suggested that the primary cell wall is composed of two interdependent networks embedded in the pectin matrix. The first network consists of cellulose microfibrils wrapped in and cross-linked with xyloglucans and the second network consists of structural proteins connected with intermolecular crosslinks. According to Carpita and Gibeaut (1993), the second network occurs when cell elongation ceases. Nicol and Höfte (1998) proposed that cell elongation would occur by the combined effect of expansins acting at the cellulose/hemicelloluse interface, of EGases cleaving xyloglucan chains and of XET allowing xyloglucan chains to move along cellulose microfibrils.

The exact composition and architecture of a cell wall varies considerably throughout development of different cell types and in response to environmental factors and stresses. For example, under drought conditions, water conservation is the first line of defence in the battle to maintain turgor. When that fails, plants compensate for water loss by changes in cell wall elasticity (Clifford *et al.*, 1998) through the deposition of cell wall proteins (Marshall and Dumbroff, 1999a, 1999b), so that despite a decrease in the cell volume, turgor is maintained.

In addition to providing mechanical strength to the plant, the cell wall also affects morphogenesis by determining the size and shape of plant cells (McNeil *et al.*, 1984). Several lines of evidence suggest that the cell wall is involved in development (Berger *et al.*, 1994; Dupree, 1996; McCabe *et al.*, 1997; Pennell, 1998; Reinhardt *et al.*, 1998; Braam, 1999; Belanger and Quatrano, 2000; Smith, 2001). The link between the cell wall and the division plane is well known (Smith, 2001) and although not proven, cell wall proteins, in particular HRGPs, are assumed to play a role in cell shape (Kieliszewski and Lamport, 1994; Cassab, 1998).

1.3.2. Cell wall proteins and plant defence

The cell wall is also involved in plant defence, acting as a physical barrier to the many different pathogens to which a plant is exposed (McNeil *et al.*, 1984). In

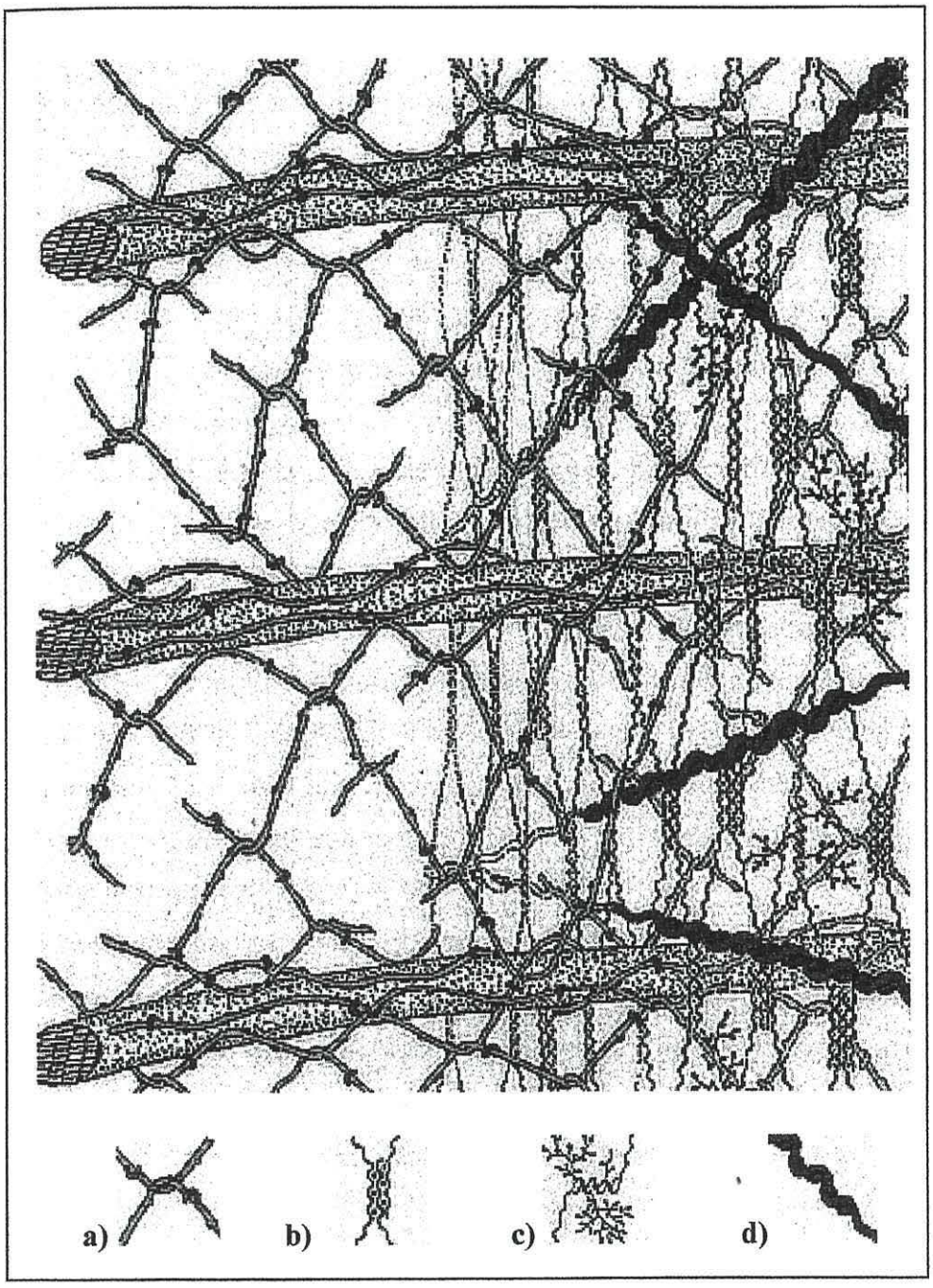


Figure 1.4. The Carpita and Gibeaut model (1993) for the structure of the plant cell wall. **(a)** Xyloglucan, **(b)** Polygalacturonic acid **(c)** Rhamnogalacturonan I with arabinogalactan side-chains, **(d)** Extensin. This illustration is taken from Carpita and Gibeaut (1993), with kind permission of the editors.

addition, signals from the cell wall elicited in response to pathogen attack initiate the plant defence response, which includes strengthening of the cell wall by the deposition of callose, lignin and HRGPs (Lamb *et al.*, 1989; Bowles, 1990; Ryan, 1990). Several authors have shown that elicitor treatment results in the rapid cross-linking of pre-existing PRPs and HRGPs within the cell wall (Bradley *et al.*, 1992; Wojtaszek *et al.*, 1995). This leads to an insoluble network of protein microfibrils, thought to enhance the effectiveness of the cell wall in either trapping pathogens in cells destined to undergo hypersensitive cell death or slowing the spread of the pathogen prior to the activation of transcription dependent defences, such as phytoalexin accumulation (Bradley *et al.,* 1992; Brisson *et al.,* 1994).

The synthesis of cell wall proteins is induced by many compounds and stimuli which are known to turn on genes involved in plant defence responses. These include jasmonic acid, wounding, pathogen attack and elicitors (Creelman *et al.*, 1992; Mouly *et al.*, 1992; Niebel *et al.*, 1993; Showalter, 1993; Brown *et al.*, 1995; Kawalleck *et al.*, 1995; Sidik, 1998; Merkouropolous *et al.*, 1999). The activation of cell wall protein genes occurs not only in tissue adjacent to the site of damage or infection but also in distant tissues and therefore requires the transmission of both intra- and inter-cellular signalling molecules (Lamb *et al.*, 1989).

The increased synthesis, deposition and subsequent cross-linking of cell wall proteins may serve to repair damaged cell walls and may lead to the formation of a more impenetrable cell wall barrier, impeding pathogen infection (Showalter, 1993). It has also been suggested that some of these proteins act as polycations, agglutinating invading bacteria to limit their spread (Leach *et al.*, 1982; Mellon and Helgeson, 1982; Swords and Staehelin, 1993).

1.3.3. Structure and biosynthesis of extensin

Extensins are the most well studied cell wall structural proteins of plants (reviewed by Showalter, 1993; Cassab, 1998; Lamport, 2001). Extensins are a family of HRGPs found in the cell walls of higher plants and are particularly abundant in dicots, where they make up to 10% of the dry weight of the cell wall depending on the cell type and its stage of development (Lamport and Catt, 1981). Extensins are rich in hydroxyproline, serine and a combination of the amino acids valine, tyrosine, lysine and histidine, but tend to contain very little of the acidic amino acids aspartate

and glutamate (José and Puigdomenech, 1993; Showalter, 1993). Extensins are therefore basic proteins with isoelectric points of around 10 (Cooper *et al.*, 1987; Varner and Lin, 1989).

In dicots, extensins are characterised by a highly repetitive pentapeptide motif Ser-$(Hyp)_4$ and an abundance of Tyr, Lys, His and Val residues arranged in repetitive motifs (Kieliszewski and Lamport, 1994). Extensins are also highly glycosylated (Shpak *et al.*, 1999, 2001), containing 50-60% (w/w) carbohydrate (Sommer-Knudsen *et al.*, 1998). The carbohydarate is thought to be important in maintaining the structure of HRGPs as linear, rod-like molecules (Stafstrom and Staehelin, 1988). The monosaccharides in extensins from flowering plants are usually arabinose (90-97%) and galactose (3-7%) (Smith *et al.*, 1984; Van Holst and Varner, 1984; Smith *et al.*, 1986). Hydroxylation of the Pro residues and glycosylation of extensins occurs post translationally in the Golgi apparatus and may involve a sequence-dependent enzymatic mechanism (Kieliszewski and Lamport, 1994; Kieliszewski *et al.*, 1995).

Extensins are secreted into the cell wall as soluble monomers where they rapidly become ionically bound to the network of acidic polysaccharides in the wall. This is followed by a more gradual process in which the extensins become covalently bound and inextractable from the cell wall (Cooper and Varner, 1983; Smith *et al.*, 1984). It is thought that the covalent insolubilisation of extensins in the cell wall may occur through the formation of isodityrosine, an oxidatively coupled dimer of tyrosine (Fry, 1982; Cooper and Varner, 1983; Waffenschmidt *et al.*, 1993). This coupling of tyrosine residues may form intra-polypeptide loops and inter-polypeptide cross-links, giving rise to an extensin network (Epstein and Lamport, 1984; Fry, 1986; Biggs and Fry, 1990). The coupling process is thought to be mediated by the enzyme peroxidase (Schnabelrauch *et al.*, 1996). However, the three dimensional details of how different extensin monomers slot into place and polymerise is purely hypothetical (Lamport, 2001).

1.3.4. The role of extensin

Despite four decades of research since the discovery of hydroxyproline as a constituent amino acid of cell wall polypeptides, direct evidence for the role of extensins in plant cell walls is still absent (Showalter, 1993; Cooper *et al.*, 1994). To

date, there is only correlative evidence based on the structural and regulatory properties they possess and the expression patterns displayed during plant development and in response to various biotic and abiotic stresses.

1.3.4.1. Structural

Lamport (1965) initially proposed that extensins played an architectural role in the cell wall, a notion supported by their biochemical and biophysical properties (section 1.3.3). The combination of amino acids comprising the extensin protein is thought to be important for secondary and tertiary structure. Ser-$(Hyp)_4$ contributes molecular rigidity and kinks (Ferris *et al.*, 2001) and the multiple Tyr residues are thought to allow both intramolecular and intermolecular isodityrosine cross-linking (Kieliszewski and Lamport, 1994). The deposition and cross-linking of extensins has therefore been proposed to increase the mechanical strength of the cell wall (Varner and Lin, 1989; Showalter, 1993).

There is much correlative evidence to support this role in that high levels of extensin are observed in tissues experiencing mechanical stress. Salvà and Jamet (2001) examined expression of the tobacco *Ext 1.4* gene during germination and adventitious lateral root formation, where expression was found to be transient in tissues experiencing mechanical constraint during their growth. For example, expression was seen in the radicle tip and hypocotyls when they grew through the seed coat and in the root primordia, although this disappeared from the root tip as soon as it emerged from the stem or primary root tissues. This pattern of expression was also observed in transgenic plantlets expressing the bean HRGP4.1 promoter-GUS gene fusion (Wycoff *et al.*, 1995). The time during which expression was observed in a given tissue corresponded to the time required for emerging from the seed i.e. during the time these tissues were submitted to mechanical constraint (Salvà and Jamet, 2001).

It has repeatedly been found that tissues subjected to constant mechanical stress initiate expression of extensin promoter-GUS reporter gene fusions. Such tissues include the nodes, endodermis and cortical cells surrounding the emergence of adventitious and lateral roots. The node is thought to experience large tensile stresses due to the weight of an axillary branch or petiole. Expression of an extensin

gene from *Brassica napus*, *extA,* in transgenic tobacco plants containing an *extA* promoter/β-glucuronidase coding sequence fusion was seen in tissues experiencing mechanical stress such as at the cortical parenchyma cells at the point where the axillary flowering branch joined the main stem, the internal and external phloem of the main stem and the region where the axillary branch joined the main stem, where most expression was seen (Shirsat *et al.*, 1996a). The expression of extensin genes from other plant species is also increased at stem nodes and other tissues under mechanical pressure (Tiré *et al.*, 1994; Wycoff *et al.*, 1995; Hirsinger *et al.*, 1999; Merkouropoulos and Shirsat, 2003). This pattern of expression was confirmed as being due to tensile stress (as opposed to a developmental effect) by hanging weights on axillary flowering stalks of low expressing transgenic tobacco plants containing the *extA* promoter-GUS reporter gene fusion (Shirsat *et al.*, 1996a). In weight-loaded plants, expression was induced.

Extensin expression has repeatedly been reported in cells associated with vascular tissue. The presence of extensin in cortical cells associated with vascular bundles and entering a new leaf trace is thought to be due to the need for extensin in cells which require a strong cell wall to withstand compression stresses imposed by neighbouring vascular tissue (Tiré *et al.*, 1994) and where tensile stress in exerted by the weight of a leaf. Localisation of extensin in the inner and outer phloem has been seen in tobacco, tomato and petunia with tissue print experiments (Ye and Varner, 1991; Ye *et al.*, 1991), in tobacco with *in-situ* hybridisations (Tiré *et al.*, 1994) and in tobacco (Shirsat *et al.*, 1991; Hirsinger *et al.*, 1999) and apple (Gittins *et al.*, 2001) using extensin promoter-GUS fusion constructs.

The expression of extensin is quite common in reproductive tissues. Hirsinger *et al.* (1999) found expression of tobacco *Ext 1.4* gene in cells at the fusion of the carpels. The external part of the zone of carpel fusion is the place where dehiscence of the capsule will occur, therefore the reinforcement of the cell wall might protect this zone from early opening during fruit growth. Extensin is involved in the formation of a tough seed coat (Cassab and Varner, 1987) and in ballistic seed dispersal mechanisms that rely on cell wall elasticity (Van Etten *et al.*, 1961). For example, expression of extensin mRNA was seen in the cells of the carpel walls and carpel wall vascular bundles when in-situ hybridisations were performed on *B.*

juncea pods (Shirsat *et al.*, 1996a). The walls of maturing rape pods are under severe tensile stress as the tissues begin to desiccate before rupturing and explosively shedding their seeds. The sclereid cells of the seed coat have also been shown to express extensin (Cassab and Varner, 1987).

The expression of extensin has been shown by many authors to be consistently high in the roots (De Loose *et al.*, 1991; Shirsat *et al.*, 1991; Garcia-Mas *et al.*, 1992; Tiré *et al.*, 1994; Ahn *et al.*, 1996; Arsenijevic-Maksimovic *et al.*, 1997; Hirsinger *et al.*, 1997; Merkouropoulos *et al.*, 1999). It was previously thought that this expression pattern was a result of the mechanical forces experienced by roots as they push their way through the soil (Tiré *et al.*, 1994; Elliott and Shirsat, 1998). However, this theory has recently been discounted by Shirsat *et al.* (2003). These authors discovered that there was no difference in expression of *extA* between plants grown hydroponically and those that were grown in a medium providing more resistance. In addition, the expression pattern of extensin in the root of tobacco plants was found to be variable (Hirsinger *et al.*, 1999), which suggests that reinforcement of the cell wall may not be necessary in the whole root. In roots, expression of gene fusions is found where secondary roots emerge. For example, the soybean *SbHRGP3* promoter-GUS gene fusion is activated in the epidermis facing the point where the lateral root is initiated but not at the tip of the growing lateral root (Ahn *et al.*, 1996); the bean *HRGP4.1* promoter-GUS gene fusion is expressed in the cortex of primary roots where lateral roots are formed (Wycoff *et al.*, 1995) and finally the tobacco *Ext1.4* promoter-GUS gene fusion is expressed in cortical cells surrounding the emergence of the lateral root (Hirsinger *et al.*, 1999). The tobacco *HRGPnt3* promoter-GUS gene fusion was found to be transiently induced in a subset of pericycle and endodermal cells involved in lateral root initiation and in the root tip (Keller and Lamb, 1989). It is possible that extensins or proteins bearing an extensin domain such as *HRGPnt3* may participate in the strengthening of the walls of cells compressed by the emerging lateral root.

Extensin has been shown to accumulate in the first four internodes of *Atriplex prostrata*, a facultative halophyte, when grown under saline conditions (Wang *et al.*, 1997). Under the same conditions, lignin is reduced, suggesting that extensin may replace lignin in providing mechanical support for cells. Lignin is generally

localised in xylem whereas extensin is found mostly in the phloem (Ye and Varner, 1991; Showalter, 1993). In younger internodes where vascular tissues are not extensively developed, extensin is enhanced by salinity stress mainly in pith parenchyma, which has little extensin deposition under normal conditions. Lignin is an important component in the secondary cell wall, while extensin is more commonly found in the primary cell wall. Wang *et al.* (1997) observed that those plants under salinity stress may stay in juvenile stages because of growth retardation and therefore the relative amount of primary cell wall is increased.

1.3.4.2. Growth control

Extensin is almost certainly involved in the growth response of a plant, although to what extent remains to be determined. It has been proposed that extensin is a negative regulator of cell growth. Monomeric extensins can be rapidly cross-linked via extensin peroxidase (Schnabelrauch *et al.*, 1996) and the resulting network increases the rigidity of the cell wall, leading to a slowing down of cell growth (Sadava and Chrispeels, 1973; Ahn *et al.*, 1998). It has been observed that high levels of HRGPs are correlated with the cessation of cell elongation (Cleland & Karlsnes, 1967; Sadava & Chrispeels, 1973; Monro *et al.*, 1974). Root elongation is considered to occur as a result of expansion of the epidermal cells in the region of elongation (Hasenstein & Evans, 1988). Once cell elongation has been completed in roots, the primary wall is locked into shape and this mechanism may involve extensin as one component (Carpita & Gibeaut, 1993) because although turgor pressure represents the driving force of cell elongation, it must be accompanied by the relaxation of the cell wall structural components to yield cell enlargement (Cosgrove, 1993). Studies with peas (Klis, 1976) and beans (Van Holst *et al.*, 1980) both showed an inverse linear relationship between growth rate and extensin deposition.

However, "extensin is not a molecular straight-jacket" (Lamport, 2001) so that cleavage of the network can result in the positive regulation of growth. Several authors have reported the existence of cell wall proteases (Gomez *et al.*, 1995; Jones and Mullet, 1995; Stano *et al.*, 1998; Grobe *et al.*, 1999; Messdaghi and Dietz, 2000). β-expansin has recently been proposed to be a protease (Grobe *et al.*, 1999) and is thought to interact with HRGPs to loosen the cell wall.

There have been several reports suggesting that the expression of HRGP genes has been correlated with cell division (Ludevid *et al.*, 1990; Smallwood *et al.*, 1994; Vera *et al.*, 1994; Hirsinger *et al.*, 1997; Ito *et al.*, 1998). It has also been shown that extensin genes are expressed at high levels in cells proliferating under hormone treatment, such as crown gall cells induced to proliferate by *A. tumefaciens* infection (Parmentier *et al.*, 1995; Hirsinger *et al.*, 1999). The study of transgenic plants carrying an *Ext 1.4*/GUS gene fusion revealed that the chimeric gene was expressed in the external part of the tumor where cell division occurs (Hirsinger *et al.*, 1999).

Transcripts from the extensin gene from *Arabidopsis thaliana*, *atExt1*, were detected in the leaves of 2- and 4-week-old rosettes but not in 6-week-old rosettes (Merkouropoulos *et al.*, 1999). The 6-week rosette defines the transition between the juvenile phase where leaf primordia initiation (with consequent cell division) ends and the vegetative phase (with extension of the apical meristem from the basal rosette) begins (Melford *et al.*, 1994). This developmental expression pattern in the young *Arabidopsis* rosette may therefore be related to cell division as extensin is expressed in dividing cells (see above). In addition, Merkouropoulos *et al.* (1999) reported that *extA* extensin gene transcripts were abundant in rapidly growing *B. napus* seedlings but were absent at later developmental stages. Mature tissues of *Nicotiana plumbaginifolia* were also shown to contain more extensin transcripts than younger tissues (Tiré *et al.*, 1994). It is likely, therefore, that actively dividing cells generating new cell walls require the synthesis of extensins, which may function to organise other cell wall polymers, in addition to providing strength.

The location of the extensin transcript throughout the plant has been studied in many species. Without exception, the extensin transcript has been found to be highly expressed in roots with lower levels in stems and petioles and very little, if any, expression in the leaves (Showalter and Varner, 1987; Gatehouse *et al.*, 1990, Shirsat *et al.*, 1991; Tiré *et al.*, 1994; Wycoff *et al.*, 1995; Hirsinger *et al.*, 1997; Merkouropoulos *et al.*, 1999). This is consistent with the fact that leaves only contain trace amounts of hydroxyproline (Cassab and Varner, 1988) but can also be explained by differences in tissue growth rates. Several transcripts that hybridise to extensin probes are present in rapidly growing (meristematic) shoot and root tissues

(Corbin *et al.*, 1987; Showalter and Varner, 1987). This is especially evident in tobacco, where an increased accumulation of HRGP mRNA is observed when cytokinin synthesis genes are introduced into transgenic plants (Memelink *et al.*, 1987). Callus and roots, therefore, contain high amounts of cell wall extensin and high levels of extensin mRNA, while leaves contain very low amounts and stems contain intermediate levels (Showalter and Varner, 1987).

1.3.4.3. Defence/response to environmental stress

A highly cross-linked network of extensins may provide anchorage for lignification and in this way create an impenetrable barrier to fungal hyphae and other pathogens (Showalter and Varner, 1989; Showalter and Rumeau, 1990). The highly basic extensins could also interact with acidic blocks of pectin, each extensin molecule "zipping" together three or four pectin molecules. This would provide a simple, reversible means of non-covalent protein-polysaccharide cross-linking in the cell wall (Cooper and Varner, 1984; Cooper *et al.*, 1984 and 1987; Cassab and Varner, 1988).

The formation of an insoluble network of protein has been proposed to enhance the effectiveness of the cell wall in either trapping pathogens in cells destined to undergo hypersensitive cell death or slowing the spread of the pathogen prior to the activation of transcription-dependent defences, such as the accumulation of phytoalexins (Esquerré-Tugayé and Lamport, 1979; Bradley *et al.*, 1992; Brisson *et al.*, 1994). A role in agglutination of phytopathogenic bacteria has also been suggested (Leach *et al.*, 1982; Mellon and Helgeson, 1982). In addition, extensin may be involved in plant protection against desiccation at wound surfaces (Cooper *et al.*, 1987).

Numerous studies have shown that extensins are produced in response to wounding (Ecker and Davis, 1987; Sauer *et al.*, 1990; Sheng *et al.*, 1991; Showalter *et al.*, 1991; Adams *et al.*, 1992; Zhou *et al.*, 1992; Rodriguez and Cardemil, 1995; Wycoff *et al.*, 1995; Ahn *et al.*, 1996; Shirsat *et al.*, 1996b; Elliott and Shirsat, 1998; Merkouropolous *et al.*, 1999), pathogens and elicitors (Hammerschmidt *et al.*, 1984; Mazau and Esquerré-Tugayé, 1986; Tiré *et al.*, 1994; Wycoff *et al.*, 1995; Merkouropolous and Shirsat, 2003) and nematodes (Niebel *et al.*, 1993). Ethylene, a

plant hormone whose level is known to increase following wounding, stimulates and may mediate wound-induced accumulation of cell wall HRGPs (Ridge and Osborne, 1970; Esquerré-Tugayé *et al.*, 1979).

The regulation of extensin synthesis in carrot roots has been studied in some detail (Chrispeels *et al.*, 1974; Chen and Varner, 1985a, 1985b; Ecker and Davis, 1987). In carrot, the extensin gene *pDC5AI* has been shown to encode two mRNAs (of 1.5kb and 1.8kb), which are transcribed from different start sites at the 5′ end of the extensin gene (Chen and Varner, 1985a). Treatment of carrot roots with ethylene caused an increase in the level of the 1.8kb mRNA, whereas wounding increased the abundance of the 1.5kb mRNA (Chen and Varner, 1985a; Ecker and Davis, 1987; Tierney *et al.*, 1988). Extensin biosynthesis in wounded carrot roots must therefore be more complicated than a simple "wound-ethylene" switch.

1.3.4.4. Other roles of extensin

A number of other possible functions have been proposed for extensin. For example, Albersheim (1978) suggested that extensin could act as a carrier protein to transport polysaccharides from the cytoplasm to the cell wall or to guide the polysaccharides into their correct position in the cell wall (Kauss and Glaser, 1974). Extensins have also been detected in the plasma membrane by immunological techniques and may function as wall-to-wall membrane linkers (Pont-Lezica *et al.*, 1993).

Immunolocalisation experiments performed by Tiré *et al.* (1994) indicated that there was an association of the extensin protein with lignin deposition in the xylem vessel cell walls. Bao *et al.* (1992) were able to immuno-detect extensin in loblolly pine in xylem cell walls of wood that had been cut and dried for three years. It has been proposed that HRGPs provide sites for lignin deposition (Whitmore, 1978).

Another possible function of extensin is in the promotion of symbiotic relationships. For example, instead of the usual induction of extensin on pathogen invasion, the expression of the *ext26* extensin gene in pea root hairs is reduced after inoculation with *Rhizobium* (Arsenijevic-Maksimovic *et al.*, 1997). Since pea is commonly involved in symbiotic relationships with *Rhizobium* species, the reduction in the amount of extensin gene transcripts may facilitate localised cell wall

degradation to favour the entry of the pathogen, resulting in the establishment of a symbiotic relationship. However, it should be emphasised that in the absence of real proof, these are all correlative relationships.

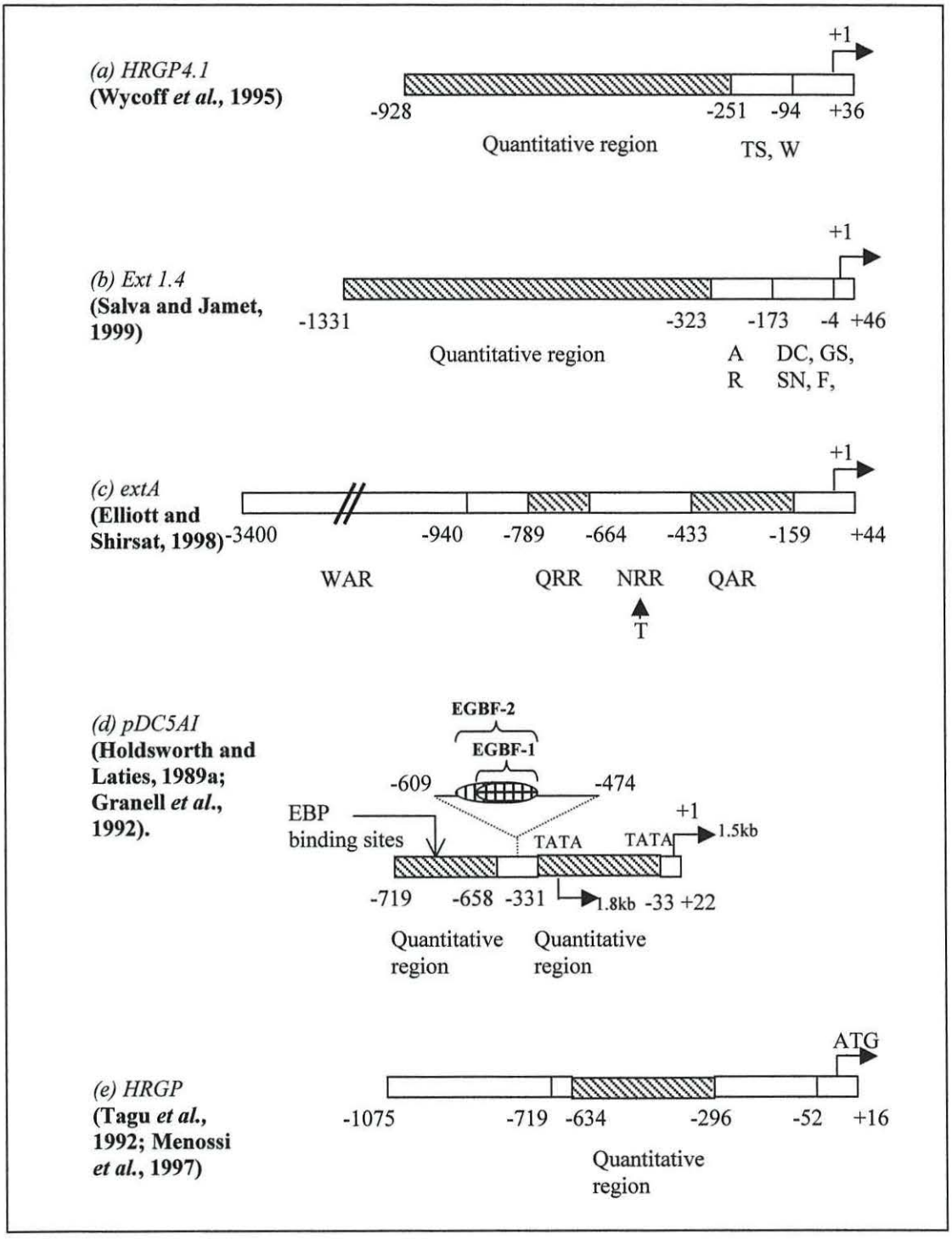
1.3.5. Extensin gene promoters

Five extensin promoters have been studied using GUS as a reporter. These are *extA* from rape (Elliott and Shirsat, 1998), HRGP from maize (Tagu *et al.*, 1992; Menossi *et al.*, 1997), *HRGP4.1* from bean (Wycoff *et al.*, 1995), *Ext 1.4* from tobacco (Hirsinger *et al.*, 1999; Salvà and Jamet, 2001) and *pDC5AI* from carrot (Granell *et al.*, 1992). Figure 1.5 shows a comparison of the structure of these extensin promoters.

HRGP4.1 is a member of a family of stress-induced extensin genes in bean (Corbin *et al.*, 1987; Wycoff *et al.*, 1995). The gene is induced in response to wounding and elicitation and is also expressed developmentally in regions where the cell wall requires strengthening, e.g. the root-tip and stem nodes (Wycoff *et al.*, 1995). The analysis of bean 5′ promoter deletions fused to the GUS reporter gene indicated that the region from –251 to +36 gave the same developmental expression pattern as the full length promoter of 928bp, although at a reduced level. The expression pattern was lost when the promoter was deleted to –94bp, implying that the basic pattern was controlled by sequences between –251 and –94 and that sequences between –928 and –251 enhanced expression. It was therefore proposed that the region between –251bp to –94bp was responsible for tissue specificity and induction by wounding (Wycoff *et al.*, 1995) (Figure 1.5a).

The upstream regulatory regions of the tobacco *Ext 1.4* extensin gene were studied by constructing a series of seven promoter-reporter fusions (Hirsinger *et al.*, 1999; and Salvà and Jamet, 2001). Fluorometric and histochemical analyses defined several regions of the *Ext 1.4* promoter. The most proximal domain between +46 and –173bp allowed expression in cells dividing under hormonal control, during germination (in the endosperm and in the hypocotyl), and in stem nodes, pollen grains, anther filaments and the external zone of carpel fusion. This region was also

Figure 1.5. The structure of five extensin gene promoters: (a) Bean *HRGP4.1* where the region involved in tissue specificity (TS) and wounding (W) is shown; (b) Tobacco *Ext1.4* where regions involved in regulation in cells dividing under hormonal control = DC, germinating seeds = GS, stem nodes = (SN), flowers = F, roots = R and in the connective tissue of anthers = A are marked; (c) Oilseed rape *extA* where four regions were identified, the negative regulatory region (NRR) which may contain an element responsive to tensile stress (T), the wound activating region (WAR), the quantitative repressor region (QRR) and the quantitative activator region (QAR); (d) Carrot *pDC5A1* where the position of the two TATA boxes, the two transcription start sites for the 1.8 and 1.5kb mRNAs, and the regions able to tissue specifically bind transcription factors, EGBF-1, EGBF-2 and EBP, are shown (e) Maize *HRGP*. Numbering refers either to transcription initiation starts (a-d) or to the translation initiation codon (e).


(a) HRGP4.1
(Wycoff et al., 1995)
+1
-928
-251
-94
+36
Quantitative region
TS, W
(b) Ext 1.4
(Salva and Jamet, 1999)
+1
-1331
-323
-173
-4 +46
Quantitative region
A
R
DC, GS,
SN, F,
(c) extA
(Elliott and Shirsat, 1998)
+1
-3400
-940
-789
-664
-433
-159
+44
WAR
QRR
NRR
QAR
T
(d) pDC5AI
(Holdsworth and Laties, 1989a; Granell et al., 1992).
EGBF-2
EGBF-1
-609
-474
EBP
binding sites
+1
TATA
TATA
1.5kb
-719
-658
-331
1.8kb
-33 +22
Quantitative region
Quantitative region
(e) HRGP
(Tagu et al., 1992; Menossi et al., 1997)
ATG
-1075
-719
-634
-296
-52
+16
Quantitative region

proposed to contain a mechanical stress responsive *cis* element because the presence of this region allowed expression upon mechanical constraint at the stem nodes, in the endosperm and hypocotyl (Salvà and Jamet, 2001). Deletion of the +46 to –4bp region, including the transcription start and the untranslated leader sequence resulted in a large decrease in the level of GUS expression, implying that this region was involved in the regulation of the gene at either a transcriptional or post-transcriptional level. A region further upstream between –173 and –323bp conferred expression in the radicle tip during emergence from the seed coat, in cortical cells around the emergence of lateral roots and in the connective tissue of anthers. Another domain between –323 to –695bp was required for expression in the piliferous zone of roots and was found to contain quantitative activator elements during germination. The most distal domain, -929 to -1331bp, allowed expression in root primordia and contained quantitative activator elements for expression in roots (figure 1.5b).

Tagu *et al* (1992) and Menossi *et al* (1997) studied the monocot maize *HRGP* promoter fused to the GUS coding sequence. The –296 to -52bp domain was shown to be required for expression in calli. The –719 to +16bp domain was functional in tissues suffering mechanical stress and in dividing cells of the endosperm. This region was also responsive to ethylene treatment and a fungal elicitor (Menossi *et al.*, 1997) and contained all the essential elements needed to define the spatial and developmental pattern of expression of the gene. Finally, the sequence between –634 and –296bp was identified as a quantitative region, responsible for an increase in the level of gene expression (Tagu *et al.*, 1992) (figure 1.5e).

In work performed by Elliott and Shirsat (1998), a series of promoter truncations (-940, -789, -664, -433, -159) of the *extA* extensin gene from *Brassica napus* (Evans *et al.*, 1990) were constructed and fused to a GUS reporter. Analysis of transgenics showed that the *extA* gene was regulated by 4 sets of positively and negatively acting *cis* regions controlling wound-inducibility, activation in response to tensile stress and quantitative expression levels (Elliott and Shirsat, 1998) (figure 1.5c). An activator region which increased expression levels 10 fold in all cell types was located between –159 to –433bp. In addition, a repressor region was identified between –664 to –789bp and a wound responsive region was found between –940 to –3500bp. Finally a region between –433 to –664bp repressed expression in non-

phloem cell types and is thought to contain an element responsive to mechanical stress (Shirsat *et al.*, 2003). The analysis of the *extA* promoter is discussed in more detail in section 1.3.7.

Granell *et al* (1992) used transient expression assays in protoplasts as a wound-induced system to study the regulation of carrot extensin gene expression. Analysis of 5′ promoter deletion constructs fused to the GUS coding sequence indicated the presence of at least two positive regulatory elements necessary for directing high levels of expression located within the extensin promoter, in addition to the negative regulatory elements previously identified in the same promoter by *in vitro* analysis (Holdsworth and Laties, 1989a) (figure 1.5d). This work is discussed in further detail in the next section (1.3.6).

However, despite the identification of distinct regions of extensin promoters that regulate similar responses, sequence comparisons between these promoter domains do not produce any homology (Salvà and Jamet, 2001). The reason why it is not yet possible to find consensus regulatory elements between all these promoters might be that all extensin genes are regulated in different ways. The extensins they encode may therefore confer different properties to the walls of the cells in which they are present.

1.3.6. Cis-elements controlling extensin gene expression

Little is known about the precise sequence of promoter elements that by binding transcription factors control the expression of extensin genes. To date, the best characterised extensin promoter is from the carrot extensin gene, *pDC5AI* as both *in vitro* and *in vivo* analysis have been performed.

Figure 1.5d shows the structure of the *pDC5AI* promoter based on promoter deletion-reporter gene analysis and *in vitro* binding assays. *pDC5AI* has been shown to encode two mRNAs (of 1.5 and 1.8kb), which are transcribed from different start sites at the 5′ end of the extensin gene (Chen and Varner, 1985a). Based on the existence of two CAAT and TATA boxes, located 35-50 nucleotides upstream of each transcriptional start site, it is thought that the promoter which drives transcription of the 1.8kb mRNA is located upstream of the promoter of the 1.5kb transcript (Ecker and Davis, 1987). These two transcripts were found to respond

differently to ethylene and wounding (Chen and Varner, 1985a; Ecker and Davis, 1987; Tierney *et al.*, 1988).

Also in carrot, Holdsworth and Laties (1989a) have shown that two root-specific binding factors, EGBF-1 (extensin gene binding factor-1) and EGBF-2 interact with the same AT-rich region in the carrot promoter. EGBF-1 is present in phloem extracts and binds to a 23bp region between –570 and –593bp (from the start site of the long transcript), whereas EGBF-2 is present in xylem extracts, binding to an additional 18bp in the 5′ direction (figure 1.5d). EGBF-1 was shown to be controlled in different ways by ethylene and wounding, suggesting that different signal pathways respond to these stimuli. The decrease in EGBF-1 activity after wounding and ethylene treatment of carrot roots corresponds with an increase in extensin mRNA levels, suggesting that EGBF-1 may act as a repressor of extensin gene transcription (Holdsworth and Laties, 1989a). If this is the case, then an inhibition of EGBF-1 should lead to the activation of transcription. The existence of a wound-induced inhibitor of EGBF-1 has been demonstrated by analysis of EGBF-1 activity in reaction mixtures containing various ratios of nuclei from wounded roots (WNE)/nuclear extracts from untreated roots (UNE) (Holdsworth and Laties, 1989b). Inhibition of EGBF-1 DNA-binding activity increased with the ratio of WNE/UNE. However, this inhibitory activity was not present in ethylene-treated roots, again indicating that wounding and ethylene control EGBF-1 activity through different factors (Holdsworth and Laties, 1989b), as wounded carrot tissue produces virtually no ethylene (Chalutz, 1973).

The upstream regulatory elements of the *pDC5AI* gene were studied further by Granell *et al.* (1992) by fusing a series of promoter truncations to the GUS coding sequence and analysing expression patterns. Several regulatory regions were identified within the first 719bp from the transcription start site of the 1.5kb mRNA (figure 1.5d). An important transcriptional regulatory element was located between the two TATA boxes, which specify the start of the two different transcripts. The region between –606bp to –474bp contained the binding sites for the previously identified factors EGBF-1 and EGBF-2 (Holdsworth and Laties, 1989a; 1989b) and gave rise to two bound complexes with nuclear protein extracts from cells (Granell *et al.*, 1992). When extracts prepared from protoplasts (wounded cells) were used, one complex was completely absent and the other was reduced, indicating that the

formation of these complexes involves the factors EGBF-1 and EGBF-2 (Granell *et al.*, 1992). The –719 to –658bp region was required for maximum expression levels as deletion of this resulted in a 48% reduction in promoter activity. This region was found to bind EBP (extensin binding protein), which is only present in nuclear extracts prepared from carrot protoplasts but not in extracts from un-wounded cells (figure 1.5d).

1.3.7. ExtA, an extensin gene from *Brassica napus*

Brassica napus (oilseed rape) is the most important oilseed crop grown in Western Europe and the fourth most important in the world after soybean, sunflower seed and cotton seed (Moore, 1983; Brogan, 1986). Each year there are huge economic losses associated with yield reduction, as a consequence of lodging and pod shatter. The strength of the cell wall is crucial to both.

Lodging occurs when the crop is knocked over by heavy rain and wind (Austin *et al.*, 1986; Jenkins *et al.*, 1996; Spence *et al.*, 1996). Pods on lodged plants are below the cutter level at harvest, are more susceptible to disease and often produce smaller seed (Austin *et al.*, 1986; Thompson and Hughes, 1986; Islam and Evans, 1994). As higher yielding varieties of oilseed rape are developed at the expense of structural tissue, it is likely that lodging will become an even more serious problem.

Pod shatter is the term given to the explosive dehiscence of oilseed rape pods. It may result in a substantial loss of seed prior to harvest and cause carry-over of the crop into the next growing season. It is estimated that between 5-10% of potential yield is lost by pod shatter in a typical crop, although this figure may be as high as 50% if harvesting is delayed by adverse weather conditions (MacLeod, 1981).

In the case of both lodging and pod shatter, strengthening of the cell walls may help to reduce yield losses. For example, pod shatter is accompanied by the degradation and separation of the cell wall of cells in the dehiscence zone, resulting in walls separating from the false septum (Austin *et al.*, 1986; Jenkins *et al.*, 1996; Spence *et al.*, 1996). Resistance to shattering depends primarily on the construction and extent of a bridge of sclerenchymous tissue between the pod walls and septum (Josefsson, 1968; Austin *et al.*, 1986). It is likely that extensins are involved in this process as they play a structural role within the cell wall. Advancing the knowledge

of extensin gene regulation could therefore contribute substantially to the improvement of oilseed rape crops.

The *extA* gene from oilseed rape is a member of a multigene family of between six to eight extensin genes (Evans *et al.*, 1990). It was isolated by screening a *B. napus* genomic library with a cDNA clone known to be expressed to high levels in the roots (Evans *et al.*, 1990). The *extA* gene encodes a transcript of 1.26kb, which follows a similar distribution pattern to other characterised extensins in that it is undetectable in the laminae, with low levels of transcript present in the petiole and stem, and high levels in the root (Shirsat *et al.*, 1991; 1996a).

The expression of *extA* has been shown to occur in plant regions subjected to tensile stress (Shirsat *et al.*, 1996a). Transgenic plants containing 940bp of the *extA* promoter fused to GUS did not express the *extA* gene in unstressed regions such as the internodes, except in the phloem. However, in a stressed situation where an axillary branch joined the main stem at the node, the *extA* gene was expressed in the thin-walled cortical parenchyma cells, in addition to the phloem. The change in GUS synthesis at the node was confirmed as being due to strong tensile stresses imposed on the cortical cells by the weight of the developing branch, by applying weights to axillary flowering stalks of transgenic plants carrying the -940bp *extA* promoter truncation fused to GUS. Weight-loading resulted in a band of non-specific expression through the tissue (Shirsat *et al.*, 1996a). *In-situ* hybridisations provided further evidence that *extA* was expressed in mechanically stressed regions of the plant as *extA* was detected in the carpel walls of maturing *Brassica* pods (Shirsat *et al.*, 1996a).

The *extA* promoter was analysed further by a series of promoter deletions (-940, -789, -664, -433, -159) fused to the GUS reporter gene (Elliott and Shirsat, 1998). Analysis of transgenic tobacco plants carrying the promoter deletion constructs by these authors revealed four distinct control regions (section 1.3.5 and figure 1.5c).

Two quantitative regulatory elements were identified. A quantitative activator region (QAR) was located between –159 to –433bp, increasing expression levels by 10 fold in all cell types when present. A quantitative repressor region (QRR) was identified between –664 to –789bp, the removal of which resulted in a 15-fold increase in expression.

In an attempt to identify a wound responsive element on the *extA* promoter, Elliott and Shirsat (1998) examined the response of transgenic tobacco plants carrying the promoter deletion constructs to a wound stimulus. It had previously been shown that rape extensin transcripts were induced in the aerial tissues following wounding (Shirsat *et al.*, 1996b). However, when dot blot hybridisations were performed on RNA extracted after application of the wounding stimulus, no induction of GUS transcripts was detected from any of the transgenics containing up to –940bp of the 5′ flanking sequence. The wound responsive region was therefore assumed to be between –940 to –3500bp of the *extA* promoter, and this was confirmed by dot blot hybridisations from RNA extracted from transgenics carrying 3500bp of the 5′ flanking sequence (Elliott and Shirsat, 1998).

Finally a region between –433 to –664bp was found to negatively regulate expression because non-specific expression, i.e. in the phloem, cortex and pith, was seen in mature internodes of transgenics containing the –159 and –433 bp truncations, whereas in the longer truncations (–664, -789 and –940 bp) expression in the stem internode, root and petiole was restricted to the internal and external phloem. The region between –433 to –664bp, able to repress expression in non-phloem cell types, was termed the negative regulatory region (NRR) (Elliott and Shirsat, 1998). This pattern of expression was only observed in the mature internodes and not in younger tissues. At the node, all promoter truncations (-159, -433, -664, -789 and –940bp) expressed non-specifically in all cell- types (cortex, pith, epidermis and phloem). It was proposed that tensile stresses experienced at the node by the weight of the axillary branches were able to overcome the repression exerted by the NRR, leading to non-specific expression. This was confirmed by weight-loading axillary flowering branches of low-expressing transgenics containing all the deletion constructs (Shirsat *et al.*, 2003). GUS expression was induced in nodal regions of low expressing transgenics containing the NRR (-664, -789 and –940bp) but not in transgenics where the NRR was absent (–433 to –664bp). Elliott and Shirsat (1998) proposed that this negative regulatory region contains a *cis*-element that is responsive to mechanical stress. This putative element was termed the negative regulatory element (NRE) (Elliott and Shirsat, 1998).

1.4. Aims of the project

Investigation of the NRR from the *Brassica napus* extensin gene, *extA,* was the focus of the current work. Previous work by Elliott and Shirsat (1998) revealed that the region between –433 to –664bp of the *extA* promoter negatively regulated phloem specific expression in non-phloem tissues. However, this negative regulation could be overcome by mechanical stress because non-specific expression was seen at the nodes, due to the weight of the axillary branch (Elliott and Shirsat, 1998), and could be induced by the application of external weights (Shirsat *et al.*, 2003).

Regulation of gene expression is usually controlled at the level of transcription. For a gene to be activated, RNA polymerase II is recruited to the transcription initiation complex on the promoter. Conversely, gene repression involves preventing RNA polymerase II from binding. This level of control is brought about by short sequences located on the 5′ region of a gene that bind transcription factors (nuclear proteins). Specific transcription factors may be present constitutively, found only in certain tissues or induced by specific signals. Once a transcription factor has bound to the DNA, it may bring about conformational changes in the DNA and/or interact with the transcription machinery to bring about gene activation or repression.

The aim of this work was to identify the *cis*-elements negatively regulating tissue specific expression of *extA* and responsive to mechanical stress, termed the NRE by Elliott and Shirsat (1998), by a combination of *in vitro* and *in vivo* analysis. The NRR was investigated by the construction of a series of nine PCR-generated promoter truncations fused to GUS within the region –433 to –664bp. Their expression patterns were analysed in transgenic tobacco plants to further localise the region directing phloem-specific expression. It is possible that the NRE binds a repressor protein, the removal of which would result in loss of normal negative control resulting in expression in all cell-types. This was investigated *in vitro* using electrophoretic mobility shift assays (EMSAs). Oligonucleotides were designed based on *cis*-elements within the NRR known to bind transcription factors from published work and on sequences shown to be important from analysis of the nine promoter truncations in transgenic tobacco plants. The binding dynamics of these sequences in relation to the current literature is discussed in detail.

2. Materials and Methods

2.1. General materials and equipment

All general laboratory chemicals and biological reagents were supplied by Acros Organics (Fisher Scientific UK, Loughborough, Leicestershire), BDH Ltd. (Poole, Dorset), or Sigma-Aldrich Company Ltd. (Poole, Dorset) and were AnalaR grade or equivalent. All bacterial reagents were supplied by Lab M (IDG UK Ltd.) (Bury, Greater Manchester).

Solutions were sterilised either by autoclaving or filter-sterilisation using 0.2μm or 0.45μm disposable filters (Nalgene, Rochester company, NY, USA).

Oligonucleotides and primers were supplied by MWG Biotech (Ebersberg, Germany).

Hybond™-N nylon membranes for Southern and Northern blots were supplied by Amersham Life Science. Hybridisations were performed in Techne hybridisation tubes in a Techne Hybridiser HB-1 oven. Radiochemicals were supplied by Amersham Biosciences UK Ltd. (Little Chalfont, Bucks). X-ray film was obtained from Genetic Research Instrumentation Ltd. (GRI) (Braintree, Essex) and developing solutions came from Photosol Ltd. (Basildon, Essex).

Plastic consumables (e.g. pipette tips and microfuge tubes) were obtained from Sarstedt (Leicester, Leicestershire). Petri dishes were obtained from Bibby Sterilin Ltd. (Stone, Staffs) and Magenta™ pots were supplied by Sigma-Aldrich Co. Ltd. (Poole, Dorset).

A variety of centrifuges were used during the course of this work. A Heraeus Sepatech Biofuge 15/15R microfuge was used for small samples, whilst large samples were spun in either a MSE Mistral 3000i refrigerated bench top centrifuge (MSE, Duxford, Cambridge), or a Beckman J2-21 centrifuge, using a JA-14 or JA-20 rotor (Beckman Instruments Ltd., High Wycome, Bucks).

The spectrophotometer used was a Unicam UV 500 (ThermoSpectronic).

PCR reactions were carried out in an M J Research Inc. PTC-150 MiniCycler (GRI Ltd., Braintree, Essex) in 0.2ml tubes supplied by Alpha Laboratories Ltd. (Eastleigh, Hampshire).

Other commercially supplied reagents and equipment are acknowledged at first reference to use.

2.2. Propagation and growth of plant material

Oilseed rape (*Brassica napus* cv. Bienvenue) and tobacco (*Nicotiana tabacum* cv. Samson) seeds were sterilised by washing in 10% ethanol for 2 minutes, rinsing in sterile distilled water and then soaking for 5 minutes in 5% hypochlorite. Finally, seeds were rinsed 5 times with sterile distilled water.

Oilseed rape seeds were germinated on moist vermiculite (Vermiperl, fine grade, William Sinclair Horticulture Ltd., Lincoln, Lincs.) in the dark at 20°C. They were then transferred into the light and watered daily with Phostrogen (Phostrogen Ltd., Deeside, Clwyd).

Tobacco seeds were germinated on moist filter paper in the dark at 20°C. Following germination, the seedlings were transferred onto half-strength Murashige and Skoog (½MS) agar ($2.35gl^{-1}$ Murashige and Skoog basal medium plant cell culture powder [Sigma-Aldrich Company Ltd, Poole, Dorset, UK], catalogue number M5519) $5gl^{-1}$ sucrose, 0.8% (w/v) agar, pH 5.8) in Magenta™ pots (Sigma-Aldrich Company Ltd.). Transgenic tobacco plants were germinated in the same way as wild type tobacco plants, except they were transferred onto ½MS agar containing 50 or $100\mu gml^{-1}$ kanamycin sulphate. This selects for plants containing the inserted DNA construct due to the presence of the *nos-nptII* selectable marker gene within the T-DNA (see figure 4.2a).

When plants were large enough, they were transplanted into pots in either a 2:1 mix of compost (John Innes potting No.1):vermiculite or grown hydroponically in full strength Hoagland's Solution (1mM KH_2PO_4, 5mM KNO_3, 5mM $Ca(NO_3)_2$, 2mM $MgSO_4$, 12.5μM H_3BO_3, 0.25μM $MnCl_2$, 1μM $ZnSO_4$, 0.38μM H_2MoO_4, 36μM FeEDTA) with constant aeration. The plants were grown at 20°C with a 16-hour light/8 hour dark cycle in a C1 transgenic plant containment facility.

2.3. DNA manipulation

2.3.1. Estimation of DNA concentration

The DNA concentration was determined quantitatively using a spectrophotometer. 1µl of the DNA sample was diluted with 500µl of sterile water and the absorbance at 260nm and 280nm determined. An absorbance of 1.0 at 260nm is equivalent to a concentration of $50\mu g\mu l^{-1}$ of double-stranded DNA. The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) was used to estimate the purity of the DNA. A 100% pure preparation would have a ratio of 1.8 and any contamination with protein or phenol would give a lower value.

The DNA concentration was also determined qualitatively on agarose gels by comparing the intensity of the unknown DNA band with a marker band containing a known quantity of DNA. DNA fluoresces in the presence of ethidium bromide and the intensity of the fluorescence is proportional to its concentration (Burden and Whitney, 1995).

2.3.2. Restriction endonuclease digestion

The restriction endonucleases and buffers were supplied by Promega UK Ltd. (Southampton, Hants) and Helena Biosciences Ltd. (Sunderland, Tyne and Wear). Restriction digests were carried out using the manufacturer's guidelines. As a rule, a 10-fold unit excess of enzyme was used with 1X the recommended restriction enzyme buffer. The total reaction mixture volume was ten times the volume of the enzyme. Reactions were usually carried out at 37°C for a minimum of 3 hours, unless otherwise stated. Reaction volumes were 30µl for plasmid DNA manipulations, as this was a convenient volume to load into the wells of an agarose gel. Genomic DNA digests were performed in larger volumes due to the greater quantities of enzyme used. In this case, reaction products had to be alcohol precipitated (see section 2.5.1) and the digested DNA re-suspended in a more convenient volume, prior to gel electrophoresis.

When plasmid DNA obtained from alkaline lysis minipreps (section 2.5.1) was digested, DNAase-free RNAase was used in the reaction at a concentration of $10\mu g\mu l^{-1}$, to remove any contaminating RNA. This was prepared by dissolving

pancreatic ribonuclease A (NBL Gene Sciences Ltd.) in 10mM Tris.HCl pH7.5, 15mM NaCl, at a concentration of 5mgml^{-1} and boiling for 15 minutes. The solution was allowed to cool slowly to room temperature and stored in small aliquots at -20°C.

2.3.3. Agarose gel electrophoresis

Electrophoresis was carried out as described by Sambrook *et al.* (1989), using 0.5X TBE buffer (45mM Tris-borate, 1mM EDTA [pH 8.0]) in either midi gels (200mm x 170mm) or in mini gels (80mm x 95mm). The concentration of agarose used in the gel varied depending on the size of fragments to be separated. Smaller fragments required a higher concentration of agarose. Ethidium bromide at a concentration of 1μgml^{-1} was included in all DNA gels.

Before the DNA was loaded on to the gel, 10X loading solution (0.42% (w/v) bromophenol blue, 0.42% (w/v) xylene cyanol FF, 50% glycerol in H_2O) was added to the sample at a concentration of 1X. Suitable size markers were also loaded. These were prepared by digesting λ DNA with either *Pst* I or *Hind* III, heating to 65°C for 10 minutes and cooling on ice.

DNA in the gels was visualised on a TM-20 transilluminator (UVP Products, San Gabriel, CA, USA) and photographed with a K61B Mitsubishi video copy processor (Mitsubishi, Japan).

2.3.4. Isolation of DNA fragments from agarose gels by electroelution

Electrophoretic elution of DNA fragments from gel slices was first described by McDonell *et al.* (1977), and allows the recovery of a desired restriction endonuclease fragment from an agarose gel. The DNA fragment can then be used in ligations or for radiolabelling.

Following digestion with the appropriate restriction enzyme(s) (section 2.3.2), and separation of the DNA fragments on an agarose gel, the DNA band of interest was sliced out of the gel, using a sharp scalpel, under a low-intensity trans-illuminator to minimise damage to the DNA by UV light. The slice was transferred to dialysis tubing (Medicell International Ltd., London) with just enough TE buffer (pH 7.6) (10mM Tris-Cl [pH7.6], 1mM EDTA) to completely cover it, and sealed at either end using mediclips, making sure that there were no air bubbles trapped. The

dialysis tubing was placed in an electrophoresis tank, with the gel slice(s) parallel to the electrodes. 0.5X TBE buffer was added until it just covered the dialysis tubing. Electrophoresis was then carried out until the DNA had completely moved into solution within the tube and no DNA was visible in the gel slice under UV light. The gel slice was carefully removed and the tubing gently massaged to release any DNA stuck to the walls. The DNA-containing solution was then transferred to a microfuge tube where it was extracted with phenol: chloroform: IAA (25: 24: 1, v/v) and then alcohol precipitated as described in section 2.5.1.

Preparation of dialysis tubing involved boiling for 20 minutes in 2% (w/v) sodium bicarbonate and 1mM EDTA (pH8.0), rinsing in distilled water and then boiling for 10 minutes in 1mM EDTA. Tubing was stored in 70% ethanol and rinsed in distilled water before use.

2.3.5. Ligations

DNA fragments with compatible cohesive or blunt termini were ligated using T4 DNA ligase in ligase buffer (Promega UK Ltd., Southampton, Hants.), according to the manufacturer's instructions. Reactions were carried out in a final volume of 10µl. The amount of vector and insert DNA to be used (insert / vector ratio) was calculated using the following formula that converts molar ratios to mass ratios Promega, 1996):

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

An insert/vector mass ratio of 1:5 was used. The DNA fragments had one blunt and one cohesive end, therefore ligation reactions were left at 22°C overnight. The ligation reaction products were then used to transform *Escherichia coli* competent cells, as described in section 2.4.2.

2.4. Transformation of *Escherichia coli*

2.4.1. Preparation of competent *E. coli* cells

A single colony of *E. coli* strain DH5α was used to inoculate 5ml of Luria broth medium (LB) ($10gl^{-1}$ bactotryptone, $5gl^{-1}$ yeast extract, $10gl^{-1}$NaCl, pH7.0, autoclaved). This was shaken at 200rpm at 37°C overnight and 1ml of this was used to inoculate 1L of LB. DH5α cells were grown in this under the same conditions to an OD_{600} of 0.5-1.0. The culture was then chilled on ice for 30 minutes and the cells harvested by centrifugation at 4,000 rpm at 4°C for 15 minutes in a Beckman JA14 rotor. The cells were then re-suspended in 500ml of sterile distilled water pre-chilled to 4°C. The centrifugation step was repeated three times before the cells were re-suspended in 20ml of sterile 10% glycerol pre-chilled to 4°C. After a further centrifugation step at 4,000rpm for 15 minutes, the pelleted cells were re-suspended in 2ml of pre-chilled 10% glycerol. Competent cells were aliquoted into 40µl fractions and stored at -80°C for up to six months. Competent bacterial cells produced by this method gave a transformation efficiency of approximately 1.0×10^6 transformants per microgram of supercoiled plasmid DNA.

2.4.2. Transformation of competent cells by electroporation

A 40µl aliquot of competent cells was mixed with 2µl of DNA ligation mixture (20ng of plasmid vector DNA and 4ng of insert DNA) in distilled water so that the total volume was 80µl. This was added to a sterile pre-chilled electroporation cuvette and the cells were electroporated using an EQUIBIO EasyjecT Plus® multipurpose electroporation system (EquiBio Ltd, Monchelsea, Kent; distributed by Flowgen, Lichfield, Staffs, UK). Conditions used were for bacterial electroporation and were as follows: V=2500, C=0025, T=0005, R=0201 in high voltage mode, where V=Volts, C=capacitance in microFarads, T=computed mathematical pulse time (T=RXC) and R= resistance in ohms. Immediately on completion of electroporation, 1ml of SOC medium ($20gl^{-1}$ bacto-tryptone, $5gl^{-1}$bacto yeast extract, $0.5gl^{-1}$ NaCl, 2.5mM KCl, pH 7.0, autoclaved, followed by 10mM $MgCl_2$, 20mM glucose, filter sterilised through a 0.22 micron filter), pre-warmed to 37°C, was

added and the electroporation mix was allowed to recover for 1 hour at 37°C before plating onto a selective medium.

2.4.3. Identification of transformants using colony PCR

When colonies were approximately 1mm in size, they were picked off the plate using a sterile pipette and added to 50µl of sterile water. This was mixed and a sterile loop was used to streak the bacteria onto a selective medium, which was left at 37°C overnight to provide a stock culture. The remaining bacterial suspension was heated to 99°C for 5 minutes to release the DNA. The cell debris was removed by centifugation at 12,000rpm for 1 minute. 1µl of the supernatant was then used in each PCR reaction mix with specific primers, under the conditions described in section 2.11.2. When the PCR reaction products were run on an agarose gel, positive colonies produced a band of the correct size. These colonies could then be used for further analysis.

2.5. Isolation of plasmid DNA from *Escherichia coli* cells

2.5.1. Alkaline lysis minipreps

This method is based on that detailed in Sambrook *et al.* (1989). It was used to prepare small quantities of DNA and was most often used to confirm the presence of an insert of the correct size, by restriction enzyme analysis.

A 5ml or 10ml (for plasmids with a low copy number such as pBI101.2) bacterial culture was grown overnight at 37°C with the appropriate antibiotic selection and the cells harvested by centrifugation at 2,500rpm for 10 minutes. The resulting pellet was re-suspended in 200µl of solution I (50mM glucose, 25mM Tris.HCl pH8.0, 10mM EDTA pH8.0) containing lysozyme at $2mgml^{-1}$ and incubated on ice for 30 minutes. 300µl of solution II (0.2M NaOH, 1% (w/v) SDS) prepared fresh was added and mixed by inversion. Following an incubation of 5 minutes on ice, 200µl of solution III (for 100ml of solution at 3M with respect to potassium and 5M with respect to acetate: 60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml distilled water) was added, mixed by inversion and

incubated on ice for a further 5 minutes. Cellular debris was removed by centrifugation at 15,000rpm.

The aqueous phase was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v). This was mixed by vortexing and the aqueous phase collected in a fresh microfuge tube following centrifugation for 3 minutes at 15,000rpm. This step was repeated if protein contaminants were still visible at the interface of the organic and aqueous phase. After a further extraction with chloroform: isoamyl alcohol (IAA) (24: 1, v/v) and centrifugation for 3 minutes at 15,000rpm, the plasmid DNA in the aqueous layer was precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 5.6) and 2 volumes of ice cold 100% ethanol. This was left at -20°C for a minimum of 1 hour, centrifuged at 15,000rpm for 30 minutes, and the DNA pellet washed in 70% ethanol. The pellet was then air dried and re-suspended in TE buffer (pH 7.6).

DNA prepared using this method still contains RNA. This was removed by adding $10\mu g\mu l^{-1}$ RNAase (see section 2.3.2) prior to restriction enzyme digestion.

2.5.2. Qiagen® minipreps

The Qiagen® plasmid midikit was used to produce small quantities of high quality plasmid DNA, and was used mainly for sequencing, according to the instructions supplied by the manufacturer. The kit is based on the alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt.

2.5.3. Qiagen® midipreps

The Qiagen® plasmid midikit was used to produce large quantities of high quality DNA.

A 100ml bacterial culture was grown, and plasmid DNA prepared from it according to the manufacturer's instructions. The procedure involved alkaline lysis of the bacterial cells and binding of the resulting DNA to an equilibriated Qiagen® P100 column, under low salt conditions. RNA, proteins and low molecular-weight impurities were removed by a medium salt wash. The DNA was eluted from the column in a high-salt buffer, desalted by precipitation with 0.7 volumes of

isopropanol and centrifuged at 18,000xg for 30 minutes. The DNA pellet was washed with 70% ethanol, air-dried and re-suspended in TE buffer (pH7.6).

2.6. Southern hybridisations

2.6.1. Southern blotting

After electrophoresis on an agarose gel (section 2.3.3), DNA was transferred to a Hybond™-N nylon membrane using a modification of the capillary transfer method described by Southern (1975).

The gel containing DNA samples was gently agitated in denaturing solution (1.5M NaCl, 0.5M NaOH) for 45 minutes, rinsed briefly with distilled water and then agitated for a further 45 minutes in neutralising solution (1M Tris-HCl pH 7.4, 1.5M NaCl). The gel was rinsed again in distilled water before soaking in 20X SSC (saline sodium citrate) (3M NaCl, 0.3M sodium citrate, pH7.0) for 5 minutes and placing it face down onto the gel apparatus.

The apparatus for performing the blot consisted of eight sponge dishcloths saturated in a tank of 20X SSC and covered with two sheets of Whatman 3MM chromatography paper pre-wetted with 20X SSC. The gel was then placed on to this and surrounded with saran wrap to seal in the moisture. The membrane was placed on top of the gel, taking care to avoid air bubbles, and 4 sheets of Whatman 3MM paper placed on top of this, followed by two nappy insert pads (Boots Plc., Nottingham). Finally, a glass plate and a 500g weight were placed on top.

The SSC is drawn through the gel by capillary action to the insert pads. This carries the DNA with it, which then becomes trapped on the nylon membrane.

The gel was blotted at room temperature overnight, after which the position of the wells was marked on the membrane. The membrane was then baked at 80°C for 2 hours to fix the DNA, and used in a hybridisation reaction.

2.6.2. Hybridisation

Blots were pre-wetted with 2X SSC before pre-hybridisation with 5X SSC, 5X Denhardt's solution (50x Denhardt's stock solution is 1% ficol, 1% polyvinylpyrrolidone, 1% BSA fraction V), 0.5% SDS and 100μgml^{-1} denatured

(boiled for 5 minutes and snap cooled on ice) salmon sperm DNA (see below). The blot was pre-hybridised at 65°C for a minimum of two hours, after which the pre-hybridisation solution was removed and replaced with hybridisation solution. This consisted of 5X SSC, 5X Denhardt's solution, 100μgml^{-1} denatured salmon sperm DNA (see below), 0.5% SDS and the denatured, labelled probe (see section 2.7). The blots were left to hybridise at 65°C overnight and washed in solutions of increasing stringency (2X SSC, 1X SSC and finally 0.5X SSC, if necessary). Washes were carried out at 65°C for 1 hour.

The membrane was then removed from the hybridisation tube, sealed in saran wrap and exposed to X-ray film with intensifying screens for the appropriate length of time at -50°C.

NB. Salmon sperm DNA was prepared by dissolving salmon sperm DNA (Sigma-Aldrich Co. Ltd) in water at a concentration of 10mgml^{-1}. The salmon sperm DNA was then extensively sonicated in an MSE Soniprep, autoclaved twice and snap cooled on ice in between before storing in 1ml aliquots at -20°C.

2.7. Radiolabelling DNA probes

The probe DNA was obtained by electroelution from an agarose gel (see section 2.3.4). The DNA fragment was then labelled with [α-^{32}P] dCTP using the random priming oligonucleotide labelling method of Feinberg and Vogelstein (1983).

100ng of probe DNA was made up to 36μl with sterile distilled water. The probe was then denatured by boiling for 7 minutes and then snap cooling on ice. In a separate tube, 10μl of OLB (see below), 1μl (5 units) Klenow polymerase fragment and 3μl [α-^{32}P] dCTP (~1110kBq) were mixed. The denatured probe DNA was then added to this tube, mixed and incubated at 37°C overnight. The labelled probe DNA was separated from the unincorporated nucleotides on a 1ml Sephadex® G50 column as described by Sambrook *et al.*, 1989.

Before the probe was added to the hybridisation solution (see section 2.6.2), it was denatured by boiling for 10 minutes and snap cooling on ice.

OLB is made from the following components:-

Solution O: 1.25M Tris.HCl, 0.125M $MgCl_2$ pH8.0

Solution A: 1ml of solution O + 18µl 2-mercaptoethanol + 5µl dATP, 5µl dGTP, 5µl dTTP, each made up as a 0.1M stock in 3mM Tris.HCl and 0.2mM EDTA, pH7.0).

Solution C: hexadeoxyribonucleotides (sodium salt, random sequence, Pharmacia Biotech Ltd, Milton Keynes, Bucks, catalogue no. 2721-66-01) in TE buffer pH 7.5 (10mM Tris.HCl pH7.7, 1mM EDTA pH8.0) at 90 OD unitsml^{-1}.

Solutions A:B:C were mixed in the ratio 10:25:15 to make OLB which was stored in 10µl aliquots at –20°C.

2.8. The production of transgenic tobacco plants

The nine promoter truncation constructs (section 4.2) in *E. coli* were transferred by conjugation to *Agrobacterium tumefaciens* by tri-parental mating. The resulting *Agrobacterium* strain was then used to transform tobacco plant cells according to the method described by Ellis *et al.* (1988).

2.8.1. Bacterial strains and tri-parental mating

The promoter truncation constructs were introduced into the plasmid vector pBI101.2 (Jefferson *et al.*, 1987) (Clontech Laboratories Inc.) in the *E. coli* strain DH5α. The other bacterial strains used were the mobilising *E. coli* strain HB101 containing the helper plasmid pRK2013 (Ditta *et al.*, 1980) and the host *A. tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983).

A 5ml overnight culture containing the appropriate antibiotic(s) for each strain was grown from a single colony from glycerol stocks. The pBI101.2/DH5α and HB101/pRK2013 strains were grown at 37°C with 50µgml^{-1} kanamycin sulphate. The *A. tumefaciens* strain LBA4404 was grown at 25°C with 100µgml^{-1} rifampicin and 500µgml^{-1} streptomycin sulphate.

The recombinant vectors were mobilised in a tri-parental mating by mixing 400µl of each overnight culture, each previously washed in LB medium three times to remove antibiotics, and incubated on an LB plate without antibiotic selection at 25°C. After an overnight incubation, bacteria were scraped off the plate, re-suspended in 10mM $MgSO_4$ and plated on to LB plates containing 50µgml^{-1}

kanamycin sulphate, 100μgml^{-1} rifampicin and 500μgml^{-1} streptomycin sulphate at different dilutions. These were incubated at 25°C until colonies were visible (2-3 days). The presence of the insert was confirmed by Southern hybridisation on DNA isolated from *Agrobacterium* colonies grown on the above selective medium.

2.8.2. Isolation of DNA from *A. tumefaciens*

A 5ml culture of *A. tumefaciens* was incubated overnight at 25°C in LB broth containing 50μgml^{-1} kanamycin sulphate, 100μgml^{-1} rifampicin and 500μgml^{-1} streptomycin sulphate. 1.5ml of the culture was centrifuged at 12,000rpm for 5 minutes and the cells re-suspended in 300μl of TE buffer (pH8.0). 100μl 5% sarkosyl and 150μl 5mgml^{-1} pronase was added to this before incubating at 37°C for 1 hour. An equal volume of phenol: chloroform: IAA (25: 24: 1, v/v) was added and gently mixed by pipetting up and down to shear the chromosomal DNA. This was spun for 5 minutes at 12,000rpm and the aqueous phase transferred to a fresh tube. The phenol: chloroform step was repeated three times before alcohol precipitating at -20°C for 1-2 hours with 0.05 volumes of NaCl and 3 volumes of ice cold ethanol. The precipitated DNA was spun down at 12,000rpm for 10 minutes, washed in 70% ethanol, and the pellet dried and re-suspended in 25μl distilled water containing RNAase.

2.8.3. Tobacco transformation and regeneration

A 25ml culture of each *Agrobacterium* strain containing each promoter truncation construct was grown in LB containing 50μgml^{-1} kanamycin sulphate, 100μgml^{-1} rifampicin and 500μgml^{-1} streptomycin sulphate at 25°C to an OD_{600} of approximately 0.8. The bacteria were pelleted by centrifugation at 1,500xg for 10 minutes, washed three times in 2mM $MgSO_4$ and re-suspended in 25ml MS2SBN medium (4.71gl^{-1} Murashige & Skoog medium [section 2.2], 20gl^{-1} sucrose, 1mgl^{-1} 6-benzylaminopurine (BAP) and 0.1mgl^{-1} α-naphthaleneacetic acid (NAA) pH5.8).

Young, fully expanded leaves were taken from wild-type *N. tabacum*, and the petioles sealed with melted dental wax. The following procedures were carried out in a laminar flow hood. Leaves were surface sterilised in 70% ethanol for 30 seconds, rinsed in sterile distilled water and soaked in 5% sodium hypochlorite with

occasional agitation for 15 minutes. They were washed three times in sterile distilled water before cutting discs from the leaves with a sterile 0.8cm cork borer, avoiding the leaf margin and major veins.

The discs were then placed on to the *Agrobacterium* suspension for 45 minutes with occasional agitation before transferring the discs on to MS2SBN plates. The plates were sealed with micropore^{TM} tape to allow gas exchange, and cultured at 20°C with a 16 hour photoperiod. As a control, 10 discs were floated on distilled water and plated on to MS2SBN medium. After two days, the leaf discs were transferred into MS2SBN medium containing 400μgml^{-1} augmentin and gently agitated overnight. They were then surface dried on sterile blotting paper before being placed on MS2SBN plates containing 200μgml^{-1} augmentin and 50μgml^{-1} kanamycin sulphate (MS2SBNKA) and cultured as before. The optimal kanamycin concentration for use was determined experimentally (see section 4.3.1 and figure 4.8). Half of the control discs were plated on to MS2BN plates and the other half were plated on to MS2SBNKA plates.

After three weeks the leaf discs were transferred onto fresh MS2SBNKA medium. Once shootlets had begun to develop around the edges of the discs (1-2 weeks later) they were excised and transferred onto ½MS (2.4gl^{-1} Murashige-Skoog medium, 5gl^{-1} sucrose, pH5.8) containing 100μgml^{-1} augmentin and 50μgml^{-1} kanamycin sulphate in magenta pots. Once rooted, plantlets of normal appearance were transferred onto MS (4.71gl^{-1} Murashige-Skoog medium, 10gl^{-1} sucrose, 0.8% agar, pH5.8) containing 100μgml^{-1} augmentin and 50μgml^{-1} kanamycin sulphate.

Plantlets were transplanted into pots in a sterile compost: vermiculite (2:1) mix when they had developed an extensive root system. The pots were covered with a clear polythene bag and left in the growth room at 20°C with a 16 hour photoperiod for three days. The bags were then gradually removed and the plants left to develop in the growth room.

2.9. Extraction of plant genomic DNA from tobacco

The method used to extract genomic DNA from tobacco plants was modified from a procedure described by Doyle and Doyle (1987), and produces DNA of sufficient quality for restriction enzyme analysis.

0.25g of leaf tissue was frozen immediately in liquid nitrogen in a microfuge tube and ground to a fine powder. This was homogenised with 1ml of CTAB extraction buffer (2% hexadecyltrimethylammonium bromide (CTAB), 100mM Tris.HCl pH 8.0, 1.4M NaCl, 20mM EDTA pH 8.0 and 0.2% (v/v) mercaptoethanol) pre-warmed to 60°C, before incubating at 60°C for 30 minutes with occasional mixing. The homogenate was split in to 2 tubes and an equal volume of chloroform:isoamyl alcohol (24: 1, v/v) was added. This was mixed on a rotating wheel for 15 minutes before centrifuging at 7,600rpm for 10 minutes in a microfuge. The aqueous phase was transferred to a fresh tube and 0.7 volumes of cold (-20°C) isopropanol added. After gentle inversion the genomic DNA was visible. This was centrifuged at 12,000rpm for 1 minute and 500µl of wash solution (76% ethanol, 10mM ammonium acetate) added to the DNA pellet. The tube was left on ice for 30 minutes before centrifugation at 12,000xg and removal of the wash solution. The genomic DNA was then air dried and re-suspended in 100µl of TE buffer (pH7.6). This was incubated for 2 hours at 37°C with 1µl of 10µgµl^{-1} DNAase free RNAase.

The DNA was precipitated with 0.2 volumes of 10M ammonium acetate and 2 volumes of ice cold 100% ethanol. After a minimum of 1 hour this was centrifuged at 12,000rpm for 2 minutes and the pellet washed with 70% ethanol. The pellet was then air dried to remove residual ethanol before re-suspending in 25µl TE buffer (pH7.6).

2.10. Histochemical analysis of GUS expression

Histochemical assays were carried out as described by Jefferson (1987). Transverse hand sections of stem internodes, nodes and petioles and lengths of root were taken from high-expressing plants (section 5.2). These were incubated in 50mM NaH_2PO_4, pH7.0 containing 1mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and 1.8mM cycloheximide at 37°C. After 14 hours, the tissue was fixed in 3% gluteraldehyde (in 50mM NaH_2PO_4, pH7.0) at 4°C overnight and cleared in 100% ethanol. Tissue was stored at 4°C in 100% ethanol.

2.11. Primer design and polymerase chain reaction (PCR)

2.11.1 Primer design.

Primers for PCR were designed to flank the region of interest according to the following guidelines:

- Avoidance of sequences that would produce internal secondary structure
- The 3' ends of primers were not complimentary to avoid the production of primer dimers.
- Primers were between 20 and 30mer.
- More than three G or Cs in a row at the 3' end of the primer were avoided
- Primers had a GC content between 40-60%.
- Primers to be used in the same reaction had similar melting temperatures (Tm). Annealing temperature is dependent on the primer with the lowest Tm. This was calculated using the following equation:

$$Tm = (G / C \times 4) + (A / T \times 2) - 5$$

2.11.2. PCR reaction

A typical PCR reaction mixture contained 1µl of DNA (at a concentration optimised for the reaction), 0.5µM of forward and 0.5µM of reverse primer, 0.2mM dNTPs, 2.5µl of 10X Mg^{2+} free buffer (10mM Tris-HCl (pH 9.0 at 25°C), 50mM KCl and 0.1% Triton® X-100), 3mM $MgCl_2$ and 0.2µl of Taq polymerase in storage buffer A (50mM Tris-HCl [pH 8.0 at 25°C]), 100mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol and 1% Triton® X-100) (Promega) in a final volume of 25µl made up with molecular biology grade water. Contamination from external DNA was minimised by aliquoting reagents used regularly in PCR, making up a master mix containing the appropriate reagents for each set of reactions and using high retention filter pipette tips (Thistle Scientific, Uddington, Glasgow). Reagents were kept on ice at all times.

PCR reactions were carried out according to the following cycling conditions, unless otherwise stated:

93°C for 1 minute

95°C for 1 minute

94°C for 1 minute
55°C for 45 seconds } x 35 cycles
72°C for 2 minutes

72°C for 5 minutes

hold at 4°C

2.12. DNA sequencing

Sequencing reactions were carried out in a CEQ 2000 DNA Analysis System (Beckman Coulter, High Wycome, Bucks) by capillary electrophoresis, according to the manufacturers instructions.

50fmol of plasmid containing the insert of interest, prepared by the Qiagen® miniprep method (section 2.5.2), was used per reaction as the DNA template. This was heated to 96°C for 1 minute and cooled to room temperature before adding 8µl of DTCS quick start master mix (manufacturer) and 2µl of 1.6µM primer in a final volume of 20µl. The pre-heat treatment step is particularly important for large plasmids, such as pBI101.2, as they tend to block up the capillaries in the sequencer.

The following thermocycling program was used:

96°C for 20 seconds
50°C for 20 seconds } x 30 cycles
60°C for 4 minutes

hold at 4°C

Following the PCR step was an ethanol precipitation step. In to each reaction mixture, 4µl of stop solution (1.5M sodium acetate, 50 mM EDTA) and 1µl of 20mgml^{-1} glycogen, supplied in the kit, and 60µl of ice cold 95% ethanol was added. This was mixed thoroughly and centrifuged immediately at 4,000rpm for 15 minutes at 4°C.

The pellet was washed twice with 200µl of ice-cold 70% ethanol and centrifuged at 14,000 rpm at 4°C for 5 minutes. The pellet was left to air dry before re-suspending in 40µl of sample loading solution (supplied in the kit). Individual sequencing reaction mixes were loaded into wells on a 96 well plate with a drop of mineral oil on the top and sequenced in the CEQ 2000 machine using the programme LFR-1. Separation was at 49°C, 4.2kV for 85 minutes with the capillary temperature at 50°C, denaturation at 120°C and injection voltage at 2kV). Data analysis was carried out using the module in the CEQ 2000 software (Beckman Coulter) and the data analysis programme "chromas."

2.13. RNA procedures

2.13.1. Preparation of equipment

The equipment and solutions for RNA work were pre-treated as described by Sambrook *et al.* (1989) to prevent contamination of the RNA with RNAases from external sources.

All laboratory glassware, plasticware and pestles and mortars to be used for RNA work were pre-treated by soaking in diethyl pyrocarbonate (DEPC) (0.1% v/v, in distilled water) at room temperature overnight. DEPC is a strong inhibitor of RNAases (Fedorcsak and Ehrenberg, 1966). After soaking it was autoclaved to remove any trace of DEPC.

Sterile disposable plastic-ware such as pipette tips are free of RNAases and therefore require no DEPC treatment. However, they were taken from an unopened bag using clean gloves and autoclaved before use.

All solutions were prepared using DEPC-treated glassware and chemicals were reserved for work with RNA. They were made up in DEPC-treated water and

autoclaved prior to use. DEPC-treated water was made by treating distilled water with 0.1% (v/v) DEPC at 37°C overnight, followed by autoclaving.

Electrophoresis tanks were designated for RNA gels only. These were cleaned with detergent, rinsed with water and then DEPC treated overnight before being rinsed thoroughly with DEPC-treated water.

2.13.2. RNA isolation and quantification

Total RNA was isolated using either a kit or by a modification of a method based on that described by Kay *et al.* (1987).

2.13.2.1. Purescript total RNA isolation kit (Gentra)

Total RNA was extracted from 100mg of frozen ground tissue as described in the manufacturer's protocol and using the reagents supplied in the kit. The procedure was modified slightly by repeating the initial centrifugation step several times to ensure that no suspended plant material remained in the supernatant containing the RNA. Once the RNA had been extracted, the pellet was re-suspended in 50µl of DEPC treated water and stored at -80°C.

2.13.2.2. Large-scale RNA extraction (modification of Kay *et al.*, 1987)

1g of tissue was ground to a fine powder in liquid nitrogen using a pre-cooled pestle and mortar. This was homogenised with 4ml of extraction buffer (25 mM Tris-Cl, pH 8.0, 25mM EDTA, pH 8.0, 75mM NaCl, 1% SDS, 0.1% (v/v) β-mercaptoethanol, made up to volume with DEPC-treated water and prepared fresh with each use). An equal volume of phenol: chloroform: IAA (25: 24: 1, v/v) was added and the mixture vortexed in a Falcon tube for 30 seconds before centrifuging for 10 minutes at 600rpm. This step was repeated at least three times and the aqueous phase transferred to a fresh tube. 10M LiCl was added to a final concentration of 2M, drop by drop while vortexing to avoid precipitation of DNA. This was left overnight at 4°C before centrifuging at 6,000rpm for 20 minutes. The pellet was washed with 500µl of 2M LiCl and centrifuged at 6,000rpm for 10 minutes. The pellet was then re-suspended in 500µl of DEPC-treated water and this was alcohol precipitated at 4°C for at least 1 hour using 50µl 3M sodium acetate, pH

5.2 and 1250µl of 100% ice-cold ethanol. This was then centrifuged at 14,000rpm for 15 minutes and the pellet carefully washed twice with 500µl 80% ethanol. The air-dried RNA pellet was re-suspended in 100 µl DEPC-treated water and stored at -80°C.

2.13.2.2. RNA quantification

The quality and quantity of each RNA sample was determined spectrophotometrically, based on the assumption that a 40µgml^{-1} solution of RNA in water has an absorbance of 1.0 at 260 nm. The ratio between the readings at 260nm and 280nm (OD_{260nm}/OD_{280nm}) was used to estimate the purity of the RNA. Pure RNA has an OD_{260nm}/OD_{280nm} of 2.0, and lower values indicate contamination by phenol or protein.

2.13.3. Formaldehyde gel electrophoresis and northern blotting

RNA was run on formaldehyde gels and transferred to Hybond™-N nylon membrane (Amersham Life Science) using the method described by Fourney *et al.* (1988).

2.13.3.1. Sample preparation

10µg of the RNA sample was added to 25µl of freshly prepared electrophoresis sample buffer (25µl deionised 100 % formamide, 5µl 10X MOPS/ EDTA buffer (50mM sodium acetate, 0.2M MOPS [3-(N-morpholino) propanesulphonic acid], 10 mM EDTA, pH 7.0), 8µl 37 % formaldehyde, 3µl DEPC-treated water, 3µl 100 % glycerol, 2.6µl 10 % (w/v) bromophenol blue). The RNA sample and buffer were mixed, heated to 65°C for 15 minutes and cooled on ice. 1µl of 1mgml^{-1} ethidium bromide (in DEPC-treated water) was added to the sample before it was loaded on to a formaldehyde gel.

2.13.3.2. Gel preparation and electrophoresis

For a 200ml gel, 2.6g agarose, 20ml 10X MOPS/EDTA and 174ml DEPC-treated water was added to a DEPC-treated flask. This was heated to dissolve the agarose, then allowed to cool to 50°C before 10.2ml of 37 % formaldehyde was added. The solution was then mixed before the gel was poured. Once set, the gel was placed in a

DEPC-treated tank containing 1X MOPS/EDTA electrophoresis buffer. The wells were flushed with buffer before the RNA samples were loaded and the gel run at 80V for 4 hours.

2.13.3.2. Transfer of the RNA to a nylon membrane

After photography, the gel was soaked in 0.05M NaOH made up in 1X SSC and then for two 20 minute periods in 10X SSC.

The RNA was transferred to a nylon membrane in 10X SSC by capillary action using the same method as for the Southern blots (see section 2.6.1). The blot was left at 4°C overnight. RNA transfer was confirmed by holding the membrane over a transilluminator. The RNA was then fixed to the membrane by UV cross-linking at 700 x 100μJ/cm^2 in a CL-1000 Ultraviolet Cross-linker (UVP Inc., Cambridge) and then baking for 2 hours at 80°C.

2.13.4. Northern hybridisation

The blots were pre-hybridised in an oven at 42°C for at least 5 hours (usually overnight) in pre-hybridisation solution (50% formamide, 5X SSC, 5X Denhardt's solution, 0.1% (w/v) SDS, 100μgml^{-1} denatured salmon sperm DNA [for the preparation of Denhardt's and denatured salmon sperm DNA, see section 2.6.2]). This was replaced with hybridisation solution (50% formamide, 5X SSC, 2X Denhardt's solution, 0.1% SDS, 100μgml^{-1} denatured salmon sperm DNA) containing the denatured, labelled DNA probe (see section 2.6.2) and left at 42°C for 36 hours.

The blots were washed twice for 10 minutes with 3X SSC, 0.1% SDS at room temperature, twice with 1X SSC, 0.1% SDS at room temperature, twice with 0.1X SSC, 0.1% SDS at 42°C and finally, twice with 0.1X SSC, 0.1% SDS at 65°C. The filter was then removed, sealed in saran wrap and exposed to X-ray film with an intensifying screen. Filters were left at -50°C for the appropriate length of time before being developed.

2.14. Electrophoretic mobility shift assays (EMSAs)

2.14.1. Nuclear protein extraction

Nuclear protein extracts were prepared from healthy *N. tabacum* petiole, stem and laminar and *B. napus* laminar and petiole (Deryckere and Gannon, 1994). This method was adapted for the isolation of plant nuclear proteins. 5g of each tissue from tobacco and rape was collected, immediately wrapped in aluminium foil and frozen in liquid nitrogen. The plant tissue was then ground to a fine powder in liquid nitrogen. The powdered material was homogenised with a polytron on full speed for three 30 second bursts, using three volumes (15ml) of Buffer 1 (Extraction Buffer) (1M 2-methyl-2,4-pentanediol, 10mM HEPES/KOH pH 7.6, 10mM magnesium chloride and 0.5% Triton X-100 with 5mM β mercaptoethanol and 1mM PMSF [phenyl methyl sulphonyl fluoride] added just before use). The homogenised material was kept on ice continually.

The homogenate was filtered through a sterile polypropylene filter (20μm pore size) and two layers of cheesecloth. The filtrate was then centrifuged at 3,000rpm for 10 minutes in a Beckman JA20 rotor and the supernatant discarded. The pellet was briefly washed in three volumes (15ml) of Buffer 2 (Resuspension Buffer) (0.5M 2-methyl-2,4-pentanediol, 10mM HEPES/KOH pH 7.6 and 10mM $MgCl_2$ with 5mM β mercaptoethanol and 1mM PMSF, added just before use). This was centrifuged at 3,000rpm in a Beckman JA20 rotor for 5 minutes. The washed nuclei were re-suspended in 500μl of solution B (25% glycerol, 15mM HEPES/KOH pH 7.6, 420mM NaCl, 5mM $MgCl_2$ and 0.2mM EDTA, with 1mM DTT and 1mM PMSF added just before use), and incubated on ice for twenty minutes. The lysed nuclei were transferred to an Eppendorf tube and centrifuged at 12,000rpm for 15 seconds in a microfuge. The supernatant containing nuclear proteins was aliquoted into single-use fractions and frozen immediately in liquid nitrogen. The extracts were stored at -80°C.

2.14.2. Protein estimation

The Bradford method was used to quantify the amount of nuclear protein prepared from each extract (Bradford, 1976). The assay was performed on 800μl of diluted

sample and 200µl of Bradford Reagent (50mg Coomassie Brilliant Blue G250 in 25ml 95% ethanol, 50ml 85% orthophosphoric acid, to 500ml with distilled water, filtered) after 15 minutes. The absorbance was read at 595nm against a blank of 800µl distilled water and 200µl Bradford Reagent and the results compared to a standard curve prepared with 0.125 to 64µg of BSA protein by a dilution series.

2.14.3. Annealing single stranded oligonucleotides

Synthesised oligonucleotides were lyophilised and were supplied as single stranded from the manufacturer (MWG Biotech). These were re-dissolved to 100µM using sterile molecular biology grade water and aliquoted into small fractions. For a 5pmolµl^{-1} stock of double-stranded oligonucleotide, 4µl of each 100µM strand, 4µl 20X annealing buffer (1M Tris-HCl pH 8.0, 2M $MgCl_2$, 5M NaCl and 0.5M EDTA, pH 8.0) and 68µl sterile deionised water was incubated at 95°C for 5 minutes and cooled slowly to room temperature for approximately 2 hours. Annealed oligonucleotides were stored in fractions at -20°C.

2.14.4. Labelling probes

10pmol of double stranded oligonucleotide was labelled using 1µl T4 polynucleotide kinase buffer, 3µl [γ-^{32}P]ATP (~11100Bq) and 1µl (10U) T4 polynucleotide kinase, to a final volume of 10µl. The reaction mixture was incubated at 37°C for 15 minutes before adding 1µl of 0.5M EDTA and 89µl TE buffer.

Percentage incorporation was checked by first spotting 1µl of probe onto a piece of Whatman GF/C glass microfibre filter and recording the counts per second (cps). A solution of 10% trichloroacetic acid was then used to wash unincorporated nucleotides from the filter. The cps was recorded again and used to obtain a value for percentage incorporation.

2.14.5. Gel shift assays

EMSAs were performed according to a modified version of Promega technical bulletin 110 (Griffiths *et al.*, 1995).

2.14.5.1. Preparation of 4% polyacrylamide gel

The apparatus used for this method was a Hoefer® vertical slab gel tank (Pharmacia Biotech Inc., San Francisco, California, USA). Before each gel was poured, the glass plates were cleaned thoroughly with ethanol and one of the plates in contact with the surface of the gel was siliconised with 'Repelcote' silicon treatment (2% solution of dichlorosilane in octamethylcyclotetrasiloxane). An 11 x 14cm acrylamide gel of 1mm thickness was poured quickly (3ml 5X TBE, 4ml 30% acrylamide [37.5: 1, acrylamide: bis acrylamide, w/w] (Bio-Rad, Hemel Hempstead, Herts, UK), 0.94ml 80% glycerol, 21.75ml distilled water, 30µl TEMED and 300µl of freshly made 10% ammonium persulphate). When the gel had set, it was covered with sterile water-moistened paper and saran wrap to prevent dehydration, and left at 4°C overnight.

2.14.5.2. Binding reactions

Each reaction mix contained 2µl of 5X binding buffer (20% glycerol, 5mM $MgCl_2$, 2.5mM EDTA (pH 8.0), 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl (pH 7.5) in deionised water with 0.5µg/µl poly(dIdC:dIdC) (Sigma-Aldrich Co. Ltd.) added just before use) and 5µg nuclear protein extract, unless stated otherwise. When cold homologous and cold non-homologous oligonucleotide was added, it was in 150-fold molar excess to the probe i.e. 15pmol. The total reaction mixture volume was made up to 10µl. The reactants were well mixed and incubated on ice for 20 minutes. 1µl of radioactive oligonucleotide probe (0.1pmol, ~1ng) was added per reaction mixture and incubated at room temperature for 20 minutes. 1µl 10X loading dye (250mM Tris-HCl (pH 7.5), 0.2% bromophenol blue, 0.2% xylene cyanol and 40% glycerol in 10ml water) was then added per reaction mixture.

2.14.5.3. Gel electrophoresis

The gel apparatus was assembled in the gel tank, which was then filled with cold 0.5X TBE buffer. The wells were flushed out with 0.5X TBE to remove air bubbles and the gel was pre-run for ½ hour at 120V. The wells were flushed out again before the samples were carefully loaded into the wells using sequencing tips. When loading was complete the gel was run at 250V for 5 minutes to run the samples into the gel. The gel was then run at 120V, until the lower dye front was approximately 3

cm from the base of the gel (2 ½ hours). During the running of the gel, a cooling system was in place to avoid overheating and disruption of the nuclear proteins. The glass plates were carefully prised apart and the radioactive gel was blotted onto 3MM chromatography paper, placed on another sheet of chromatography paper, covered in saran wrap and dried down onto the paper in a Model 443 Slab drier (Bio-Rad) at 80°C for 2 hours. The gel was exposed to photographic film with an intensifying screen for an appropriate length of time.

2.14.6. Quantification of band density.

A Bio-Rad Personal Molecular Imager FX® (Bio-Rad) was used to perform density analyses on bands. A dried polyacrylamide gel was exposed to an intensifying screen for 16 hours and the program "Quantity One" was used to analyse the image.

2.15. SDS PAGE (Sodium dodecylsulphate polyacrylamide gel electrophoresis)

2.15.1. Preparation of a 10% SDS-PAGE gel and buffers

Glass plates were cleaned with 70% ethanol and assembled with 0.75mm spacers in the gel-casting unit, following the manufacturer's instructions (Bio-Rad). For the resolving gel, 2.5ml of buffer (3M Tris-Cl, pH 8.8 and filtered through a nitrocellulose filter to remove debris) and 3.2ml of 30% acrylamide (29.9g acrylamide: 0.8g Bis) were mixed and the volume made up to 10ml with sterile distilled water. The above mixture was degassed under vacuum for 10 minutes (oxygen inhibits polymerisation), before adding 100µl of 10% SDS, 10µl TEMED and 100µl 5% ammonium persulphate (prepared fresh). The gel mixture was poured immediately, followed by a thin layer of water-saturated butanol. When the gel had set, the butanol was poured off and the gel surface rinsed with distilled water.

For the stacking gel, 2.5ml of buffer (0.25M Tris, pH 6.8), 0.5ml acrylamide (29.9g acrylamide: 0.8g Bis) were mixed in a final volume of 3ml and the mixture degassed under vacuum for 10 minutes, before adding 50µl 10% SDS, 10µl TEMED and 75µl 5% ammonium persulphate (prepared fresh). The solution was then poured over the top of the resolving gel, the combs inserted and the gel allowed to set.

The solution for the lower tank consisted of 2.5M Tris, pH 8.5. The stock solution for the upper tank buffer consisted of $30gl^{-1}$ Tris base, $144gl^{-1}$ glycine, $10gl^{-1}$ SDS, pH8.5. This was diluted 10X before use and filtered through nitrocellulose filters.

2.15.2. Sample preparation and electrophoresis

5X sample buffer/loading buffer (2.0ml 0.25M Tris, pH6.8, 0.8ml glycerol, 1.6ml 10% SDS, 0.2ml 0.05% bromophenol blue, to 6ml with distilled water) was made up before use and stored at 4°C in 0.75ml aliquots. 0.25ml β-mercaptoethanol was added to each aliquot just before use. The loading buffer was then diluted to 1X with the nuclear protein sample, so that approximately 20μg of nuclear protein extract was run in each track. Before loading the gel the mixture was boiled for three minutes to denature the proteins. This also applied to the molecular weight standard, which was prepared using the manufacturers instructions (Sigma-Aldrich Co. Ltd.).

Samples were electrophoresed at 200V for approximately 1 hour.

2.15.3. Silver staining

Gels were fixed overnight in 40% ethanol and 10% acetic acid then gently agitated three times in 10% ethanol for five minutes each time. This was followed by three 5 minute washes in distilled water and a 30 minute wash in 0.2% silver nitrate. After a brief wash in distilled water, the gel was bathed in developing solution (2.5% sodium carbonate, 0.1% formaldehyde) for 30 seconds or until the solution turned brown and bands were visible. A brief wash in 1% acetic acid stopped the reaction proceeding further. The next step involved three 5 minute washes in distilled water before the gel was treated with Farmer's reducer (0.6g sodium thiosulphate, 0.3g potassium ferricyanide, 0.1g sodium carbonate in a final volume of 200ml) for 10 to 30 seconds. This was followed by three 10 minute washes in distilled water before returning to the 30 minute wash in 0.2% silver nitrate and the whole process from this step repeated once again.

When the staining process was complete, the gel was dipped into gel dry solution (30% methanol, 2% glycerol), carefully placed between two sheets of cellophane, avoiding air bubbles, and then dried in a gel drier for about two hours at 80°C.

3. The detection of DNA-protein interactions on three sequences within the NRR

3.1. Introduction

Identifying the DNA sequences that mediate regulated transcription is an essential step in understanding the processes that lead to expression of a gene. So far, very little is known about the precise sequence of promoter elements that control the expression of extensin genes. To date, the most well characterised promoter is from the carrot extensin gene, *pDC5AI,* as both *in vitro* and *in vivo* analysis has been carried out (Chen and Varner, 1985a and 1985b; Ecker and Davis, 1987; Tierney *et al.*, 1988; Holdsworth and Laties, 1989a and 1989b; Granell *et al.*, 1992) (see section 1.3.5 and 1.3.6). Extensive *in vivo* analysis of the extensin gene promoter from *Brassica napus*, *extA,* has also been undertaken (section 1.3.7). Previous work carried out by Elliott and Shirsat (1998) identified a region of the *extA* promoter (the NRR) between –433 and –664bp, thought to contain a negative regulatory element (the NRE). Negatively acting regulatory elements controlling cell specific expression may be common to cell wall structural protein genes because a negative regulatory element directing vascular-specific expression has also been found in the *GRP 1.8* gene promoter from bean, which also codes for a structural cell wall protein (Keller and Baumgartner, 1991). A similar mechanism of gene repression has also been seen in the response of the *pDC5A1* carrot extensin to wounding (Holdsworth and Laties, 1989b) (section 1.3.6).

It is possible that the NRR from *extA* acts by binding a nuclear protein, resulting in the repression of extensin in non-phloem cells. Repression may be overcome by the application of externally applied tensile stress or at stressed regions of the plant such as the nodes. The mechanism may involve the dissociation of a repressor protein, the inhibition of its binding or a conformational change leading to a loss of normal negative control and allowing expression in all cell-types of the stressed region.

In this chapter electrophoretic mobility shift assays are used to investigate the binding capabilities of three putative *cis*-elements located in the *extA* NRR.

3.1.1. The electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) or gel retardation/gel shift assay is an *in vitro* method used for the initial characterisation of sequence-specific DNA binding proteins (Lane *et al.*, 1992). Gel shift assays were first used in early work on rRNA-protein interactions (Jones and Berg, 1966). Since its use in studies on transcriptional regulation in bacteria (Fried and Crothers, 1981; Garner and Revzin, 1981), the technique has been widely used in molecular biology. EMSAs have been used to elucidate the regulation of plant genes by many factors, including light (Gilmartin *et al.*, 1990), UV light (Kaulin *et al.*, 1986), anaerobic stress (Ellis, 1987), pathogens (Yang *et al.*, 1999) and wounding (Holdsworth and Laties, 1989a; Granell *et al.*, 1993).

3.1.1.1. The gel shift principle

A labelled DNA fragment is mixed with protein under conditions that favour specific complex formation. The addition of excess non-specific competing DNA, such as poly(dIdC:dIdC), to the binding reaction mix should inhibit non-specific binding of proteins present in crude nuclear extracts to the labelled probe, which can result in the production of spurious complexes. Poly(dIdC:dIdC) contains inosine and cytosine residues. The large mass of alternating copolymer presents a large number of non-specific binding sites for DNA binding proteins in crude extracts but presents considerably fewer sequence-specific binding sites relative to heterologous DNA (Carthew *et al.*, 1985; Singh *et al.*, 1986).

The sample, containing an equilibrium mixture of free DNA, free protein and complexes is then electrophoresed through a non-denaturing polyacrylamide gel matrix. All species migrate under the influence of the electric field according to their own characteristic mobility, which is determined by size, shape and charge. The mobility of a protein-DNA complex is only slightly affected by changes in the size of the DNA fragment used as a probe. Figure 3.1 shows the principle behind the gel mobility shift assay. Binding of protein to a DNA fragment leads to a reduction in the electrophoretic mobility of the DNA because stable peptide-DNA complexes migrate through polyacrylamide gels more slowly than free DNA fragments.

Although some dissociation of complexes during non-denaturing gel electrophoresis occurs, complexes within the gel are remarkably stable. At least two

factors stabilise protein-DNA complexes. One is the low ionic strength of the buffer which can increase binding affinity and the second factor is an important aspect of electrophoresis known as the "caging effect." The DNA-protein complex migrates through the gel as a discreet band, despite the fact that the time required to run the gel can exceed the typical complex half-life by orders of magnitude (Carey, 1991). This is because once a complex enters the gel, the gel matrix provides a cage in that if a complex suddenly dissociates, the components tend to recombine rather than diffuse away from each other. Therefore, although a complex may dissociate many times during the gel run, re-association is strongly favoured so that the observed bands ultimately represent the fraction of free and bound probe present when the gel was loaded.

Other useful attributes of the technique include the sensitivity of separation methods for detecting DNA-protein interactions, the small amount of material required and the ability to detect low abundance interactions. Eukaryotic transcription factors are rare proteins, varying from 10^3 molecules per cell (retinoic acid receptors) to 10^5 molecules per cell (GATA-1 factor in erythroid cells) (Nicolas *et al.*, 1999). However, the EMSA is an extremely sensitive technique, allowing femtomole quantities of DNA-binding proteins to be detected routinely (Buratowski, 1996).

The specificity of the reaction is determined by the addition of excess unlabelled DNA to the binding reaction mix. DNA homologous to the probe will compete for binding whereas non-homologous or mutated should not compete (figure 3.2). Non-specific interactions will be competed out with both specific and non-specific DNA.

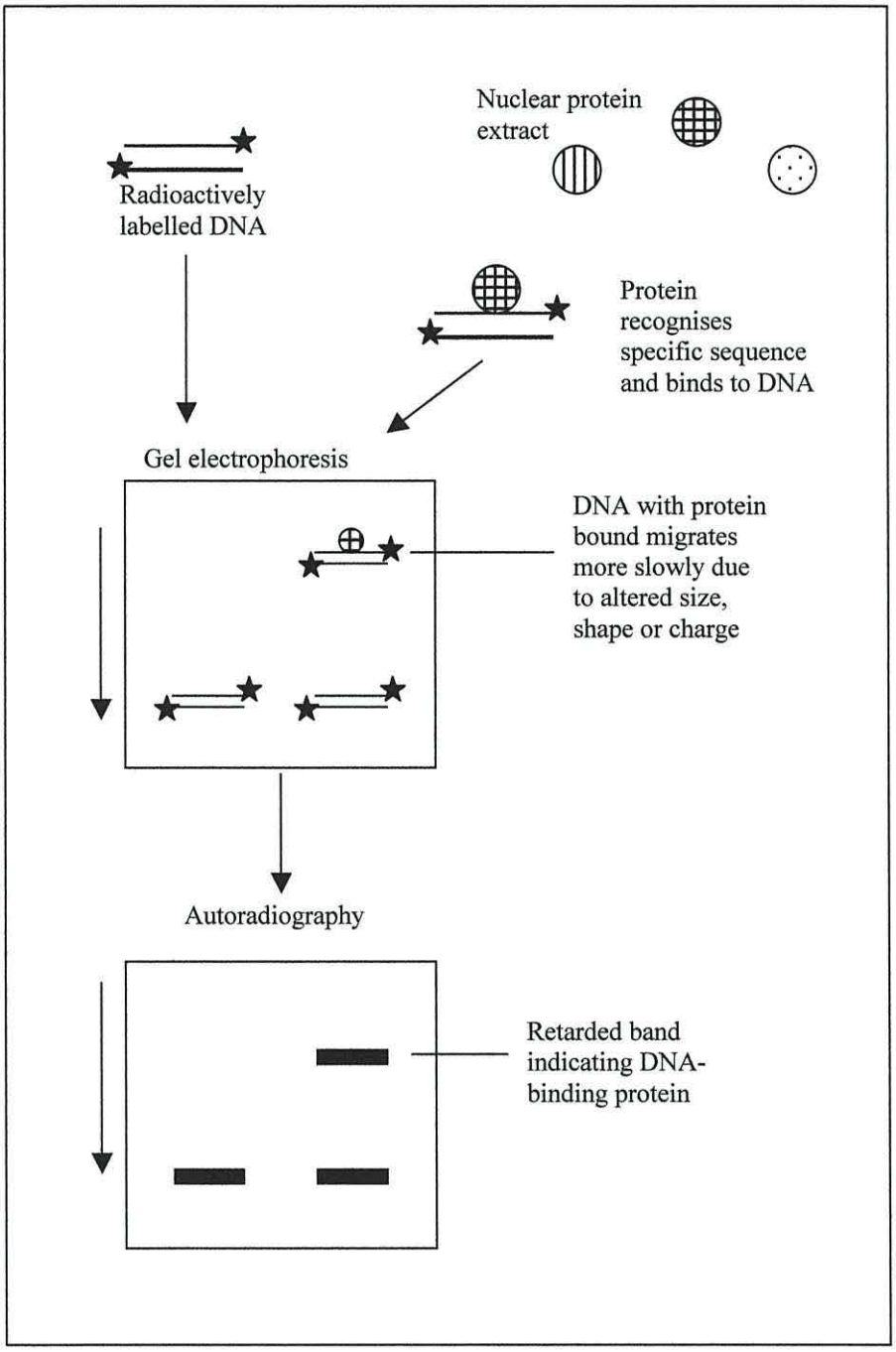


Figure 3.1. Schematic of the principle of the gel mobility shift assay. Binding of a nuclear protein to the radioactively labelled DNA causes it to move more slowly upon gel electrophoresis and therefore results in the appearance of a retarded band upon autoradiography. ★ = radioactive labelling. ⊕ = specific binding protein. —— = double-stranded DNA sequence.

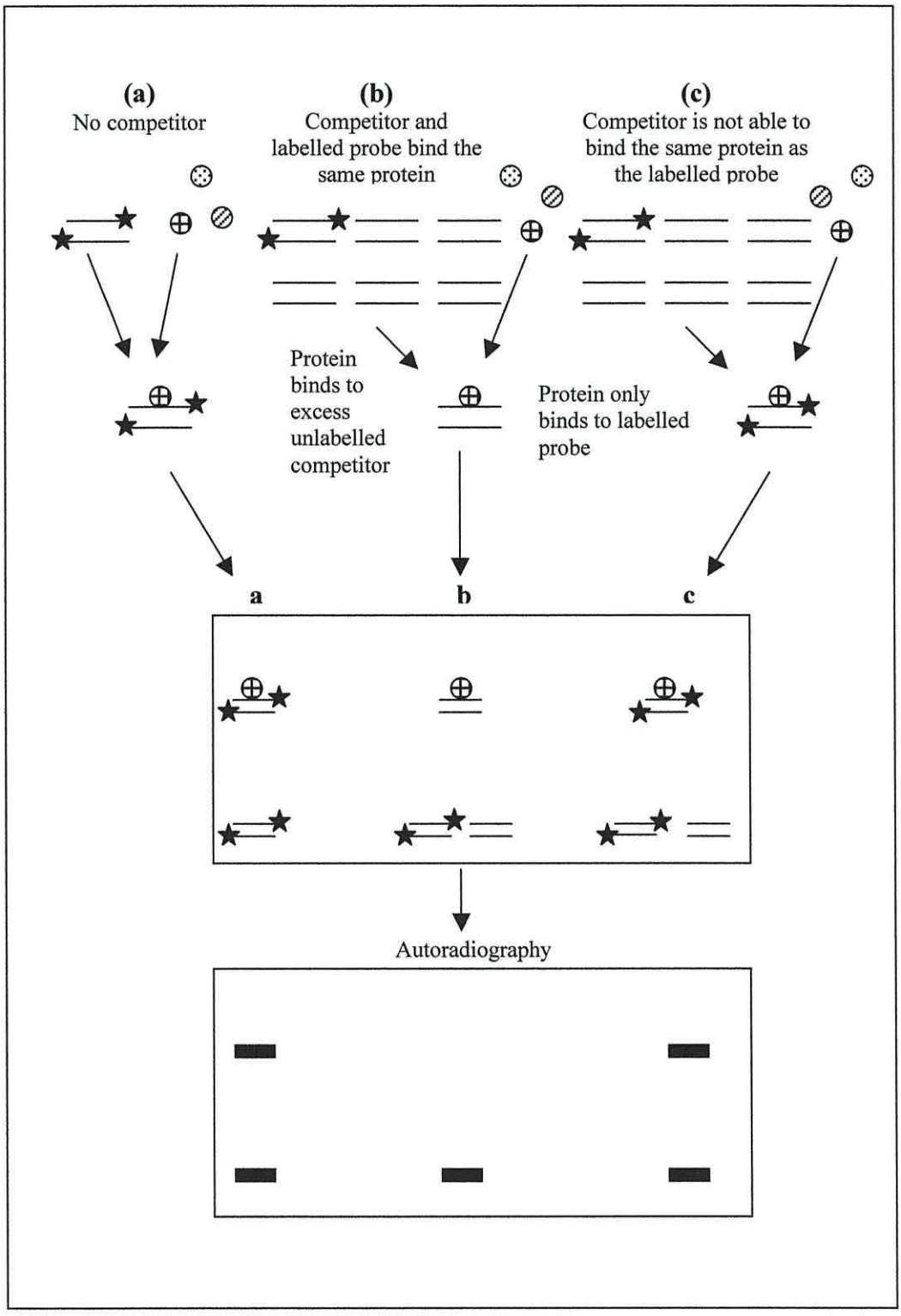


Figure 3.2. Schematic diagram to show the use of unlabelled competitor DNA in the DNA mobility shift assay. If the unlabelled competitor is capable of binding the same protein as the labelled probe, and the competitor is in sufficient excess, then a retarded band will not be observed (b). However, if the unlabelled competitor does not bind the same protein as the labelled probe, then a retarded band will be observed (c). ★ = radioactive labelling. ⊕ = specific DNA-binding protein. ⎯⎯ = double-stranded DNA sequence.

3.2. EMSAs to establish whether three sequences showing homology to plant consensus binding elements are able to bind nuclear proteins from rape and tobacco tissues

3.2.1. Selection of possible binding sites for plant nuclear proteins within the NRR

Conserved sequence motifs in similarly regulated genes have frequently been taken to imply that those sequences may be intimately involved in the mechanism of regulation (Quatrano *et al.*, 1993). Based on this assumption, three putative binding sites for plant transcription factors within the *extA* NRR (-664 to -433bp region) (figure 3.3) have been previously identified (Elliott, 1998):

- A GTGG motif in the negative regulatory region of the *GRP1.8* bean promoter has been shown to specifically bind a bZIP nuclear protein (VSF-1) (Torres-Schumann *et al.*, 1996). The *extA* promoter also contains this G-box half site (5′-TAGTGGAT-3′) located between –479bp to –472 bp within the region containing the NRR. This is shown in figure 3.3 as Vs-2. The sequence bound by VSF-1 conferred xylem specific expression.
- The sequence ATTTGCAT is a conserved enhancer element in animals and the SV40 virus (Sive and Roeder, 1986; Ninomiya *et al.*, 1987) and has also been shown to act as an enhancer in tobacco plants (Kawaoka *et al.*, 1992). Two sequences within the *extA* NRR showed strong identity to the enhancer element. Enh-1 (ATTCGCAT) is located between –650bp and –643bp and Enh-2 (TTTTGCAT) is located between –472bp and –465bp, adjacent to the Vs-2 element in figure 3.3.
- The sequence ATTAAATTTTAAATT from the soybean lectin gene has been shown to bind nuclear proteins from certain tissues during seed development (Jofuku *et al.*, 1987). The sequence ATTAAGTTATTAATT is located from –604 to –590bp on the *extA* promoter and shows 80% homology to the lectin sequence. This was designated Lec-2 in figure 3.3.

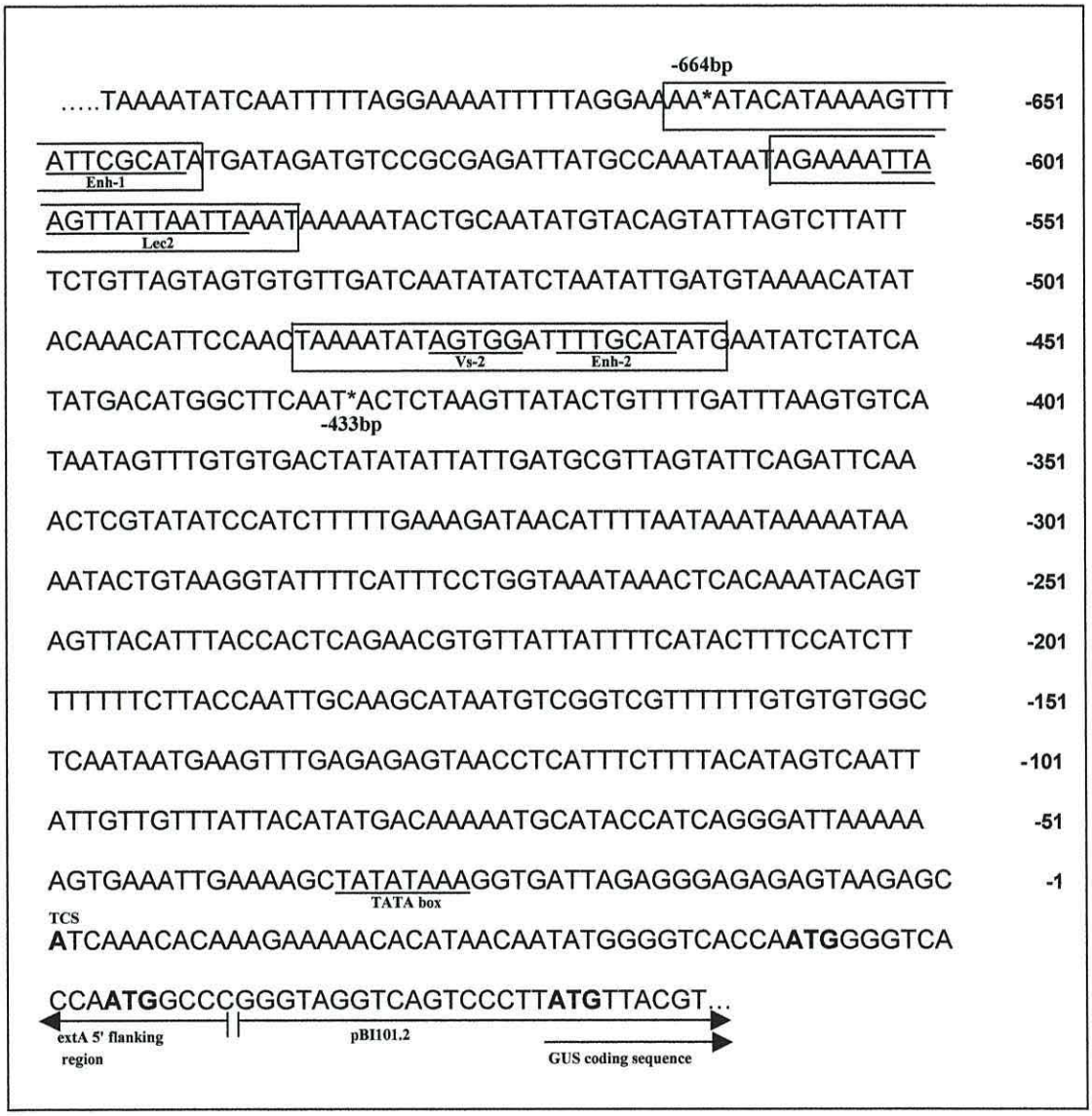


Figure 3.3. The *extA* promoter up to –686bp showing the positions of the synthetic oligonucleotides (boxed) within the NRR (-664 to –433bp), encompassing putative binding sites (underlined).

To investigate the possibility that these sequences also bound nuclear proteins in *extA*, three 25-mer single stranded oligonucleotides encompassing the putative binding sites and their complementary strands were synthesised (Table 3.1). These oligonucleotides were then annealed (section 2.14.3) so that they became double stranded. Figure 3.3 shows the position of the synthetic oligonucleotides containing the putative binding sites in the *extA* promoter.

Probe	Oligo	Sequence	Position in the *extA* gene promoter
Enh-1	Enh-1+	5′ -AAATACATAAAAGTTTATTCGCATA- 3′	-670 to -646 bp
	Enh-1-	3′ –TTTATGTATTTTCAAATAAGCGTAT- 5′	
Lec-2	Lec2+	5′-AGAAAATTAAGTTATTAATTAAATA- 3′	-609 to -585 bp
	Lec2-	3′ -TCTTTTAATTCAATAATTAATTTAT- 5′	
Vs-2/Enh-2	Vs-2/Enh-2+	5′ -TAAAATATAGTGGATTTTGCATATG- 3′	-486 to -462 bp
	Vs-2/Enh-2-	3′ -ATTTTATATCACCTAAAACGTATAC- 5′	

Table 3.1. Oligonucleotides used in EMSA experiments. Putative binding sites are underlined.

3.2.2. Nuclear protein extracts

Protein extracts suitable for EMSAs may be prepared from isolated nuclei or whole cells. Generally the use of nuclear extracts is preferable because they contain a high concentration of transcription factors. The range of salt concentration used in extraction buffers varies considerably but is always high to enable proteins to dissociate from the DNA. The concentration used in the following experiments was 420mM, taken from a standard and well-used protocol (Deryckere and Gannon, 1994).

Nuclear protein extractions were carried out according to the method detailed in section 2.14.1. Extracts were taken from *B. napus* and *N. tabacum* tissues to establish whether the binding proteins were active in a heterologous system. Extracts were always made from tissue of approximately the same age (mature tissues) to minimise errors caused by developmental effects. Laminar and petiole tissues were taken from oilseed rape, whereas laminar, petiole and internode tissues were taken

from tobacco due to the different physiology of this plant. Nuclear protein extracts were aliquoted into single use fractions and frozen immediately in liquid nitrogen before being stored at –80°C for a maximum of six months.

The concentration of protein in the nuclear extracts was quantified using the Bradford method (Bradford, 1976). A standard curve was prepared each time the assay was performed (section 2.14.2). Extracts varied in concentration. Laminar extracts tended to be high in nuclear protein, compared to petioles and internodes. This was probably due to the difficulty experienced in grinding the more solid tissues in liquid nitrogen. Quantities of nuclear extract from petioles and internodes added to the binding reaction mixtures were therefore adjusted accordingly.

3.2.3. SDS-PAGE

SDS-PAGE gels (see section 2.15) were run to check the integrity of the nuclear protein extracts and as a method of comparing the relative concentration of the extracts, in addition to the Bradford assay. Figure 3.4 shows a typical example of an SDS-PAGE gel. Any degradation of the extract may have been seen as a smear on the gel or as an extra band.

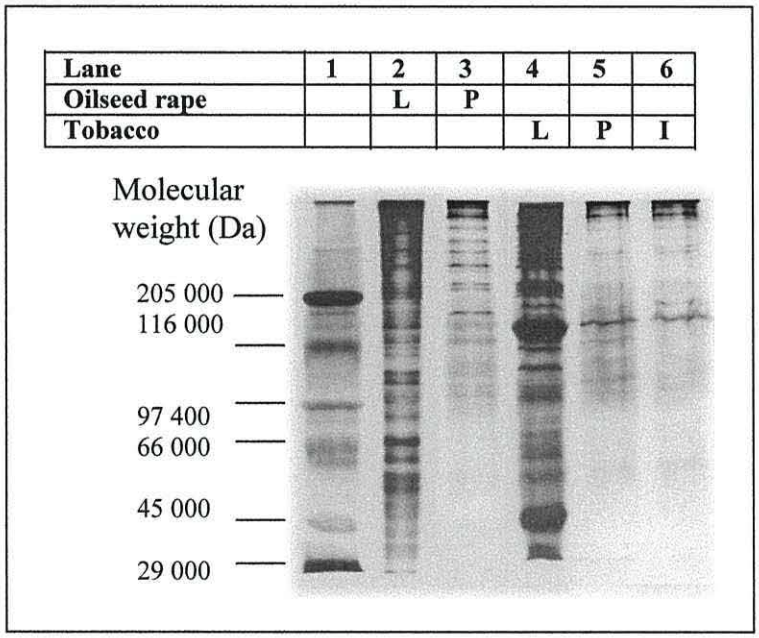


Figure 3.4. SDS-PAGE gel comparing crude nuclear protein extract from rape and tobacco tissues. Laminar tissues contain a higher concentration of nuclear protein than petiole and internode tissues. **1.** High molecular weight standard marker (Sigma). **2.** rape laminar (L) extract. **3.** Rape petiole (P) extract. **4.** Tobacco laminar extract. **5.** Tobacco petiole extract. **6.** Tobacco internode (I) extract.

3.2.4. The binding reaction

The conditions used in the binding reaction mixtures were modified from Promega technical bulletin 110 (Griffiths *et al.*, 1995). The binding reaction was performed according to section 2.14.5.2. This involved the incubation of nuclear protein extract with 1μg of poly(dIdC:dIdC) non-specific DNA in 2μl of binding buffer containing salt at a concentration to favour specific interactions (250mM). Non-specific proteins bind to DNA at a low salt concentration and specific proteins bind at a high salt concentration.

The 'cold' homologous oligonucleotide (CHO) competitor was the same sequence used as the probe, except that it was not radiolabelled. If the interaction in question was specific, then the intensity of the normal shift should be significantly reduced when it was added, if not out-competed completely.

When used, the 'cold' non-homologous oligonucleotide (CN-HO) was an unrelated 25bp non-specific DNA sequence from the *Stomoxys calcitrans* midgut defensin I promoter region (5′-CCA GAT ATA CAT ATC TAG TAA TAT G-3′). The strength of binding on the addition of CN-HO was compared with the strength of binding when CHO was added. The addition of CN-HO should have no effect on the intensity of the shift. However, the addition of too much DNA can interfere with the kinetics of the binding reaction, therefore a parallel reduction in intensity of the shift when CN-HO is added may be put down to interference rather than direct competition for binding. However, it is also possible that non-specific binding could occur to the CN-HO, which would result in a reduction of band intensity of this shift. This was tested experimentally by radio-labelling the non-specific sequence and using this to probe the nuclear protein extracts.

All binding reactions were carried out on ice using fresh nuclear extracts.

3.2.5. EMSAs to examine binding in all tissue extracts to the three 25bp synthetic oligos from the –433 to –664bp region

Very strong binding was seen to the three synthetic oligonucleotide probes when rape laminar nuclear protein extracts were used in the reaction mixture (figure 3.5, 3.6 and 3.7, all lane 6). In addition, this protein had a similar affinity for all three sequences and a similar mobility in the gel when bound to all three oligos (figure 3.8). However, when the other extracts were used (tobacco laminar, petiole, and

internode and rape petiole), no binding was observed (figure 3.5 and 3.7, lanes 3, 4, 5 and 7 and figure 6 lanes 2, 4, 5 and 7). When the amount of nuclear protein from all tissues (except rape laminar) was increased from 5 to 7μg, there was still no shift with these tissues (figure 3.9, lanes 6-18). These results are significant because the laminar is the tissue containing the least amount of extensin mRNA under non-stressed conditions (Shirsat *et al.*, 1991). If the *extA* gene is negatively regulated then the presence of a retarded band with this tissue might be expected because when the *extA* gene is "switched off," a repressor protein could be bound to the NRE, preventing expression, which is what these gel shifts appear to show.

Titrating the amount of rape laminar nuclear protein extract in the reaction mixture enables the abundance and nature of the bound protein to be established. Figure 3.10 shows an example of a protein titration with *Enh-1* oligo used as a probe. Lane 3 shows the "normal" shift when 5μg of rape laminar nuclear extract is used in the reaction mixture. A shift is first detected in lane 5 where 1μg of extract was used and this increases in intensity as more protein is added. The electrophoretic mobility of the interaction also decreases with the addition of more nuclear protein. This sequential reduction in mobility implies that the protein in question is forming a complex with another or more than one other protein, although a sequential decrease in mobility was not always as marked when the assay was repeated (figure 3.11).

The binding of a nuclear protein from rape laminar extracts to all three sequences tested could either be the result of a single binding protein recognising specific sequences on all the synthetic oligonucleotides, the result of three separate single proteins binding to specific recognition sequences on each oligonucleotide or the protein in question could be non-specific. The specificity of a bound protein is usually determined by performing competition experiments (section 3.1.1.1). In all cases, binding was not out-competed, or even reduced, by a 150-fold molar excess of cold (non-labelled) homologous oligonucleotide (figure 3.5, 3.6 and 3.7, all lane 11). These results suggest that binding is either non-specific or that a larger excess of CHO, in relation to the probe, is required to out-compete binding. Most of the following experiments have therefore concentrated on establishing the specificity of the binding protein in question.

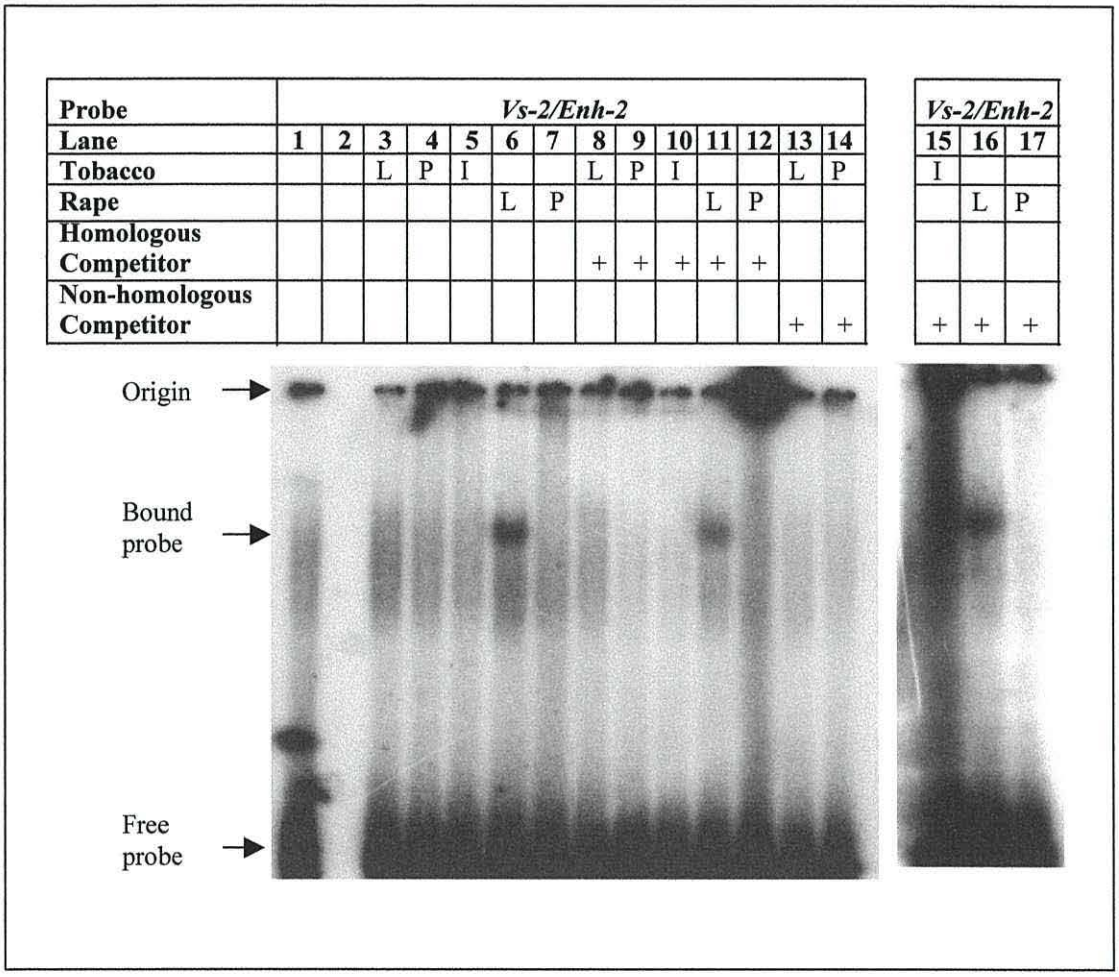


Figure 3.5. EMSA with 5µg of nuclear protein (N.P.) extract from tobacco and rape tissues using *Vs2/Enh-1* as the probe. **1.** Negative control (no protein). **2.** Blank. **3.** Tobacco laminar (L) N.P. **4.** Tobacco petiole (P) N.P. **5.** Tobacco internode (I) N.P. **6.** Rape laminar N.P. **7.** Rape petiole N.P. Lanes 8-12 contain samples where a 150-fold molar excess of cold homologous oligonucleotide (CHO) has been used in the binding reaction mixture as competitor DNA, in this case unlabelled *Vs-2/Enh-1* fragment. **8.** Tobacco laminar N.P. **9.** Tobacco petiole N.P. **10.** Tobacco internode N.P. **11.** Rape laminar N.P. **12.** Rape petiole N.P. Lanes 13-17 contain samples where a 150-fold molar excess of cold non-homologous oligonucleotide (CN-HO) has been used in the binding reaction mixture as competitor DNA, in this case an unlabelled 25bp sequence from *Stomoxys calcitrans*. **13.** Tobacco laminar N.P. **14.** Tobacco petiole N.P. **15.** Tobacco internode N.P. **16.** Rape laminar N.P. **17.** Rape petiole N.P.

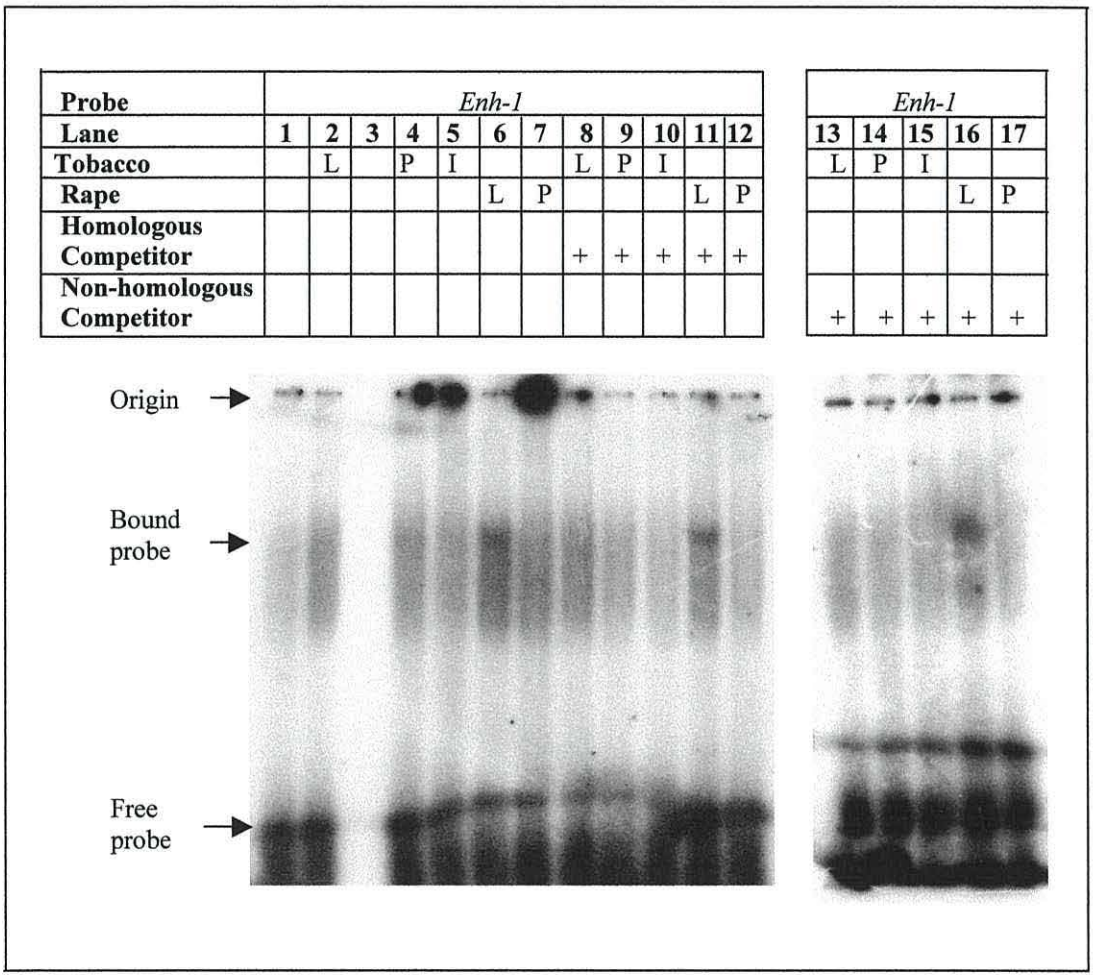


Figure 3.6. EMSA with 5µg of nuclear protein (N.P.) extract from tobacco and rape tissues using *Enh-1* as the probe. **1.** Negative control (no protein). **2.** Blank. **3.** Tobacco laminar N.P. **4.** Tobacco petiole N.P. **5.** Tobacco internode N.P. **6.** Rape laminar N.P. **7.** Rape petiole N.P. Lanes 8-12 contain samples where a 150-fold molar excess of cold homologous oligonucleotide (CHO) has been used in the binding reaction mixture as competitor DNA., in this case unlabelled *Enh-1* fragment. **8.** Tobacco laminar N.P. **9.** Tobacco petiole N.P. **10.** Tobacco internode N.P. **11.** Rape laminar N.P. **12.** Rape petiole N.P. Lanes 13-17 contain samples where a 150-fold molar excess of cold non-homologous oligonucleotide (CN-HO) has been used in the binding reaction mixture as competitor DNA, in this case an unlabelled 25bp sequence from *Stomoxys calcitrans*. **13.** Tobacco laminar N.P. **14.** Tobacco petiole N.P. **15.** Tobacco internode N.P. **16.** Rape laminar N.P. **17.** Rape petiole N.P.

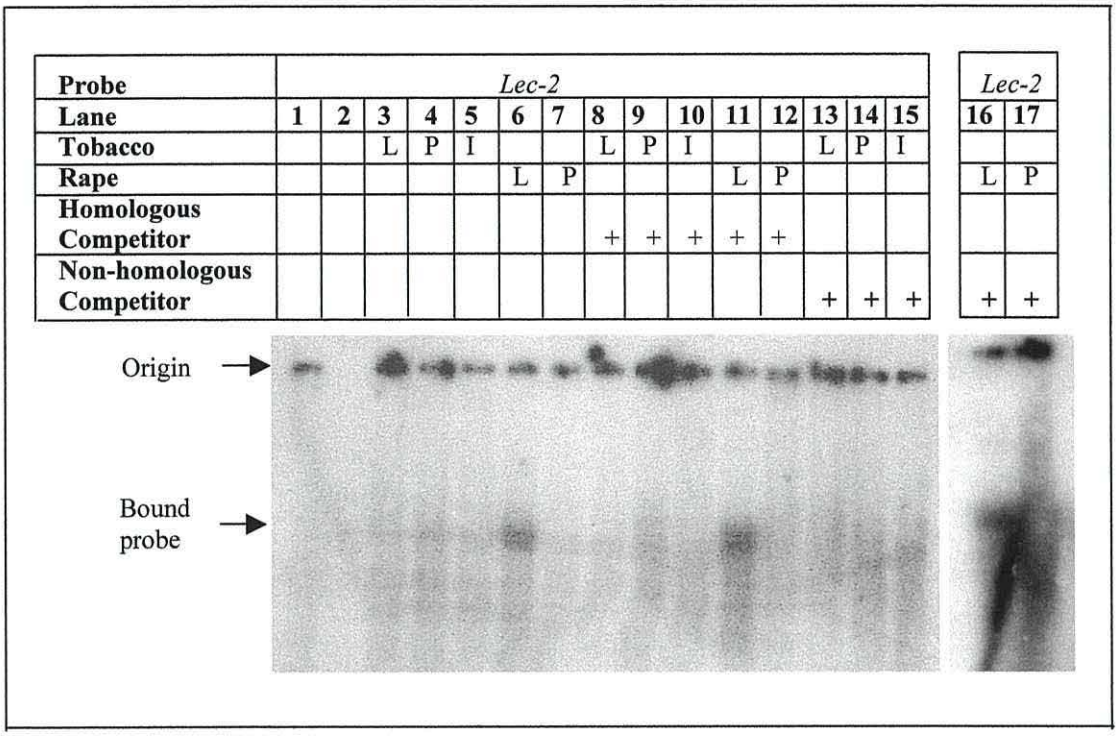


Figure 3.7. EMSA with 5µg of nuclear protein (N.P.) extract from tobacco and rape tissues with *Lec-2* as the probe. **1.** Negative control (no protein). **2.** Blank. **3.** Tobacco laminar N.P. **4.** Tobacco petiole N.P. **5.** Tobacco internode N.P. **6.** Rape laminar N.P. **7.** Rape petiole N.P. Lanes 8-12 contain samples where a 150-fold molar excess of cold homologous oligonucleotide (CHO) has been used in the binding reaction mixture as competitor DNA, in this case unlabelled *Lec-2* fragment. **8.** Tobacco laminar N.P. **9.** Tobacco petiole N.P. **10.** Tobacco internode N.P. **11.** Rape laminar N.P. **12.** Rape petiole N.P. Lanes 13-17 contain samples where a 150-fold molar excess of cold non-homologous oligonucleotide (CN-HO) has been used in the binding mixture as competitor DNA, in this case an unlabelled 25bp sequence from *Stomoxys calcitrans*. **13.** Tobacco laminar N.P. **14.** Tobacco petiole N.P. **15.** Tobacco internode N.P. **16.** Rape laminar N.P. **17.** Rape petiole N.P.

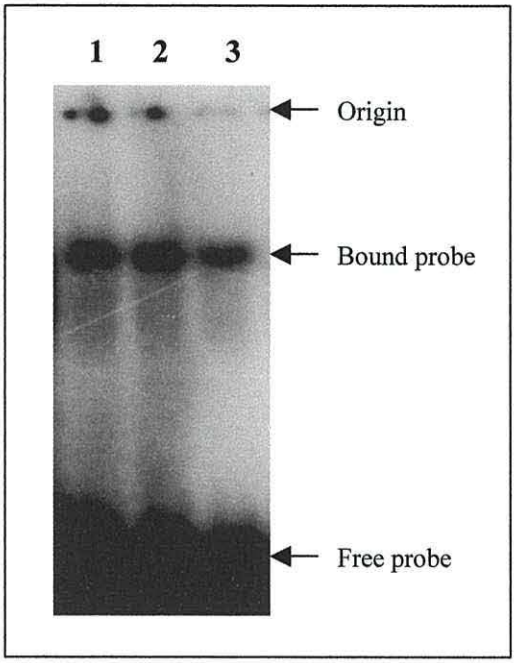


Figure 3.8. EMSA to show that all probes bind a rape laminar nuclear protein with a similar mobility in the gel – maybe even the same protein. 5μg of rape laminar nuclear protein extract was used per reaction mix. Probes used were as follows: **1.***Vs-2/Enh-2* **2.** *Lec2* **3.** *Enh1.*

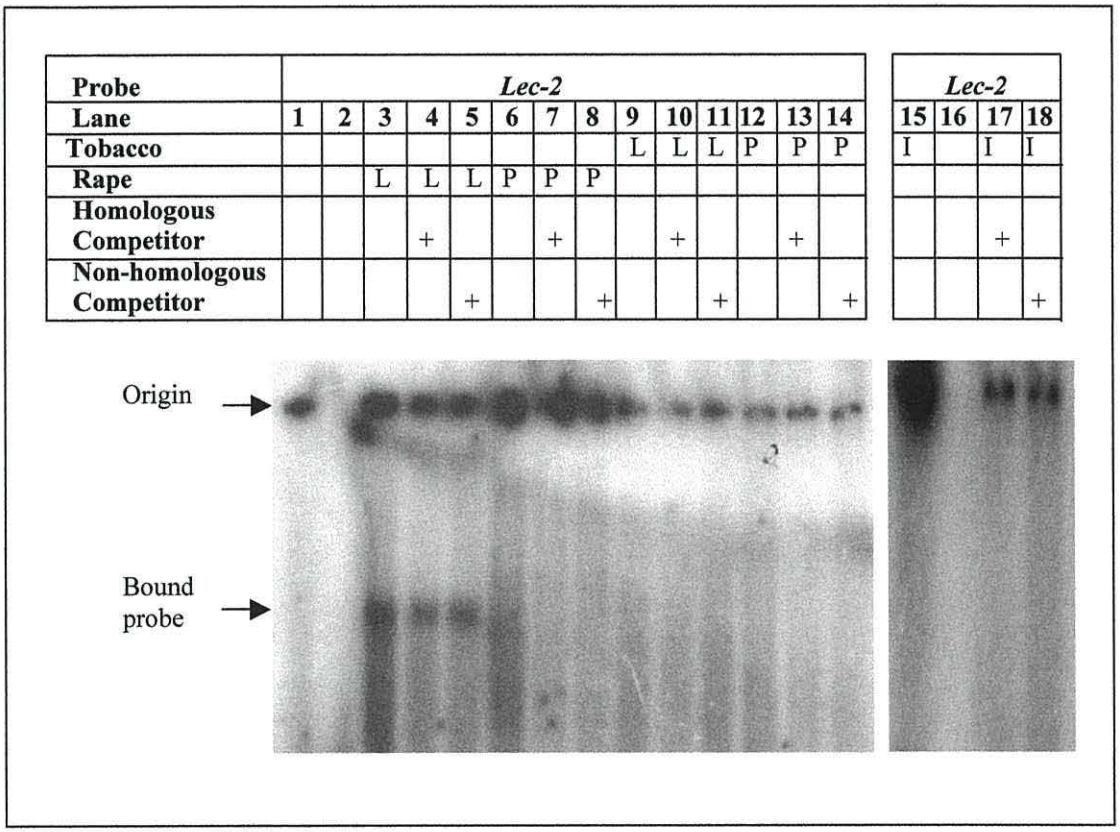


Figure 3.9. EMSA with nuclear protein from tobacco and rape tissues and *Lec-2* as the probe when nuclear protein was increased from 5μg to 7μg in all reaction mixtures except those with rape laminar, where 5μg was used. Reaction mixtures containing cold (non-radioactive) homologous *Lec-2* oligonucleotide (CHO) and cold non-homologous oligo (CN-HO) were run in lanes adjacent to reaction mixtures where no competitor DNA was added, for easy comparison. **1.** Negative control (no protein). **2.** Blank. **3.** Rape laminar N.P. **4.** Rape laminar N.P and 150-fold molar excess CHO. **5.** Rape laminar N.P and 150-fold molar excess CN-HO. **6.** Rape petiole N.P. **7.** Rape petiole N.P and 150-fold molar excess CHO. **8.** Rape petiole N.P and 150-fold molar excess CN-HO. **9.** Tobacco laminar N.P. **10.** Tobacco laminar N.P and 150-fold molar excess CHO. **11.** Tobacco laminar N.P and 150-fold molar excess CN-HO. **12.** Tobacco petiole N.P. **13.** Tobacco petiole N.P and 150-fold molar excess CHO. **14.** Tobacco petiole N.P. and 150-fold molar excess CN-HO. **15.** Tobacco internode N.P. **16.** Blank. **17.** Tobacco internode N.P and 150-fold molar excess CHO. **18.** Tobacco internode N.P and 150-fold molar excess CN-HO.

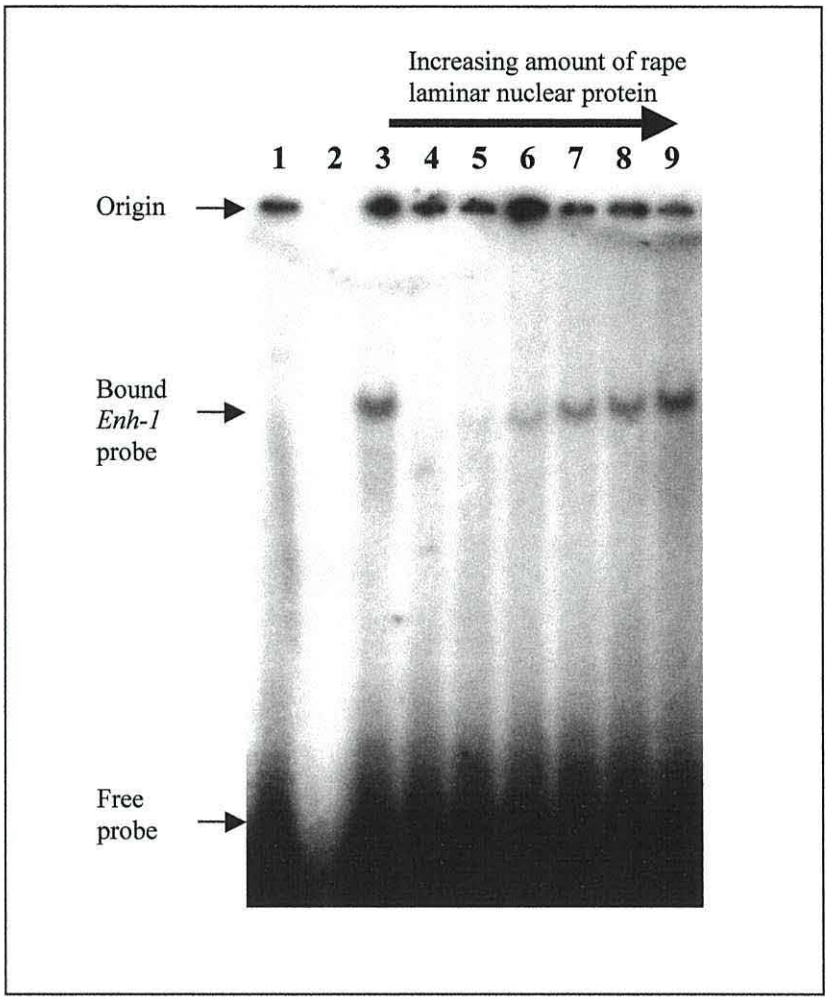


Figure 3.10. Rape laminar nuclear protein (NP) titration with *Enh1* as the probe. **1.** Negative control (no protein). **2.** Blank. **3.** Positive control (5μg rape laminar N.P). **4.** 0.5μg N.P. **5.** 1μg N.P. **6.** 2μg N.P. **7.** 3μg N.P. **8.** 4μg N.P. **9.** 5μg N.P.

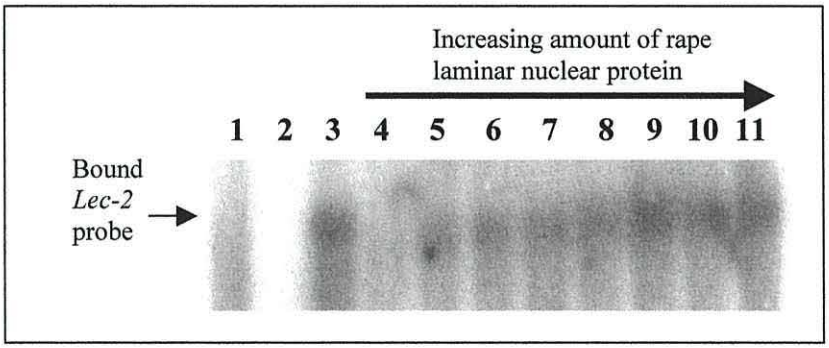


Figure 3.11. Rape laminar nuclear protein (NP) titration with *Lec-2* as the probe. **1.** Negative control (no protein). **2.** Blank. **3.** Positive control (5μg rape laminar N.P). **4.** 0.5μg N.P. **5.** 1μg N.P. **6.** 2μg N.P. **7.** 3μg N.P. **8.** 4μg N.P. **9.** 5μg N.P. **10.** 6μg N.P. **11.** 7μg N.P.

3.2.5.1. Establishing binding specificity to the three probes

Figure 3.12 shows an example of an EMSA where an increasing amount of unlabelled (cold) homologous oligo (Lec-2) was added to the binding reaction mixture before the addition of the radiolabelled (hot) probe (*Lec-2*). An extremely large excess of unlabelled Lec-2 is required to out-compete binding. As more competitor is added, the intensity of binding becomes weaker. However, lane 13 shows that even when a 600-fold molar excess of competitor is added, a shift is still visible, although greatly reduced.

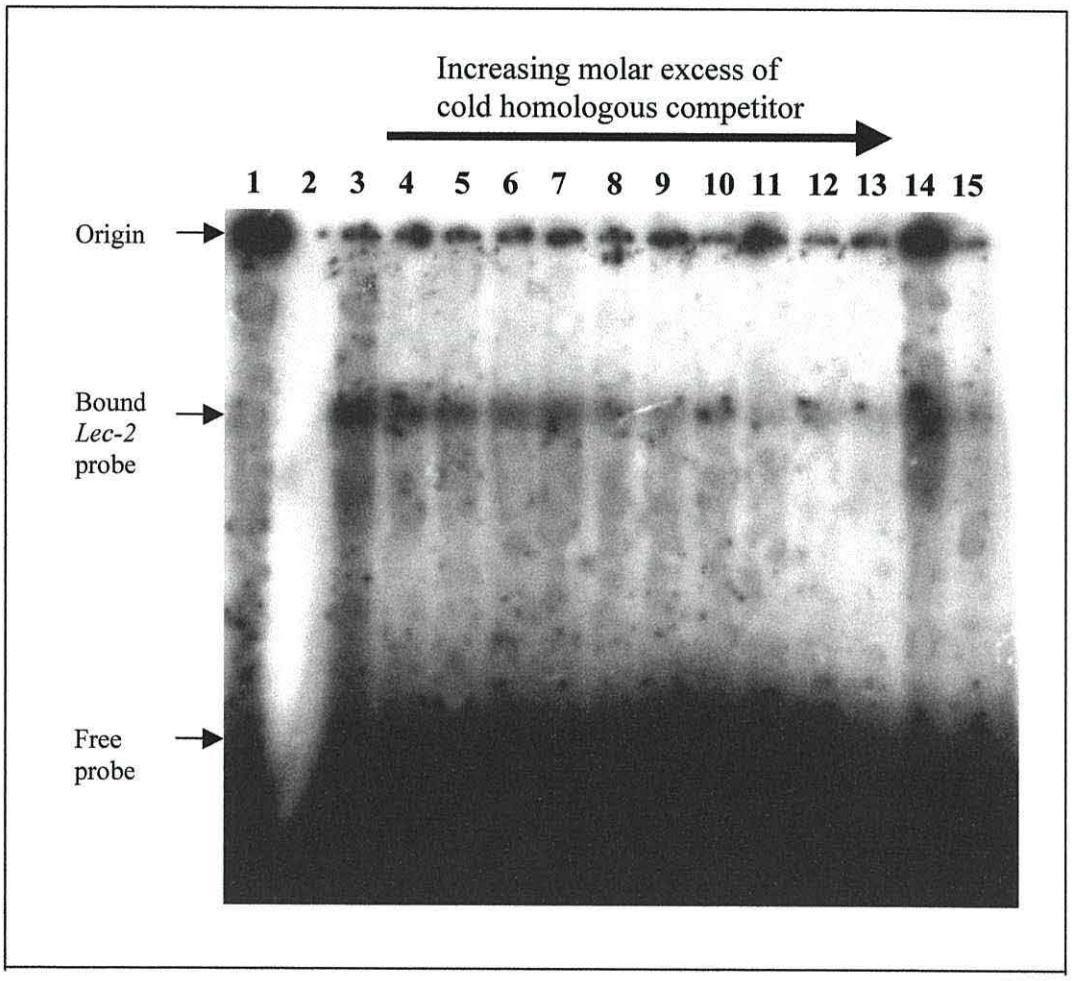


Figure 3.12. Competition experiment using *Lec-2* as a probe and rape laminar nuclear protein extract. **1.** Negative control (no protein). **2.** Blank. **3.** Positive control (5μg rape laminar N.P and no competitor). Lanes **4-13** contain 5μg rape laminar N.P and an increasing molar excess of cold homologous oligo (CHO) (*Lec-2*) in the binding reaction mix. **4.** 150X. **5.** 200X. **6.** 250X. **7.** 300X. **8.** 350X. **9.** 400X. **10.** 450X. **11.** 500X. **12.** 550X. **13.** 600X. Lanes 14 and 15 contain 3μg of rape laminar nuclear protein. **14.** 3μg rape laminar NP. **15.** 3μg rape laminar N.P. and 150-fold molar excess of CHO (*Lec-2*).

Much of this reduction in binding will be due to interference with the kinetics of the binding reaction, through the addition of excess DNA. Figure 3.13 shows that the addition of excess non-homologous oligonucleotide does indeed cause a reduction in binding intensity. Although there is clearly a decrease in band intensity with the addition of excess competitor, it was difficult to judge the effect of the two types of competitor (specific and non-specific) qualitatively. A phosphoimager was therefore used to quantify the band intensity when the two types of competitor were added. Figure 3.14 shows an example of such an analysis when *Lec-2* was used as a probe. Table 3.2 shows that the addition of a 150-fold molar excess of unlabelled Lec-2 caused a reduction in the intensity of binding by almost 50% (lane 2). The addition of cold non-homologous oligonucleotide also caused a decrease in binding intensity, although this was not as great as that observed with the specific competitor. The reduction in binding in this case could have either been caused by interference from excess DNA in the reaction mixture or non-specific binding to the CN-HO, albeit to a lesser degree than to the CHO.

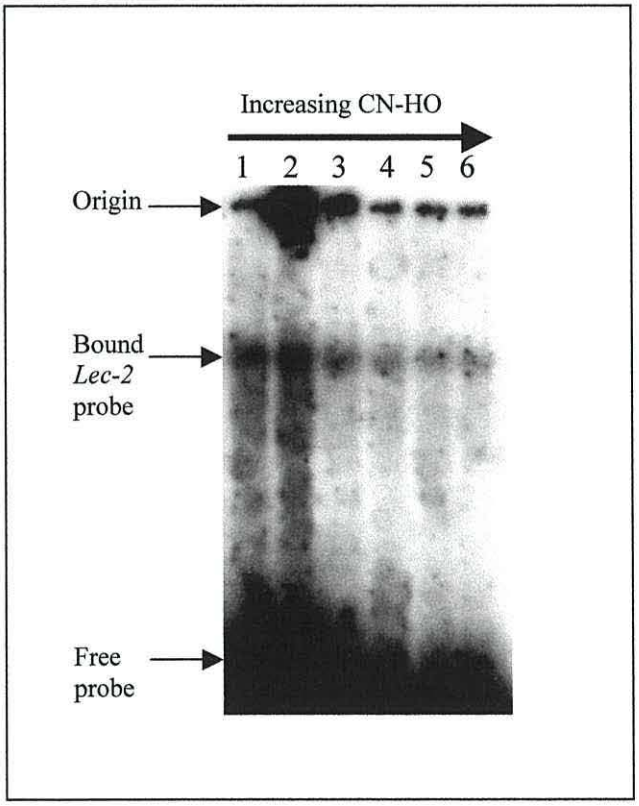


Figure 3.13. EMSA to show the decrease in shift intensity when an increasing amount of cold non-homologous olionucleotide competitor (an unrelated sequence from *S. calcitrans*) was added to the binding reaction. This is most probably caused by interference of reaction kinetics by excess DNA. Lanes 1-6 contain 5µg of rape laminar nuclear protein extract, *Lec-2* probe and an increasing molar excess of cold non-homologous oligonucleotide. **1.** 150X. **2.** 200X. **3.** 300X. **4.** 400X. **5.** 500X. **6.** 600X.

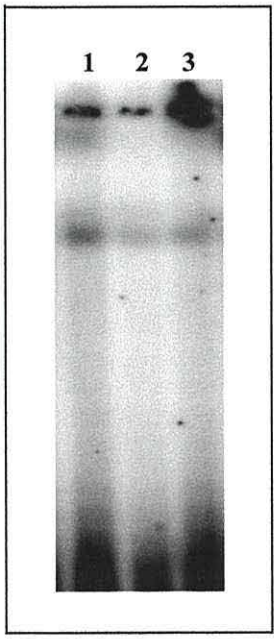


Figure 3.14. EMSA illustrating the quantitative differences in binding to a *Lec-2* probe when a 150-fold molar excess of cold homologous oligonucleotide (CHO) (**lane 2**) and cold non-homologous oligo (CH-HO) (**lane 3**) are added to a binding reaction containing 5μg of rape laminar nuclear protein. **Lane 1** shows the true shift when no competitor is added.

LANE	**1**	**2**	**3**
COMPETITOR	-	CHO	CN-HO
DENSITY OF BAND (counts/mm^2)	2900000	1600000	2300000
MINUS BACKGROUND (counts/mm^2)	2800000	1500000	2200000
% OF BAND 1	100	52	78

Table 3.2. Phospho-imager analaysis of density of bands in Figure 3.14

It is therefore possible that the promiscuous binding of the protein in rape laminar extracts to all three sequences within the NRR could be attributed to a non-specific binding protein. To examine whether this protein would also bind to an unrelated 25bp DNA sequence, the non-specific competitor DNA from *S. calcitrans* was labelled and used as a probe. Figure 3.15 shows that binding does not occur to this sequence (lane 4 compared to lane 3), indicating that binding is specific to the sequences in the NRR.

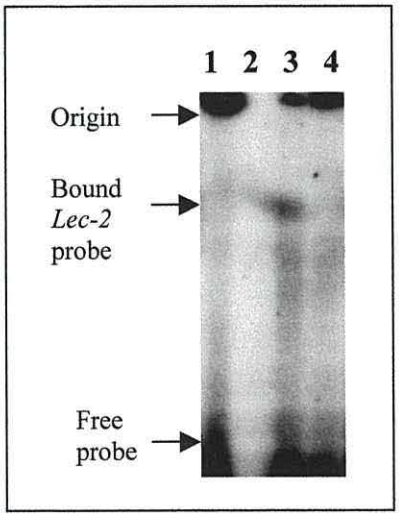


Figure 3.15. EMSA to show that radioactively labelled competitor DNA, an unrelated sequence from *Stomoxys calcitrans* does not bind nuclear proteins from oilseed rape laminar. 5μg rape laminar nuclear extract was used in each binding reaction. **1.** Negative control (no protein). **2.** Blank. **3.** *Lec-2* probe. **4.** Non-homologous oligonucleotide probe, a 25bp sequence from *S. calcitrans*

Another way of establishing binding specificity to a probe is to increase the amount of the copolymer duplex poly(dIdC:dIdC) in the binding reaction mixture, which should soak up non-specific binding proteins. Figure 3.16 shows the results of a poly(dIdC:dIdC) titration. Increasing the amount of this competitor appears to have no effect on binding intensity, even when increased 8X. Lanes 4-8 show that the addition of poly(dIdC:dIdC) has no effect on binding patterns, as binding in the absence of poly(dIdC:dIdC) in lane 3 is no different. There is a greater need for non-specific competing DNA when larger fragments are used as probes as there is more binding opportunity.

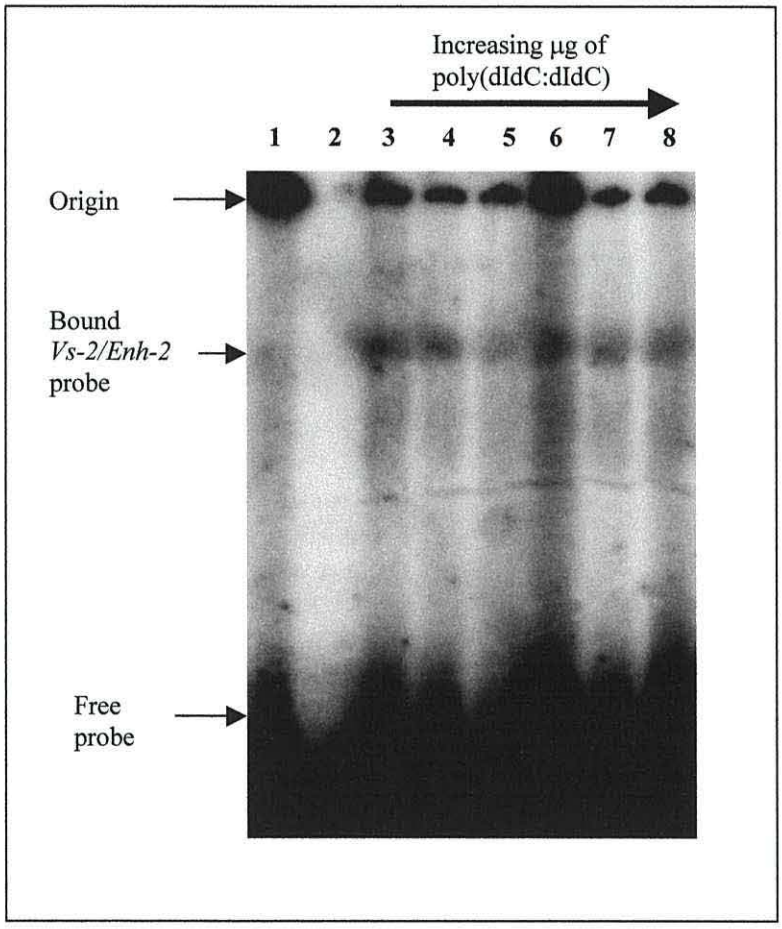


Figure 3.16. Poly(dIdC:dIdC) titration with rape laminar nuclear protein extract. *Vs-2/Enh-2* was used as the probe in all reaction mixes. **1.** Negative control (no protein). **2.** Blank. **3-8.** Increasing amount of poly(dIdC:dIdC) in the binding buffer with 5µg of rape laminar nuclear protein. Poly(dIdC:dIdC) concentrations are given in µg. **3.** 0µg **4.**0.5µg. **5.** 1µg. **6.** 2µg. **7.** 3µg. **8.** 4µg.

It appears that the difficulty in out-competing binding is not the result of a non-specific binding protein but that the nuclear protein(s) binding to the three oligonucleotide sequences is/are extremely abundant. This was confirmed by the fact that reducing the amount of rape laminar nuclear protein extract to 3μg in the binding reaction mixture also reduced the intensity of the shift when a 150-fold molar excess of homologous competitor DNA was added (figure 3.12, lane 14 and 15).

3.3. Summary

In summary, *Lec2, Vs-2/Enh-2* and *Enh-1* probes containing elements previously identified as being able to bind nuclear proteins by sequence homology (Elliott, 1998) all bind an oilseed rape laminar nuclear protein, or proteins of similar mobility, to the same extent. However, it is not known whether these putative elements are involved in the binding of this protein/these proteins, simply that oligonucleotides containing them cause a retarded band in EMSAs. Binding did not occur when rape petiole nuclear protein or extracts taken from tobacco tissues were used. The proteins in question are extremely abundant within the laminar tissue because a large molar excess (greater than 600-fold molar excess) is required to out-compete binding when 5μg of nuclear extract is used. This/these protein(s), although binding promiscuously to the three NRR sequences, appear(s) to be relatively sequence-specific because binding does not occur to an unrelated 25bp sequence from the *S. calcitrans* promoter.

4. The construction of *extA* promoter truncation-GUS fusions in transgenic tobacco plants

4.1. Introduction

Tobacco species are a model plant transformation system to study homologous and heterologous gene expression (Horsch *et al.*, 1985). This is because they are easy to transform with *Agrobacterium tumefaciens* and shoot regeneration from infected leaf cells is a relatively rapid and simple process (reviewed by Draper and Scott, 1991). Several extensin promoters have been studied in this way (Granell *et al.*, 1992; Tagu *et al.*, 1992; Wycoff *et al.*, 1995; Menossi *et al.*, 1997; Elliott and Shirsat, 1998; Hirsinger *et al.*, 1999; Salvà and Jamet, 2001) (see section 1.3.5). However, little is known about promoter elements that control the expression of extensin genes. There is so much variation between extensin promoters that it is difficult to infer potentially active *cis* elements from sequence comparisons to already characterised promoters (Jamet *et al.*, 2000).

Despite this, the results presented in chapter 3 demonstrated that three oligonucleotides containing *cis*-elements from the *extA* NRR, shown to bind nuclear proteins in other plant systems (Jofuku *et al.*, 1987; Kawaoka *et al.*, 1992; Torres-Schumann *et al.*, 1996), were able to bind nuclear proteins from rape leaf extracts. Binding did not occur to an unrelated sequence from *S. calcitrans*, indicating that the interactions were sequence-specific. However, as the bound proteins were of a similar mobility, bound to each putative element with a similar affinity and were difficult to out-compete, questions were still raised over the degree of specificity of these interactions. The most effective way to determine if these regulatory elements were active *in vivo* was to analyse the activity of promoter truncations fused to a reporter gene in transgenic plants.

4.1. Construction of the promoter truncations

A series of nine internal promoter truncations within the NRR of the *extA* promoter were constructed in order to identify the exact element responsible for negative regulation of the *extA* gene and able to direct tissue-specific expression.

4.1.1. pAS41

The plasmid pAS41 had been constructed previously (Shirsat, unpublished). Figure 4.1 shows the construction of this plasmid. A 984bp *Hae* III fragment from the *extA* 5'-flanking sequence was ligated into the *Hinc* II site of the pUC18 plasmid. pUC18 is a small, high copy number *E. coli* plasmid containing portions of pBR322 and M13mp19 and carries a 54bp multiple cloning site polylinker (Yanissch-Perron *et al.* 1985).

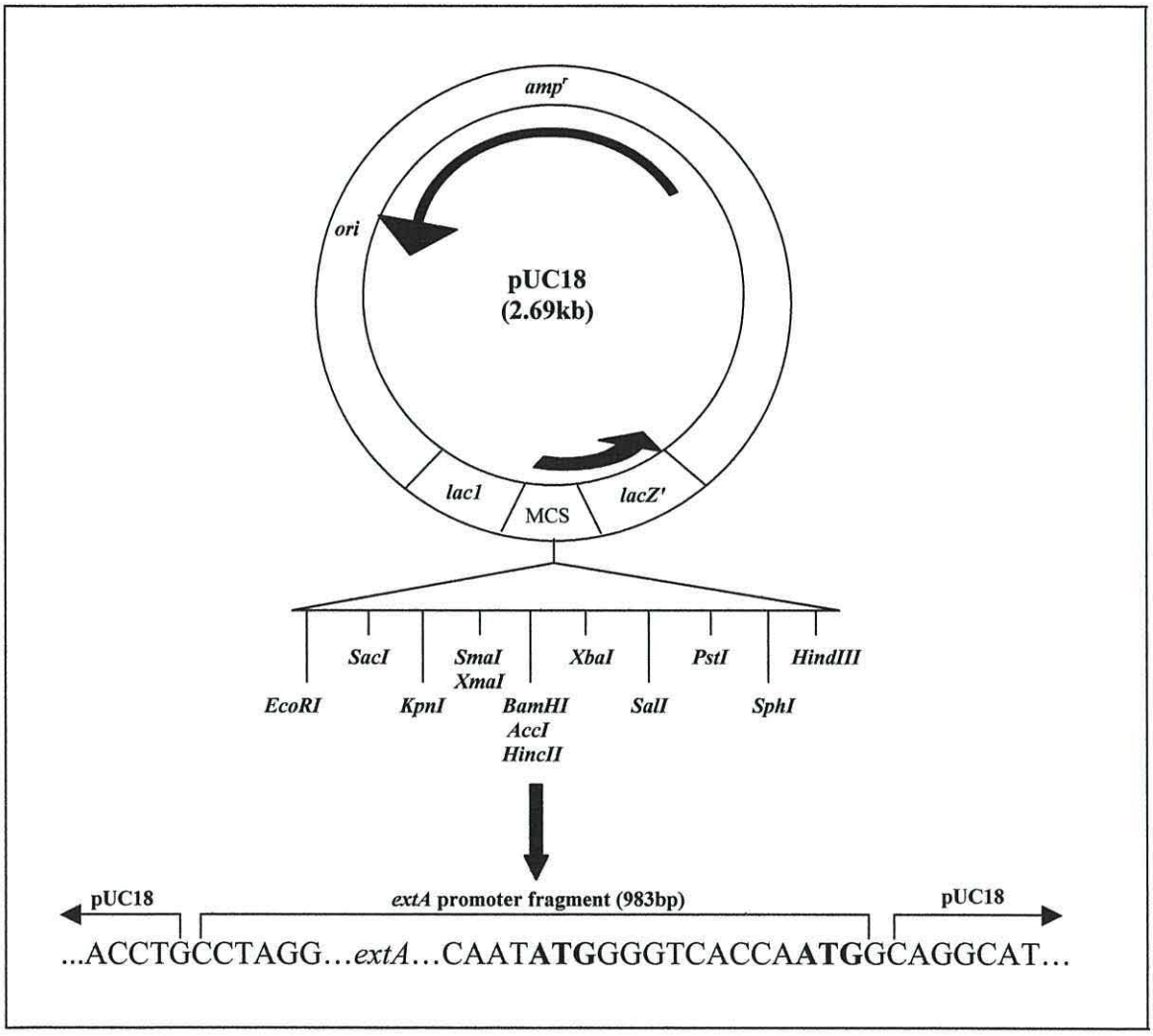


Figure 4.1. Diagram showing the construction of the plasmid pAS41 – a pUC18 plasmid containing 983bp of the *extA* extensin promoter fragment. The 983bp *Hae* III promoter fragment was excised from a genomic clone and ligated into the *Hinc* II site of pUC18. MCS = multiple cloning site.

4.1.2. PCR and cloning strategy

Figure 4.2 shows the construction of the nine promoter truncations. The plasmid pAS41 containing 940bp of 5' flanking sequence and 45bp of the coding sequence from *extA* was used as a template for PCR (section 2.11).

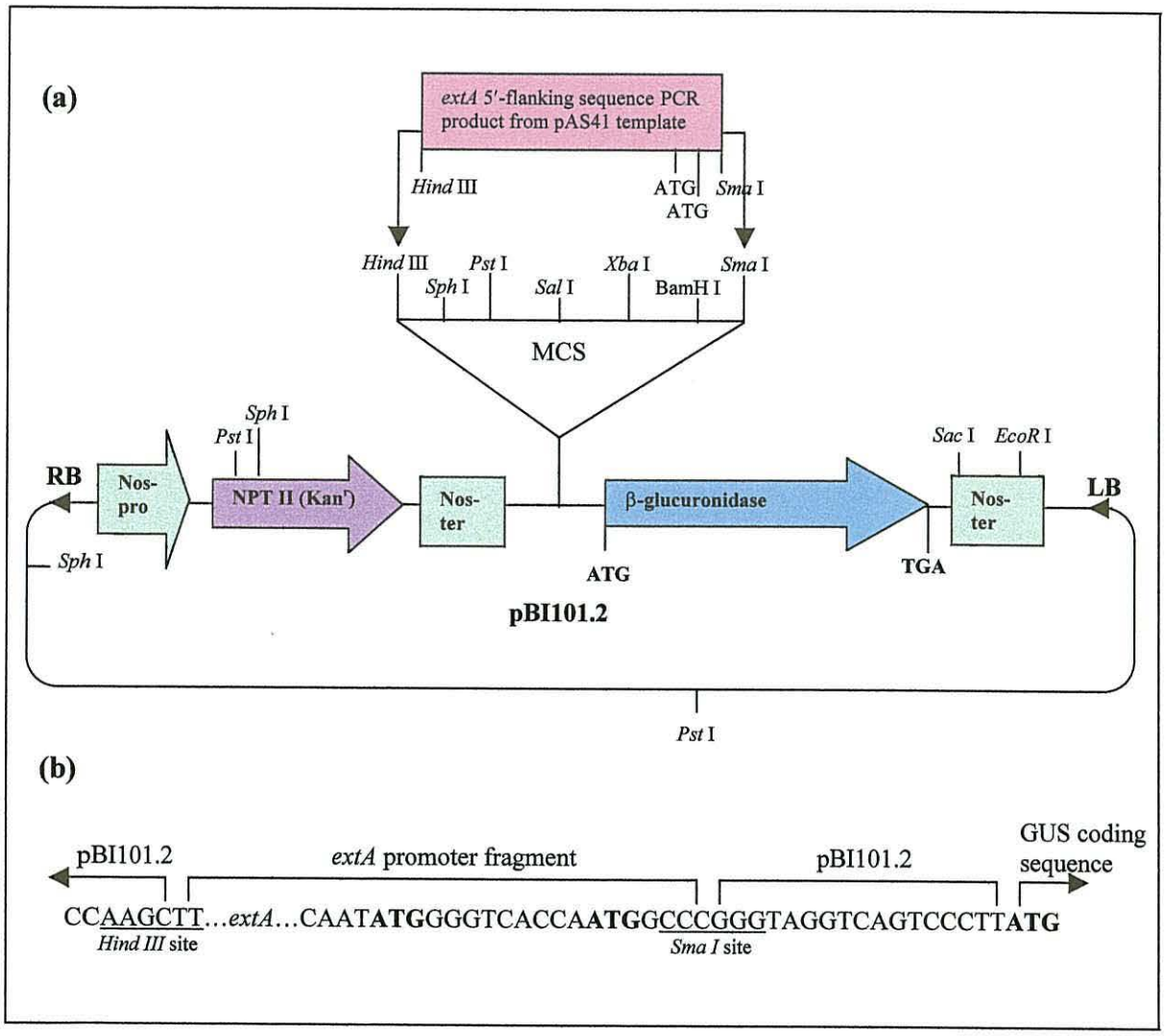


Figure 4.2. The construction of plasmids pHT2-10.

(a). Diagrammatic representation of the construction of plasmids pHT2-10. *extA* promoter truncation fragments produced by PCR from the pAS41 template were ligated into the *Hind* III and *Sma* I sites of the binary vector pBI101.2 in a translational fusion to the *E. coli* β-glucuronidase gene (GUS). The right and left border (RB and LB respectively) of the T-DNA region are shown, together with the neomycin phosphotransferase II coding region (NPTII), which confers kanamycin resistance. *nos*-pro is the nopaline synthase gene promoter and *nos*-ter is the nopaline synthase gene terminator.

(b). The fusion junction of all 3′ ends of the *extA* promoter truncations with pBI101.2, showing the presence of three in-frame ATG initiator codons (in bold).

Nine forward primers were designed to span the NRR between –664 and –433bp and to incorporate the known transcription factor binding sites (figure 4.3a and b). Forward primers were approximately 25bp apart. Restriction enzyme sites were engineered into forward and reverse primers to facilitate cloning into pBI101.2, a promoterless Bin 19-based β-glucuronidase (GUS) vector (Clontech). All forward primers contained a *Hind* III site at the 5′-end, preceded by a GC-rich tail of 6bp to aid restriction enzyme digestion. The reverse primer was common to all constructs. Here, a *Sma I* site was engineered at the 3′-end proceeded by a GC-rich sequence, as for the forward primers. The primers used were as follows:

-636*extA* (pHT2)

5' - **GTCGCAA**AGCTTGATGTCCGCGAGATTATGC - 3'

-619*extA* (pHT3)

5' - **GTCGCAA**AGCTTGCCAAATAATAGAAAATTAAGTTA - 3'

-586*extA* (pHT4)

5' - **GTCGCAA**AGCTTTAAAAATACTGCAATATGTAC - 3'

-569*extA* (pHT5)

5' - **GTCGCAA**AGCTTGTACAGTATTAGTCTTATTTCTGT - 3'

-547*extA* (pHT6)

5' - **GTCGCAA**AGCTTGTTAGTAGTGTGTTGATCAATAT - 3'

-524*extA* (pHT7)

5' - **GTCGCAA**AGCTTATCTAATATTGATGTAAAACATATA - 3'

-505*extA* (pHT8)

5' - **GTCGCAA**AGCTTCATATACAAACATTCCAACTAAAA - 3'

-481*extA* (pHT9)

5' - **GTCGCA**AAGCTTTATAGTGGATTTTGCATATGAATA - 3'

-452*extA* (pHT10)

5' - **GTCGCA**AAGCTTCATATGACATGGCTTCAATACT - 3'

R2+32*extA* (reverse primer for all constructs)

5' - **GTCGCA**CCCGGGCCATTGGTGACCCCATATTT - 3'

Underlined nucleotides constitute restriction enzyme sites. Nucleotides in bold are lost once restriction digestion has taken place and are therefore not incorporated into the new construct.

The proof-reading Taq polymerase, Tfu (MBI) was used instead of standard Taq polymerase to minimise PCR induced mutations. Tfu is a high-fidelity thermostable DNA polymerase, originally isolated from the Archaean species *Thermococcus fumicolans*. Tfu DNA polymerase exhibits a very efficient 3'-5' exonuclease proofreading activity and is stable at 92°C. Standard Taq DNA polymerase has an error rate of 1.6×10^{-5} whereas proofreading Taq has an error rate of 0.9×10^{-5}, with error rate measured according to Flaman *et al.* (1994). PCR reactions were carried out according to the manufacturer's instructions (0.5U of Tfu polymerase in 10% incubation buffer, 1μM of each primer, 0.4mM dNTPs, 100ng of pAS41 template DNA in a total volume of 25μl). The cycling conditions are shown in section 2.11.2.

Figure 4.4 shows an agarose gel containing the different sized PCR products, corresponding to the nine internal *extA* promoter truncations. Bands corresponding to PCR products of the correct size (Table 4.1) were excised from the gel and the DNA recovered by electroelution (section 2.3.4). Promoter truncations were purified by extraction with phenol, followed by ethanol precipitation (see section 2.5.1). The resulting DNA was then digested with *Sma* I (three hours at 25°C) and *Hind* III (three hours at 37°C). The restriction fragments were then isolated on an agarose gel as before and ligated into the pBI101.2 cloning vector at the *Hind* III and *Sma* I sites

(figure 4.2). The ligation mixture was used to transform *E. coli* competent cells (section 2.4.2), which were then plated onto LB media containing $50\mu g\mu l^{-1}$ kanamycin sulphate.

Positive colonies were identified using colony PCR (section 2.4.3). Colony PCR was performed using forward primers that were specific to each particular truncation and the reverse primer was common to all (see above). Figure 4.5 shows an example of a typical gel used to identify colonies containing the desired insert. In this case the plasmid containing the –505bp promoter truncation fragment was introduced into competent cells and therefore the –505*extA* forward primer was used for detection of colonies containing this fragment. Lane 1 contains the master mix (section 2.11.2) with no DNA. Any contamination from external DNA would appear as a band in this lane. Lane 2 contains 10ng pAS41 plasmid DNA in the master mix. This band, in addition to being a positive control, acts as a size marker for comparison with the other bands. Lanes 3 to 11 contain plasmid DNA from colonies picked from selective media, following the transformation event. It is clear that colony A (lane 3) and colony G (lane 9) both contain a band of the correct size and are therefore suitable for further analysis.

Minipreps were performed on positive colonies thought to contain the binary vector with the promoter truncations to yield plasmid DNA (section 2.5.1). These were then digested with *EcoR* I and *Hind* III and run on an agarose gel (figure 4.6, lanes 3-11). The resulting band sizes (table 4.1) were compared with those produced by an identical digestion of pBI101.2, without the presence of any inserts (figure 4.6, lanes 2 and 12).

4.2.3. Sequencing of the promoter truncation constructs

All constructs were sequenced to confirm that the promoter truncations had joined to the binary vector in frame (section 2.12). Sequencing was therefore carried out across the fusion junction using the following forward primer, part of the *extA* promoter sequence at position –171bp:

5' - **CGGTCGTTTTTTGTGTGT - 3'** (-171 *extA* F)

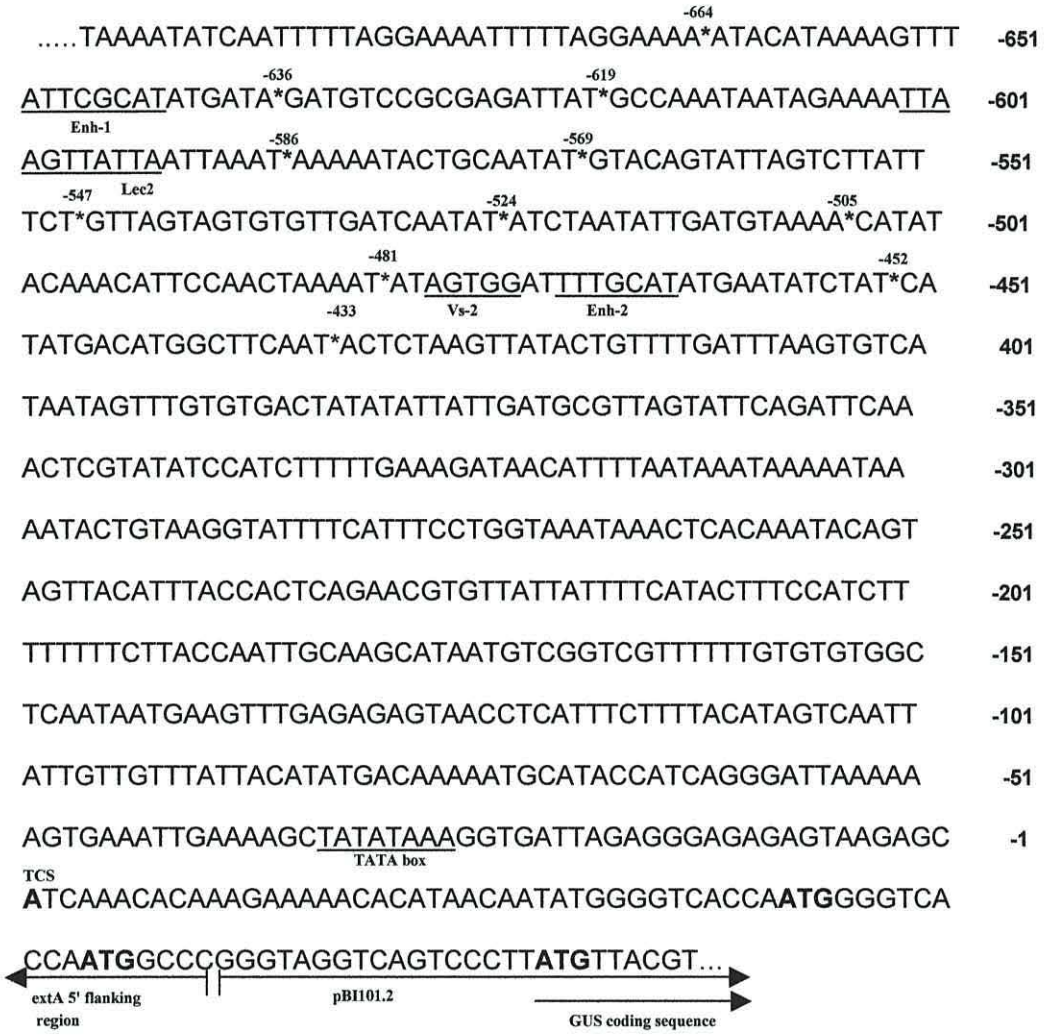
Sequencing revealed that all constructs were in frame. An example of such a sequence is shown in Appendix I.

Figure 4.3. The NRR sequence of the *extA* promoter fused to the GUS coding sequence showing the end points of the promoter truncations and a diagrammatic representation of the promoter truncation constructs.

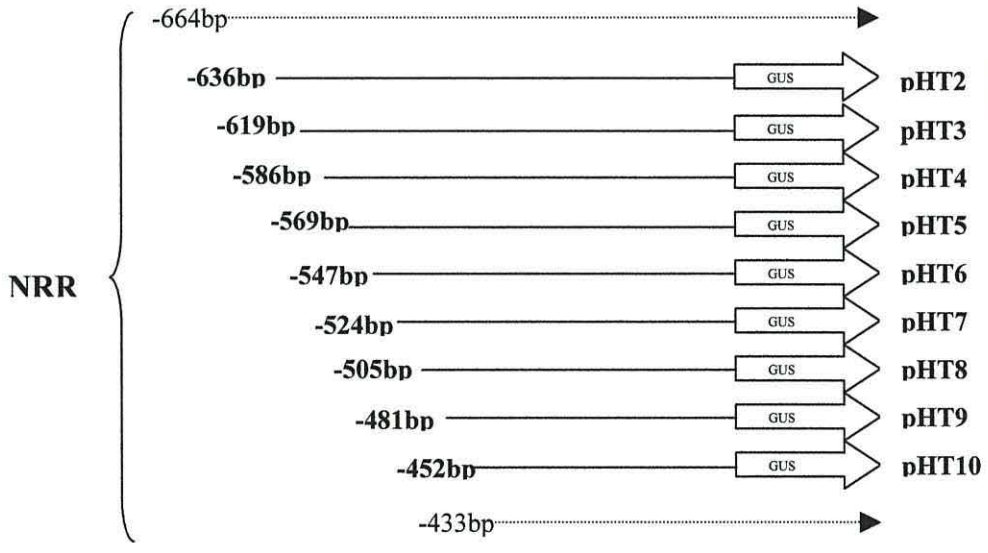
(a). The NRR sequence of the *extA* promoter fused to the GUS coding sequence showing the end points of the promoter truncations (*). Nucleotides are numbered relative to the transcription start site (TCS).

(b). A diagrammatic representation of the clones containing the *extA* promoter truncations fused to GUS, within the NRR.

(a)


-664
.....TAAAATATCAATTTTTAGGAAAATTTTTAGGAAAA*ATACATAAAAGTTT -651
-636 -619
ATTCGCATATGATA*GATGTCCGCGAGATTAT*GCCAAATAATAGAAAATTA -601
Enh-1
-586 -569
AGTTATTAATTAAAT*AAAAATACTGCAATAT*GTACAGTATTAGTCTTATT -551
-547 Lec2 -524 -505
TCT*GTTAGTAGTGTGTTGATCAATAT*ATCTAATATTGATGTAAAA*CATAT -501
-481 -452
ACAAACATTCCAACTAAAAT*ATAGTGGATTTTGCATATGAATATCTAT*CA -451
-433 Vs-2 Enh-2
TATGACATGGCTTCAAT*ACTCTAAGTTATACTGTTTTGATTTAAGTGTCA 401
TAATAGTTTGTGTGACTATATATTATTGATGCGTTAGTATTCAGATTCAA -351
ACTCGTATATCCATCTTTTTGAAAGATAACATTTTAATAAATAAAAATAA -301
AATACTGTAAGGTATTTTCATTTCCTGGTAAATAAACTCACAAATACAGT -251
AGTTACATTTACCACTCAGAACGTGTTATTATTTTCATACTTTCCATCTT -201
TTTTTTCTTACCAATTGCAAGCATAATGTCGGTCGTTTTTTGTGTGTGGC -151
TCAATAATGAAGTTTGAGAGAGTAACCTCATTTCTTTTACATAGTCAATT -101
ATTGTTGTTTATTACATATGACAAAAATGCATACCATCAGGGATTAAAAA -51
AGTGAAATTGAAAAGCTATATAAAGGTGATTAGAGGGAGAGAGTAAGAGC -1
TATA box
TCS
ATCAAACACAAAGAAAAACACATAACAATATGGGGTCACCAATGGGGTCA
CCAATGGCCCGGGTAGGTCAGTCCCTTATGTTACGT...
extA 5' flanking region
pBI101.2
GUS coding sequence

(b)


NRR
-664bp
-636bp GUS pHT2
-619bp GUS pHT3
-586bp GUS pHT4
-569bp GUS pHT5
-547bp GUS pHT6
-524bp GUS pHT7
-505bp GUS pHT8
-481bp GUS pHT9
-452bp GUS pHT10
-433bp

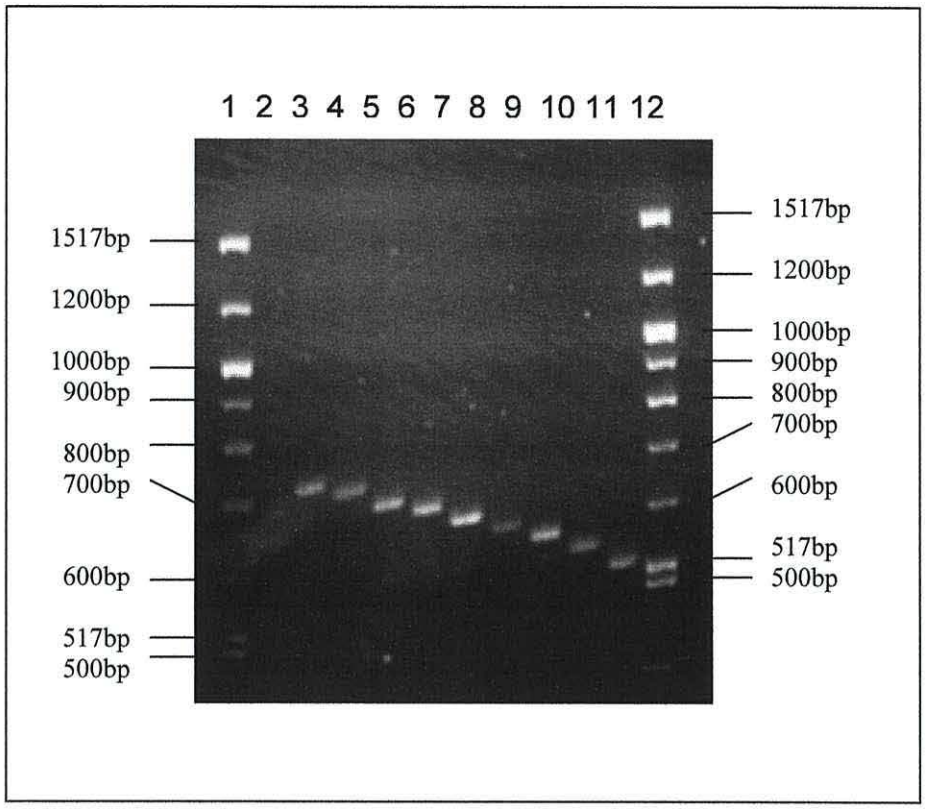


Figure 4.4. 2.5% agarose gel showing the decreasing size of PCR products from the *extA* promoter NRR used to create the nine internal promoter truncation constructs, pHT2-10. Lanes **1** and **12** contain a 100bp DNA ladder (New England Biolabs [UK] Ltd. Hitchin, Herts). Band sizes are shown. Lane **2** contains the negative control (no template DNA). Lanes **3** to **11** contain PCR products from the pAS41 plasmid template used to create the following constructs: Lane **3.** pHT2; lane **4**. pHT3; lane **5**. pHT4; lane **6**. pHT5; lane **7**. pHT6; lane **8**. pHT7; lane **9**. pHT8; lane **10**. pHT9; lane **11**. pHT10.

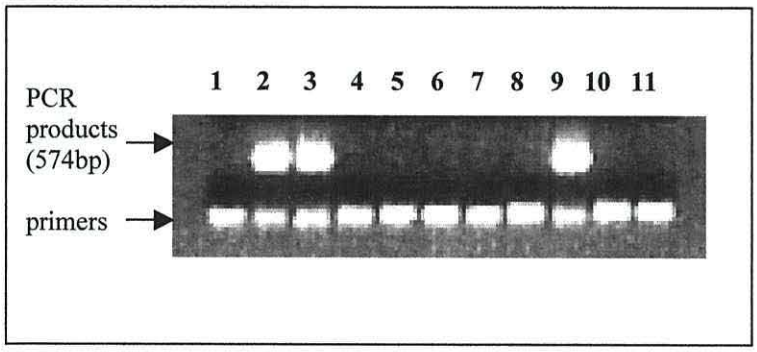


Figure 4.5. 0.8% agarose gel showing results of colony PCR performed on colonies from transformation with –505bp of promoter truncation restriction fragment. Primers used were –505*extA* and R2+32*extA*. Lane **1.** negative control – no DNA; lane **2.** positive control – 10ng pAS41; lane **3.** colony A; lane **4.** colony B; lane **5.** colony C; lane **6.** colony D; lane **7.** colony E; lane **8.** colony F; lane **9.** colony G; lane **10.** colony H; lane **11.** colony I.

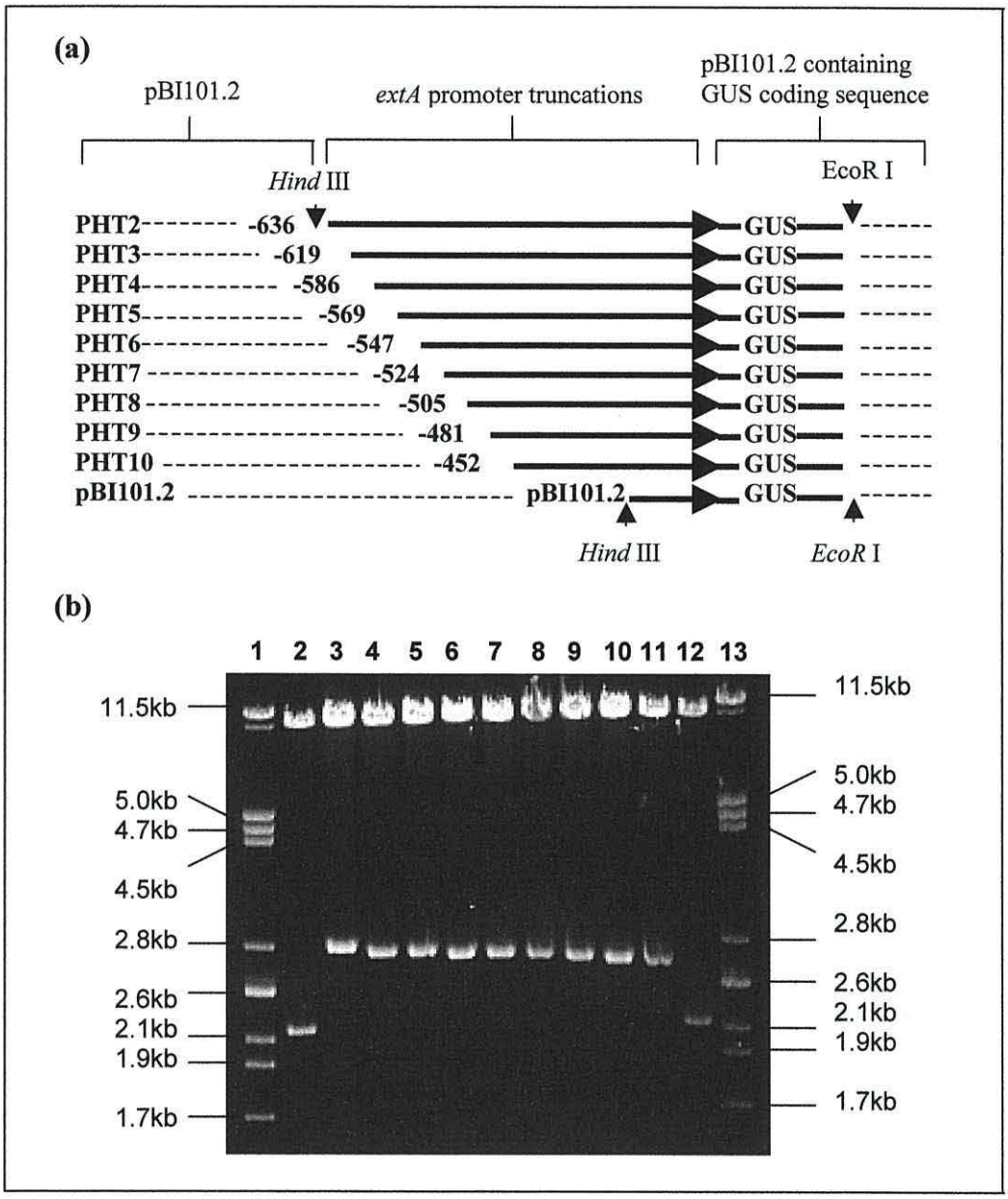


Figure 4.6. Digestion of the binary vector containing the nine *extA* promoter truncations with *Hind* III and *EcoR* I.

(a). Diagram to show the *Hind* III/*EcoR* I fragments containing the nine promoter truncations fused to GUS and the *Hind* III/*EcoR* I fragment from pBI101.2, without the presence of the *extA* promoter. The thick solid lines represent these fragments seen in (b).

(b). 0.8% agarose gel showing the gradual decrease in size of the promoter truncation constructs when the binary vector, pBI101.2 containing the promoter truncations, is digested with *EcoR* I and *Hind* III. Lanes **1** and **13** contain the DNA size marker λPst I. Band sizes are shown. Lanes 2 to 11 contain 1μg DNA restricted with *EcoR* I and *Hind* III from the following plasmids: lane **2**. pBI101.2; lane **3**. pHT2; lane **4**. pHT3; lane **5**. pHT4; lane **6**. pHT5; lane **7**. pHT6; lane **8**. pHT7; lane **9**. pHT8; lane **10**. pHT9; lane **11**. pHT10; lane **12**. pBI101.2.

4.3. Regeneration of transgenic tobacco plants

4.3.1. Transformation of *Nicotiana tabacum*

Each of the pBI101.2–based plasmids containing the *extA* promoter truncations (pHT2-10) were mobilised from *E. coli* into the *Agrobacterium tumefaciens* strain LBA4404 in a triparental mating, using the helper plasmid pRK2013 (section 2.8.1). pBI101.2 contains the *NPTII* gene (figure 4.2a), therefore *Agrobacterium* strains containing the promoter truncation constructs could be selected for by their resistance to kanamycin sulphate.

DNA was prepared from each of the resulting *Agrobacterium* strains (section 2.8.2), cleaved with *Sac* I and *Hind* III and analysed by Southern hybridisation to confirm the presence of the inserts. The results of the Southern blot can be seen in figure 4.7. The size of the fragment in each case corresponds to the *Sac* I/*Hind* III fragment in the original promoter truncation construct (table 4.1). This indicated that the triparental mating was successful and each *Agrobacterium* strain contained an intact promoter truncation construct.

Primer	5' position of *extA* promoter truncation relative to TCS	Size of PCR product (bp)	Resulting plasmid	Size of *Hind* III /*Sma* I fragments (bp)	Size of *Hind* III /Sac I fragment (kb)	Size of *Hind* III /*EcoR* I fragment (kb)
-636extA	-636	705	pHT2	689	2.164	2.874
-619extA	-619	688	pHT3	672	2.147	2.857
-586extA	-586	655	pHT4	639	2.114	2.824
-569extA	-569	636	pHT5	620	2.097	2.805
-546extA	-546	615	pHT6	599	2.074	2.784
-524extA	-524	593	pHT7	577	2.052	2.762
-505extA	-505	574	pHT8	558	2.033	2.743
-401extA	-481	550	pHT9	534	2.009	2.719
-452extA	-452	521	pHT10	505	1.980	2.690

Table 4.1. Table showing band sizes of PCR products and restriction digestion products for each construct.

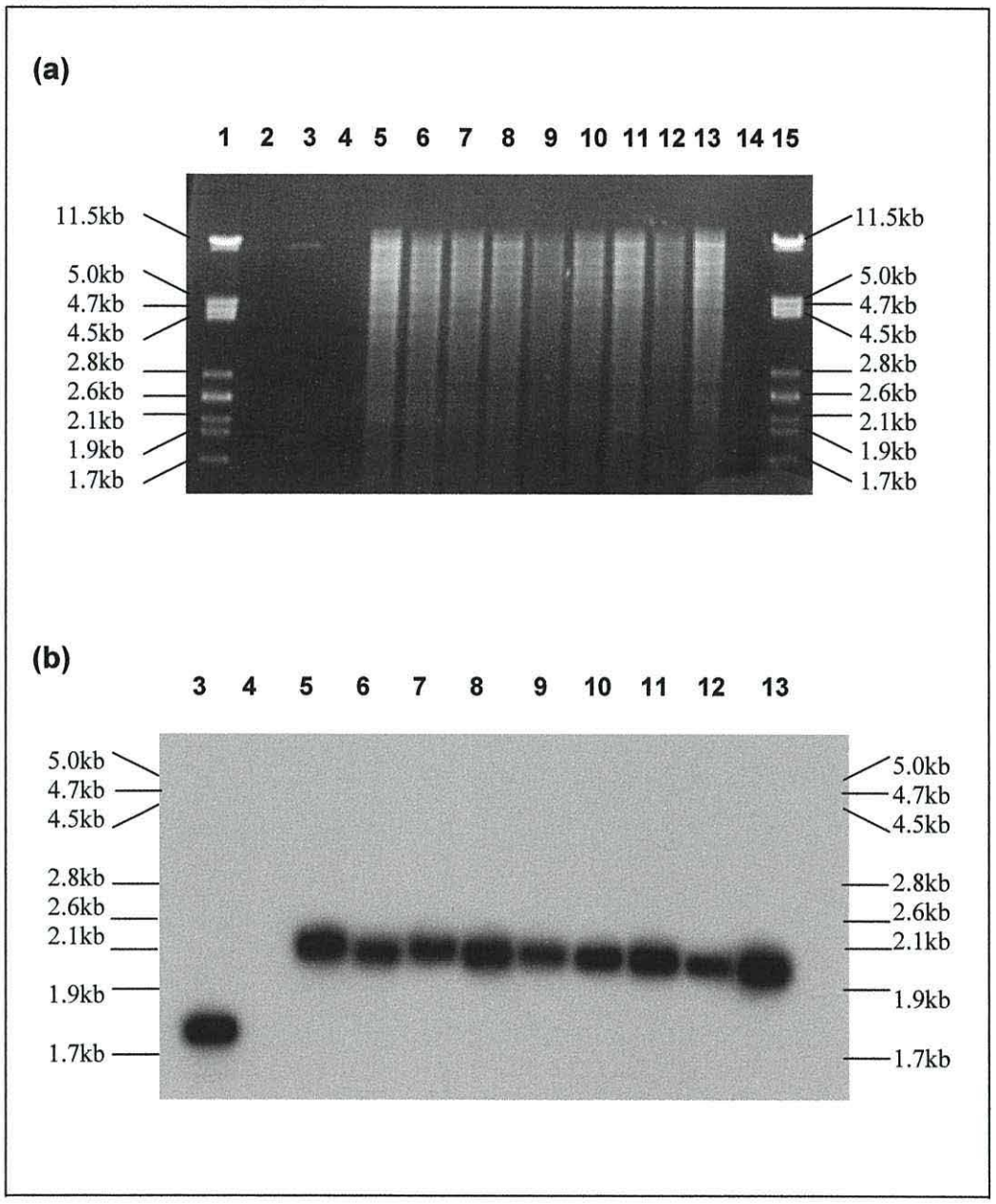


Figure 4.7. An agarose gel and autoradiograph of *Agrobacterium* DNA showing the presence of the promoter truncation constructs.

(a). 0.8% agarose gel of *Agrobacterium* genomic DNA cleaved with *Sac* I and *Hind* III from constructs containing the nine promoter truncations. Lanes 1 and 15 contain the DNA size marker *λPst I.* Band sizes are shown. Lane **3** contains the untransformed plasmid pBI101.2 cleaved with *Sac* I and *Hind* III for comparison. Lanes 5 -13 contain cleaved *Agrobacterium* DNA from the following promoter truncation clones: Lane **5.** pHT2; lane **6.** pHT3; lane **7.** pHT4; lane **8.** pHT5; lane **9.** pHT6; lane **10.** pHT7; lane **11.** pHT8; lane **12.** pHT9; lane **13.** pHT10.

(b). An autoradiograph of the above gel after Southern blotting and hybridisation to the GUS coding sequence. The positions of the size markers are shown.

The *Agrobacterium* strains containing the promoter truncation constructs were then used to transform *Nicotiana tabacum* leaf discs (section 2.8.3). The T-DNA inserted into the tobacco genome contained the *extA* promoter truncation-GUS fusion and the *NPTII* kanamycin resistance gene (figure 4.2a), therefore plants were selected for by their resistance to kanamycin sulphate.

The kanamycin concentration in the selective media was titrated from 10 to 100μgml^{-1} kanamycin sulphate to achieve optimum selection. Leaf discs were taken from wild-type plants and examined for callus formation after three weeks. Figure 4.8 illustrates that, even at low concentrations, the presence of kanamycin sulphate affects callus formation (figure 4.8b and c), although is not able to prevent it entirely. After three weeks, no callus was observed on discs where over 30μgml^{-1} kanamycin sulphate was used in the medium. However, after four weeks very small amounts of callus could be seen on discs containing 30μgml^{-1}kanamycin sulphate (figure 4.8d), presumably as a result of the antibiotic loosing its selective powers over time. This emphasised the importance of changing media regularly. Based on these results selective media included kanamycin sulphate at a concentration of 50μgml^{-1} because callus tissue was never seen at this concentration, even after four weeks.

Between 15 and 33 kanamycin-resistant transgenic tobacco plants were regenerated from each promoter truncation. Transgenic tobacco plants were numbered. For example the thirty three pHT2 regenerants were referred to as T2.1 – T2.33.

4.3.2. Identification of plants containing rearrangements

In *Agrobacterium*-mediated transformations, mutations or rearrangements in the transgene occurring prior to, during or after infection may affect expression (Shirsat *et al.*, 1989; Breyne *et al.*, 1992), therefore it is important to identify such plants and to eliminate them from the analysis. There can also be significant variation in the level of expression between individuals containing the same gene due to the point of integration in the genome (position effect) or as a result of differences in T-DNA copy number.

In order to establish the copy number of a transgene, the amount of DNA hybridising to restriction fragments within the genomic DNA would need to be compared to the amount hybridising to gene copy equivalents run on the same

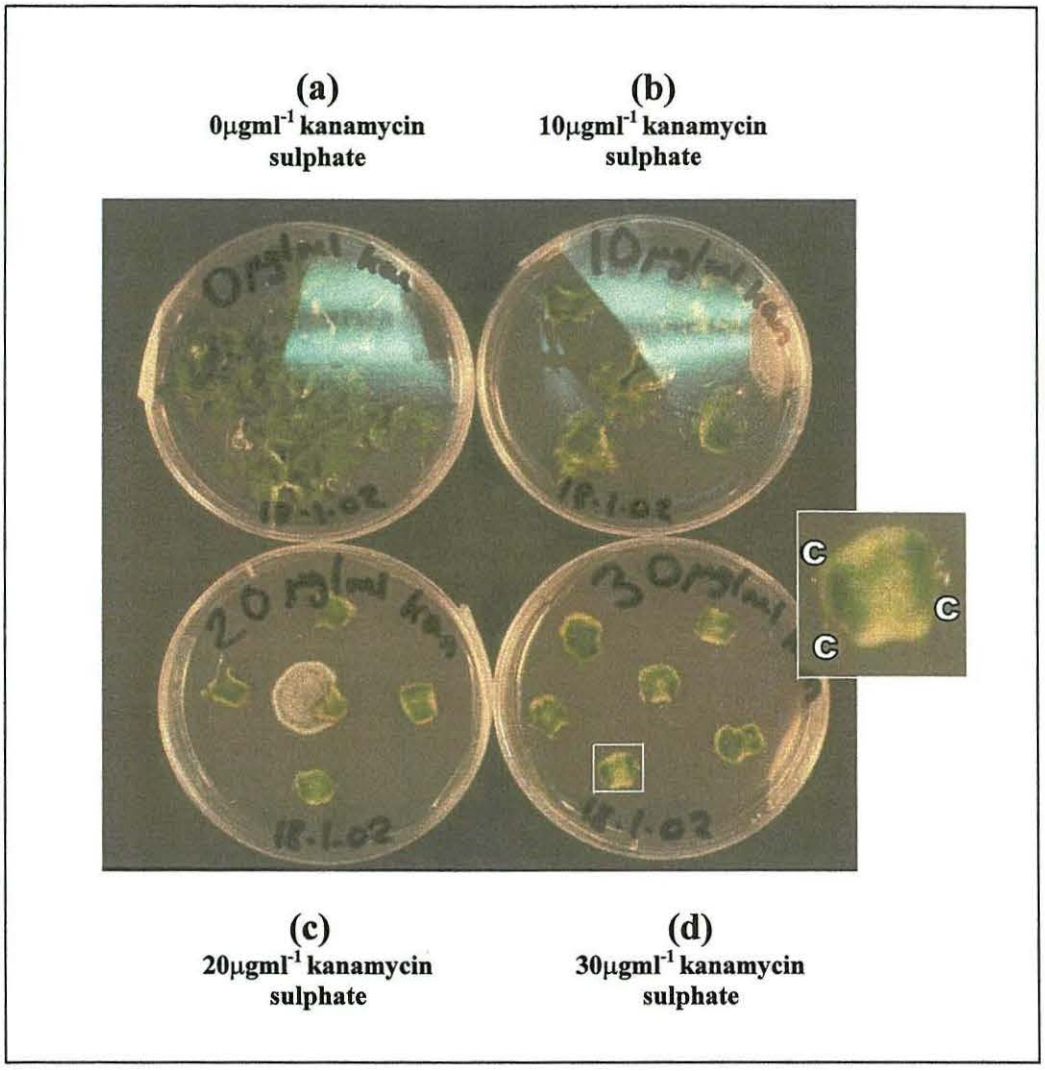


Figure 4.8. The effect of decreasing the kanamycin sulphate concentration in the culture medium on the formation of callus tissue from leaf discs of wild-type tobacco plants. Leaf discs are shown four weeks after being placed on the medium.

(a). Young plantlets have emerged from callus tissue in the absence of kanamycin sulphate.

(b). The addition of 10μgml^{-1} kanamycin sulphate to the medium reduces the time taken for callus to form.

(c). At 20μgml^{-1} kanamycin sulphate, only a very small amount of callus is visible.

(d). The enlarged leaf disc shows that there is very slight callus (C) formation around the edge of the disc at a kanamycin sulphate concentration of 30μgml^{-1}.

agarose gel. Gene copy equivalents could be obtained by cleaving pBI101.2 with *EcoR* I/*Hind* III to isolate the GUS coding sequence of the T-DNA. The amount of digested plasmid DNA for a single copy equivalent is calculated as follows:

For 1μg of genomic DNA:-

$$1 \text{ gene copy} = \frac{\text{size of standard gene fragment (bp)}}{\text{haploid size of genome (bp)}}$$

where the size of the standard gene fragment is the size of the vector containing the gene (pBI101.2 = 12200bp) and the hapoid size of the *Nicotiana tabacum* genome is 3.7×10^9bp. This figure would then be multiplied by 10 to give the gene copy number equivalent for 10μg of genomic DNA. For the equivalents of multiple gene copies, the amount of DNA loaded onto the gel would be multiplied by the number of multiple genes. Unfortunately, this method is often not very informative as it is extremely difficult to load pg quantities of DNA onto a gel with any degree of accuracy. However, the work detailed in this chapter did not involve a quantitative analysis of the transgenics, therefore the identification of gene copy number was not necessary.

To identify plants containing rearrangements, genomic DNA was isolated from each transgenic plant, as described in section 2.9. The restriction enzymes *EcoR* I and *Hind* III were used to cleave 15μg of genomic DNA and this was electrophoresed on a 0.8% agarose gel, Southern blotted and hybridised to the GUS coding sequence.

A plant containing an insert with no rearrangements produced a single band, following autoradiography, corresponding to the size of the *extA* promoter-GUS fragment of the inserted DNA. Multiple bands indicated that rearrangement had taken place. Rearrangements proved to be rare. The plants containing the most were the T9s, where 6 out of 15 transgencis had rearrangements of the transgene. This is in the range of 1-40% reported in other plant transformations (Herman *et al.*, 1990).

Two typical gels and autoradiographs are shown in figure 4.9. Here, digests of DNA from all T9 plants are shown. From the autoradiographs it is clear that plants T9.1, 9.2, 9.4, 9.5, 9.6, 9.10, 9.12, 9.14 and 9.15 (lanes 6, 7, 9, 10, 11 from

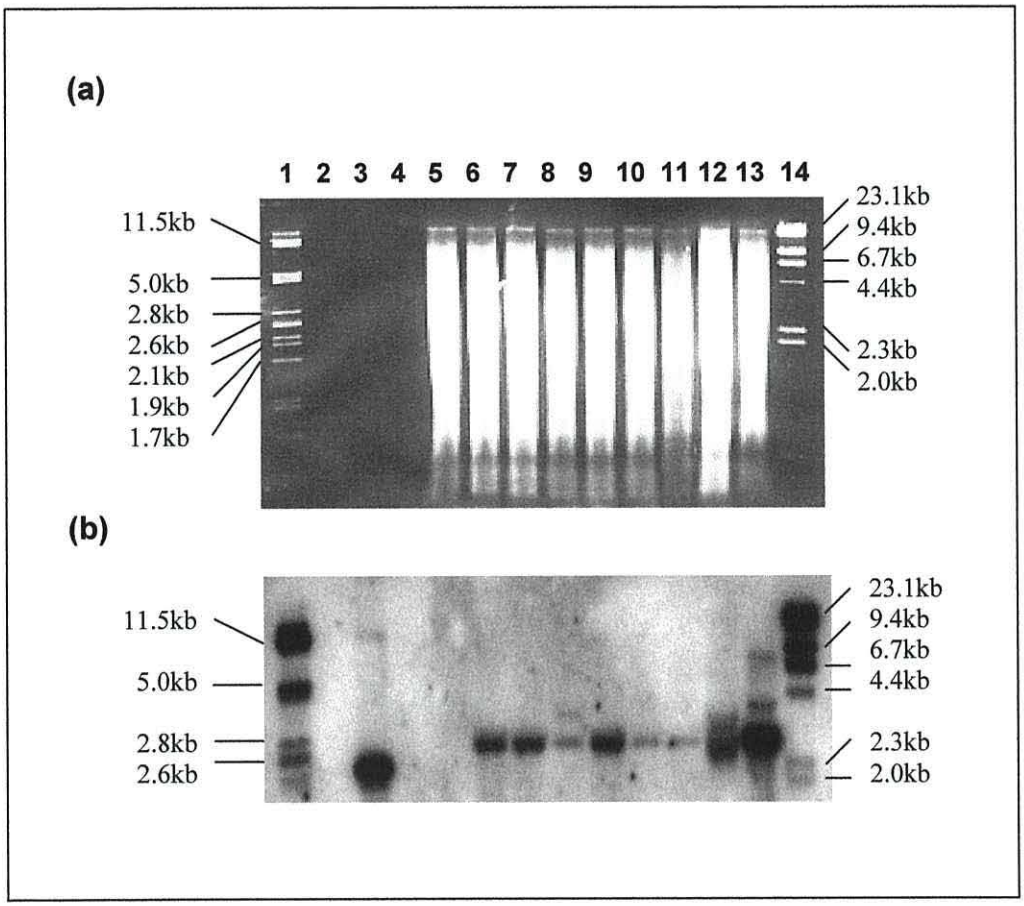
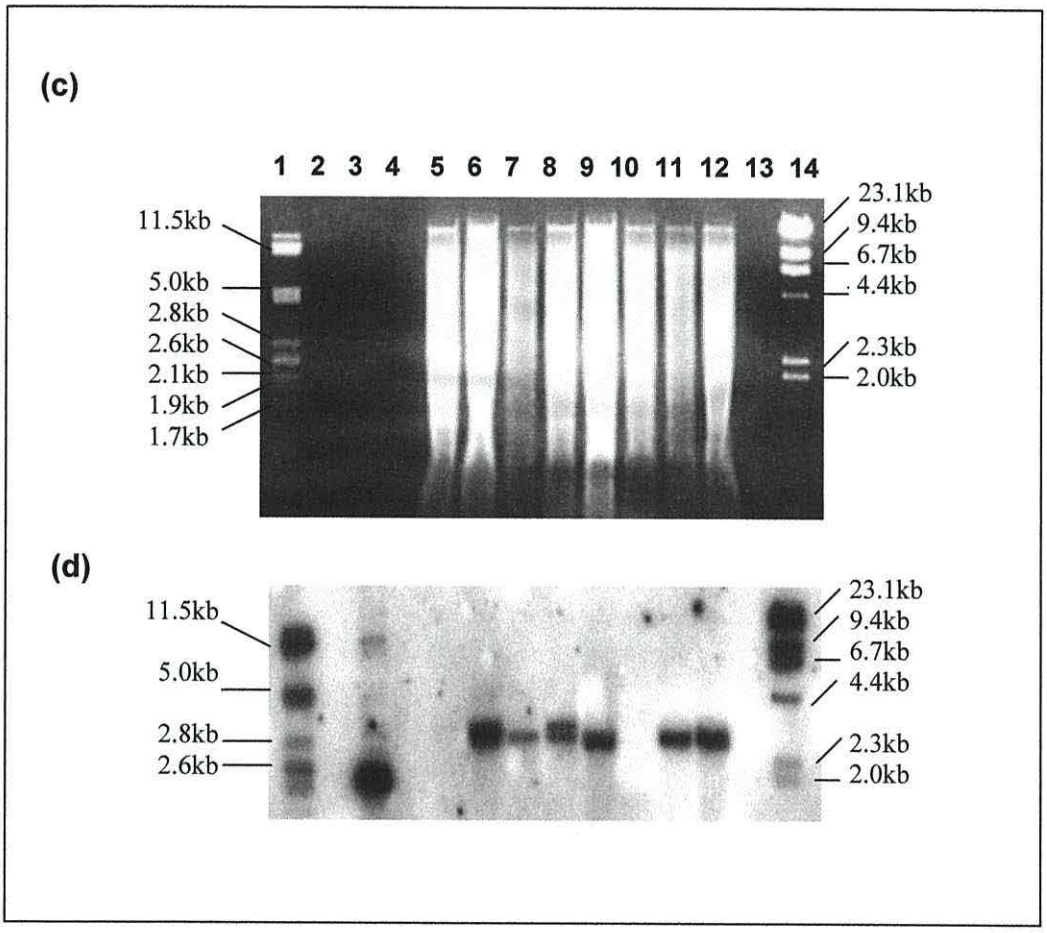


Figure 4.9. Agarose gels and autoradiographs of genomic DNA from T9 transgenic tobacco plants containing –489bp of the *extA* promoter fused to GUS, and used to identify the presence of rearrangements.

(a). 0.8% agarose gel of plant genomic DNA from T9 transgenic tobacco plants cleaved with *EcoR* I and *Hind* III. Lanes **1** and **14** contain the DNA size markers *λPst* I and *λHind* III respectively. Band sizes are shown. Lane 3 contains 40pg of pBI101.2 digested with *EcoR* I and *Hind* III for comparison. Lanes 5 - 13 contain 15μg of restricted DNA from the following plants: Lane **5.** untransformed tobacco; lane **6**. T9.1; lane **7**.T9.2; lane **8**.T9.3; lane **9**.T9.4; lane **10**.T9.5; lane **11**.T9.6; lane **12**.T9.7; lane **13**.T9.8. Lanes **2** and **4** were left blank.

(b). An autoradiograph of the above gel which was Southern blotted and hybridised to the GUS coding sequence. The positions of the size markers are shown.



(c). 0.8% agarose gel of plant genomic DNA from T9 transgenic tobacco plants cleaved with *EcoR I* and *Hind III*. Lanes **1** and **14** contain the DNA size markers *λPst* I and *λHind* III respectively. Band sizes are shown. Lane **3** contains 37pg of pBI101.2 digested with *EcoR* I and *Hind* III for comparison. Lanes 5 - 12 contain 15μg of restricted DNA from the following plants: Lane **5.** untransformed tobacco; lane **6**. T9.9; lane **7**. T9.10; lane **8**.T9.11; lane **9**.T9.12; lane **10**.T9.13; lane **11**.T9.14; lane **12**.T9.15. Lanes **2**, **4** and **13** were left blank.

(d). An autoradiograph of the above gel which was Southern blotted and hybridised with the GUS coding sequence. The positions of the size markers are shown.

figure 3.9b and lanes 7, 9, 11, 12 from figure 3.9c respectively) contain an un-rearranged *extA* promoter-GUS fusion, because a single band of the correct size is present. The difference in intensity of the bands is due to differences in copy number of the transgene. Plants T9.3, 9.7, 9.8, 9.9, 9.11 and 9.13 (lanes 8, 12 and 13 from figure 3.9b and lanes 6, 8 and 10 from figure 3.9c respectively) have rearranged copies of the transgene, as multiple bands of different sizes can be seen. These gels also show that the observed hybridisation is not due to homology between the GUS probe and tobacco DNA as untransformed tobacco DNA (lane 5, figure 3.9b) did not hybridise.

A summary of number of rearrangements per transgenic line can be seen in Table 5.1.

4.3.3. Genetic transmission of kanamycin resistance

Between 7 and 15 tobacco plants containing an un-rearranged promoter truncation construct were identified by Southern hybridisation. All of these plants exhibited normal morphology and set viable seed following self-pollination. Seeds obtained from three randomly selected plants from each promoter truncation construct were tested for kanamycin resistance by germination in the presence of $100\mu gml^{-1}$ kanamycin sulphate. The seedlings from each parent plant segregated into kanamycin resistant and sensitive phenotypes. Resistant seeds germinated normally and continued to grow in the presence of kanamycin, whereas sensitive seeds germinated but the seedlings were unable to expand and were slowly bleached.

4.4. Summary

A series of 5' internal promoter truncations of the NRR (-664 to -433bp) fused to the GUS coding sequence were made, in order to identify the *cis* element responsible for negative regulation of *extA*. These truncations terminated at positions -636, -619, -586, -569, -547, -524, -505, -481, -452 relative to the transcription start site and the resulting plasmids were named pHT2-10 respectively. The truncated fusion genes were tri-parentally mated into *Agrobacterium tumefaciens* and transgenic tobacco plants generated. Plants containing an intact, un-rearranged copy of the inserted promoter truncation were used for the analysis of *extA* expression.

5. Cellular localisation of GUS expression in the transgenic tobacco plants

5.1. Introduction

Tobacco is a suitable heterologous system to analyse the expression patterns of the *extA* promoter truncations as the *B. napus extA* gene maintains the same pattern of expression in tobacco observed in oilseed rape, in that the *extA* transcript is undetectable in tobacco laminae, is present at low levels in the stems and at high levels in the roots (Shirsat *et al.*, 1996a). In addition, *extA* does not hybridise to untransformed tobacco RNA and the observed transcript size of 1.26kb in transgenic tobacco is the same as that expected for the *B. napus extA* transcript. This demonstrates that the above expression pattern was not a result of the 1.65kb product of the endogenous tobacco extensin gene (Tiré *et al.*, 1994).

Previous *extA* promoter truncation analysis in transgenic tobacco plants led to the hypothesis that the NRR from *extA* represses expression in non-phloem cell types because transgenics containing *extA* promoter deletion-GUS reporter gene fusions lacking this region (the –433bp deletion constructs) expressed in cortical and pith parenchyma cells, in addition to the phloem (Elliott and Shirsat, 1998) (section 1.3.5 and 1.3.7). This expression pattern was dependent on the age of the tissue. The localisation of reporter gene expression is only influenced by the NRR when the tissue reaches a certain age or stage of development. This developmental regulation was seen in young stem internodes and petioles of transgenics, where GUS activity was present in all cell types regardless of the presence of the NRR, suggesting that the NRR is overridden or inactive in young tissue (Elliott, 1998).

In this chapter, histochemical analysis was performed on transgenic plants containing the nine *extA* promoter truncation-GUS gene fusions (chapter 4) in order to identify at what position in the NRR loss of negative control occurs, leading to non-specific expression of the gene fusion.

5.2. Histochemical assays

The *extA* promoter-driven GUS reporter (*uidA*) gene present in the transgenic plants encodes a hydrolase, β-glucuronidase (GUS). This enzyme catalyses the cleavage of a number of β-glucuronides, many of which are commercially available as fluorometric, spectrophotometric and histochemical substrates (Jefferson *et al.*, 1986). The substrate used in the following histochemical analysis was 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). This substrate is hydrolysed by the GUS enzyme, resulting in a blue precipitate at the site of enzyme activity (Jefferson, 1987).

Histochemical assays were performed according to Jefferson (1987), with the addition of cycloheximide in the reaction buffer at a concentration of 1.8mM (see section 2.10). Cycloheximide is a protein synthesis inhibitor and should negate any wound-induced effects on *extA*-driven GUS expression caused by sectioning. This was an important additive to the buffer because the expression of *B. napus* extensin genes is controlled by wounding (Shirsat *et al.*, 1996b).

5.3. Variation in the localisation of GUS between transgenics containing the different promoter truncations

5.3.1. Identification of transgenics for histochemical analysis

The expression levels of GUS from all the promoter truncations varied considerably between plants. This phenomenon has been reported many times and is thought to be due to differences in gene copy number, differences in the position of the transgene within the tobacco genome or methylation of introduced DNA (Shirsat *et al.*, 1989). High expressers were identified qualitatively by sectioning the stem of all transgenics (figure 5.1, section 1), incubating in X-gluc for an equal amount of time and comparing the intensity of the blue staining. Table 5.1 shows the percentage of high, medium and low expressers of transgenics that were used for analysis. Expression levels were judged qualitatively in relation to the highest expresser containing that particular construct. Using the T2 plants as an example, out of 33 transgenics produced, 3 contained rearranged copies of the transgene, 14 plants were picked for

histochemical analysis (not containing any rearrangements) and out of these 14 plants, 6 were high expressers (43%). Rearrangements and expression levels were expressed as a percentage for ease of comparison, as different numbers of plants were analysed for each promoter truncation construct.

Transgenics expressing the GUS transgene to high levels were used in further histochemical analysis.

Transgenic plant	T2	T3	T4	T5	T6	T7	T8	T9	T10
Truncation of *extA* promoter (bp)	-636	-619	-586	-569	-547	-524	-505	-481	-452
Number of transgenics produced	33	32	15	15	15	15	15	14	15
% rearrangements	3	9	0	0	0	0	0	36	0
Number of transgenics analysed	14	11	15	15	11	11	7	7	9
% High expressers	43	46	7	27	37	18	57	57	22
% Medium expressers	14	18	20	13	0	64	14	0	22
% Low expressers	43	36	73	60	63	18	29	43	56
Number of plants used for histochemical analysis	8	7	3	6	4	9	5	4	4

Table 5.1. A summary of transgene expression levels and percentage rearrangements based on histochemical analysis of transgenic plants and Southern analysis of transgenic tobacco genomic DNA (section 4.3.2). The position of each promoter truncation is given relative to the transcription start site.

5.3.2. Histochemical analysis of transgenic plants containing the nine promoter truncations

Transverse sections of internode and petiole tissues were taken from transgenics expressing the GUS gene to high levels and were used for histochemical analysis. Figure 5.1 shows the positions at which sections were taken from a mature axillary branch of a transgenic tobacco plant. As the age of the tissue has been shown to

affect the localisation of GUS (Elliott, 1998), care was taken to assay tissue of a similar age.

Although the intensity of expression varied between different transgenics containing the same promoter truncation, the pattern of GUS expression remained constant.

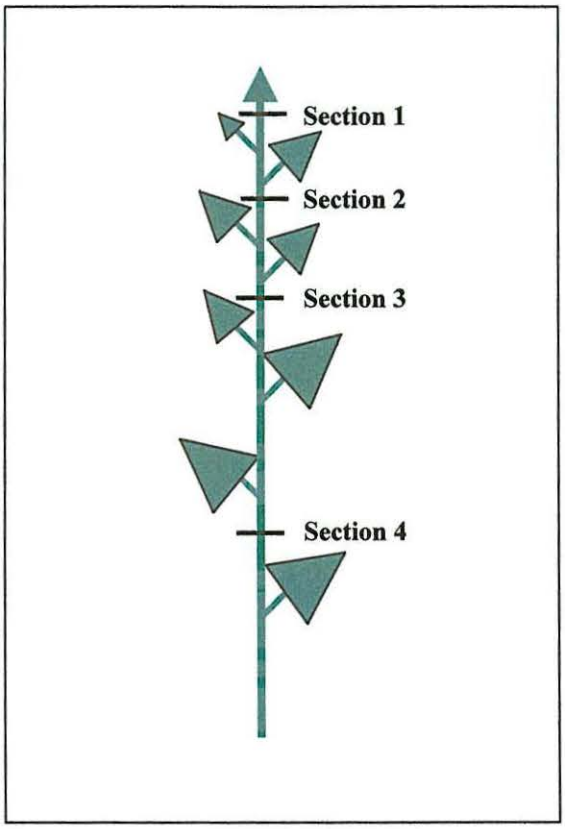


Figure 5.1. Schematic diagram of a transgenic tobacco plant to show the positions at which transverse stem sections were taken. These sections were incubated in X-Gluc to determine expression patterns of the *extA* promoter-GUS gene fusions throughout the plant.

Representative internode sections from an axillary branch of each promoter truncation are shown in figure 5.2. As previously observed (Elliott, 1998), GUS expression was seen constitutively with all promoter truncations when young tissue was assayed (figure 5.2, sections 1 and 2). In mature stem internodes of plants containing the *extA* promoter truncations extending to -636, -619, -586 and -569bp, GUS was localised in the external and internal phloem, as is the case for the full-length promoter (Elliott and Shirsat, 1998) (figure 5.2, sections 3 and 4). However,

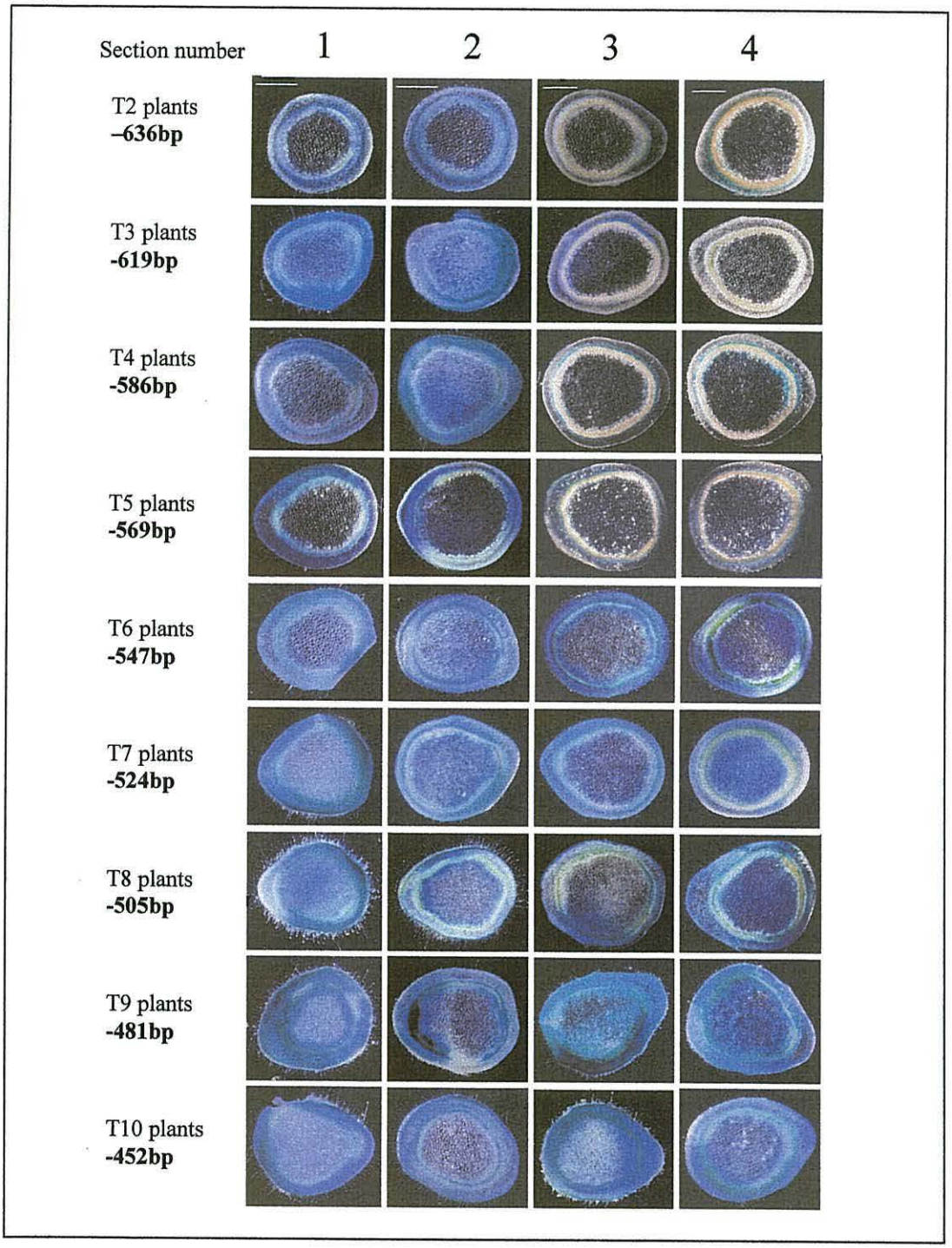
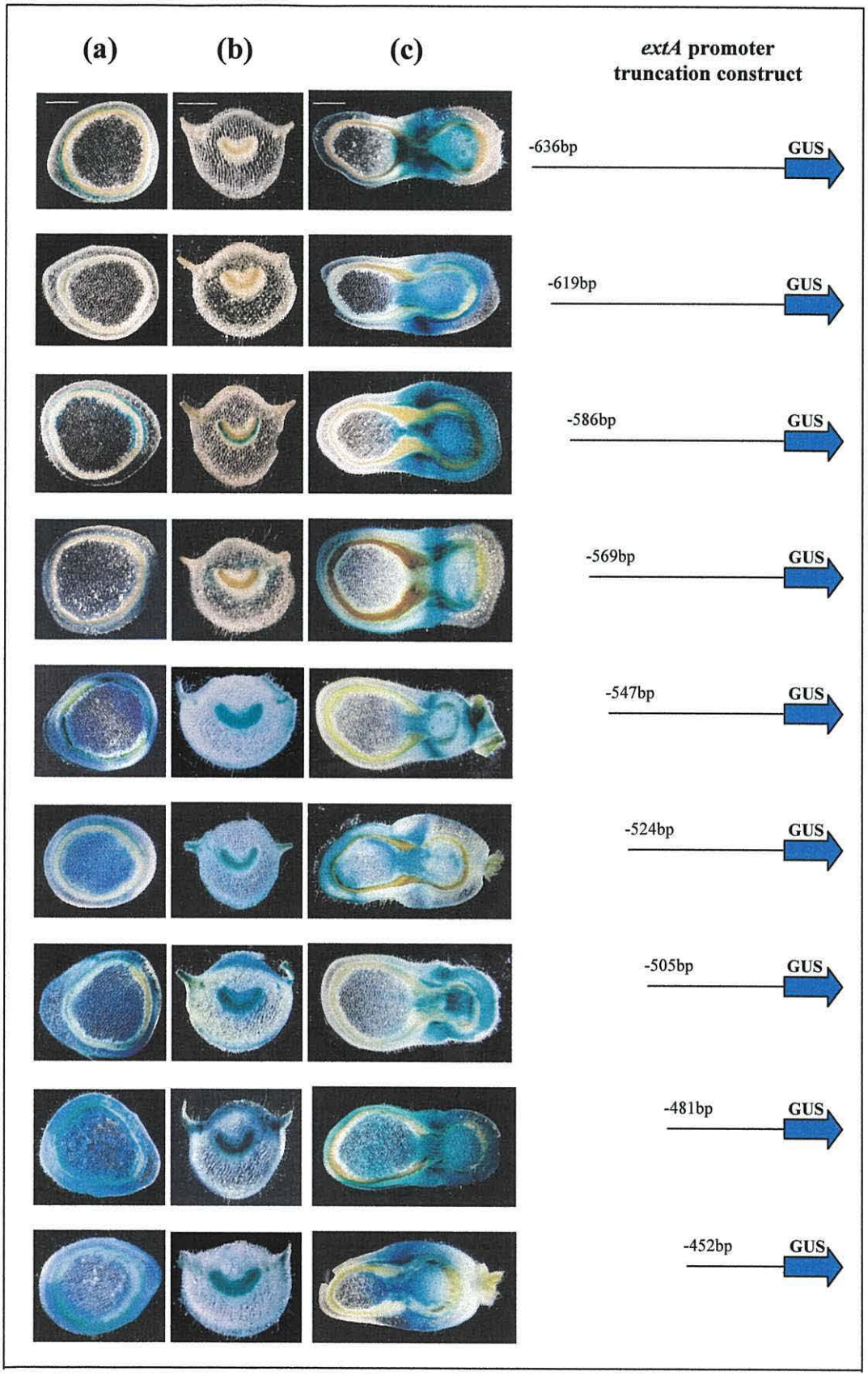


Figure 5.2. Transverse sections taken from internodes of transgenic tobacco plants containing the nine different *extA* promoter truncation constructs fused to the GUS reporter gene. Sections were taken from different aged internodes from the stem. Section 1 was taken from the youngest stem tissue and section 4 was taken from the oldest. The exact position of each internode section is shown in figure 5.1. These sections were subjected to GUS histochemical analysis in the presence of 1.8mM cycloheximide. The sections presented are representative of results obtained by examining 10 sections per plant from at least three individual transgenics from each promoter truncation. Bar = 2mm.

Figure 5.3. Histochemical analysis of **(a)** mature stem internodes **(b)** mature petiole **(c)** nodes from transgenics containing the nine *extA* promoter truncations fused to GUS. The sections presented are representative of results obtained by examining 10 sections per plant taken from at least three individual transgenics from each promoter truncation. Bar = 2.5mm


(a)
(b)
(c)
extA promoter truncation construct
-636bp
GUS
-619bp
GUS
-586bp
GUS
-569bp
GUS
-547bp
GUS
-524bp
GUS
-505bp
GUS
-481bp
GUS
-452bp
GUS

this changed significantly in plants containing the -547, -524, -505, -481 and -452 promoter truncations, where strong GUS activity was seen in the cortical parenchyma and pith of the stem internode, in addition to the phloem (figure 5.2, sections 3 and 4). This pattern is seen more clearly in figure 5.3a, in relation to a schematic of the promoter truncations.

The same pattern of expression was observed in mature petioles. Figure 5.3b shows that GUS expression in petiole from plants containing the *extA* promoter truncations extending to -636, -619, -586 and -569 is localised to the phloem, whereas expression becomes constitutive in petiole from plants containing the -547, -524, -505, -481 and -452bp promoter truncations.

This suggests that the negative regulatory element, which represses expression in non-phloem cell types, is located between -569 and -547bp of the *extA* promoter (figure 5.4).

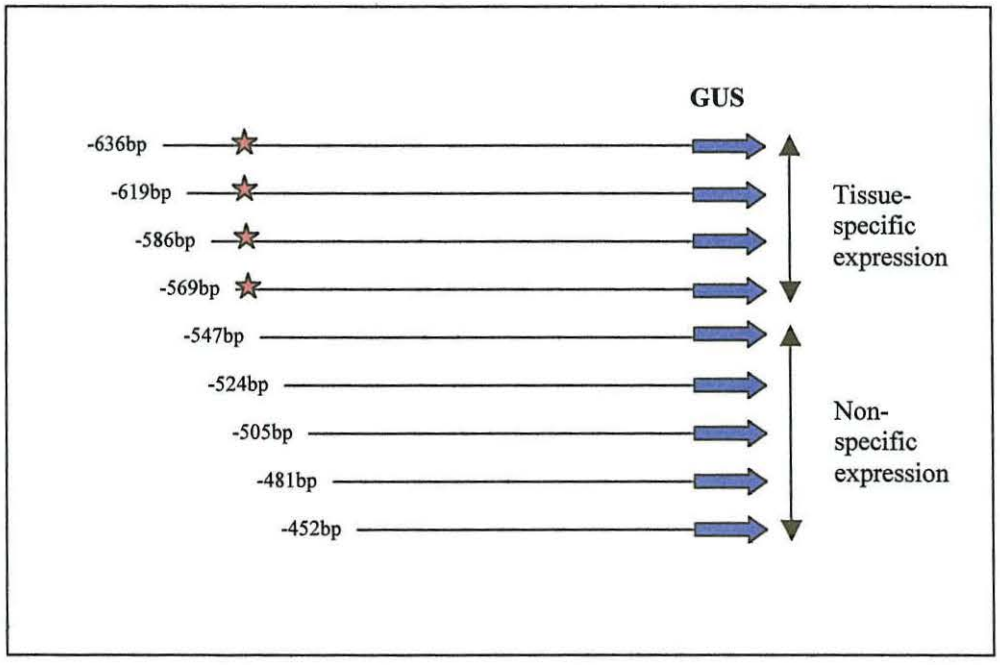


Figure 5.4. Schematic to show the position of the NRE (☆) based on histochemical analysis of the nine *extA* promoter truncation constructs in transgenic tobacco plants. GUS expression in mature petiole and internode becomes non-specific in plants containing the –547bp construct.

5.4. The effect of tensile stress on the expression of *extA* in transgenic tobacco containing the promoter truncation constructs

5.4.1. GUS is synthesised in regions of the plant under tensile stress

Figure 5.3c shows that nodal regions of transgenics express the *extA*-GUS gene fusion non-specifically. There is a particularly strong band of expression at the junction, where maximum tensile stress is exerted by the weight of the axillary branch on the main stem. This is in agreement with the findings of Shirsat *et al.* (1996a) and Shirsat *et al.* (2003). This pattern of expression is consistent throughout the promoter truncation constructs in tobacco, despite the presence of the NRE in the longer truncation constructs (-636 to -569bp). This may be because the effect of tensile stress acts to overcome repression by the NRE (Shirsat *et al.*, 2003). Such expression patterns can also be seen at the base of the infloresence, which has to carry the weight of several flower stalks and seed-pods. Figure 5.5 shows an example of expression patterns where several flower stalks join the main stem.

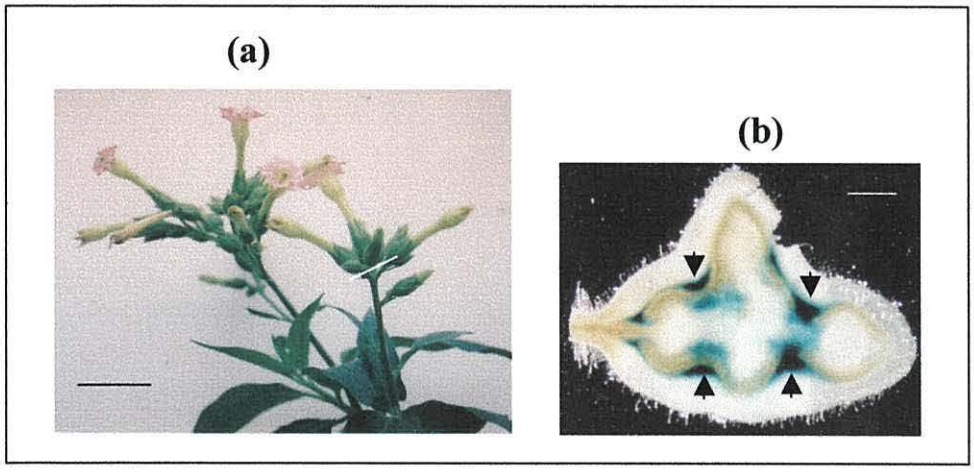


Figure 5.5. GUS is synthesised at the junction of the inflorescence with the main stem in regions that are under tensile stress.

(a) A transgenic tobacco inflorescence showing the position of the section in (b). Bar = 3cm.

(b) Transverse section at the junction of the inflorescence with the main stem. Arrows show where the strongest GUS expression is seen. This corresponds to areas experiencing the greatest tensile stress. Bar = 2.5 mm.

5.4.2. The effect of weight-loading transgenic plants containing the constructs

Work by Shirsat *et al.* (1996a) demonstrated that the application of external weights to axillary branches of transgenic tobacco plants expressing the -940bp *extA* promoter-GUS fusion gene to low levels induced expression at the node. When this was repeated with transgenics containing *extA* promoter deletion-GUS fusion constructs, weight-loading only induced a band of GUS expression in the nodes of transgenics containing 664bp of the *extA* 5′ region (i.e. containing the NRR). GUS expression was unable to be induced by weight-loading the axillary branch of low-expressing transgenics containing 433bp of the *extA* promoter, which lacked the presence of the NRR (Shirsat *et al.*, 2003). Such evidence suggests that the negative control exerted by the NRR is overcome by tensile stress.

To further test this hypothesis, transgenic tobacco plants containing the nine promoter truncation constructs were subjected to tensile stress by suspending 10g weights from the axillary branches for 36 hours, as detailed in Shirsat *et al.* (1996a). Plants expressing the transgene to low levels were chosen to ensure that the effects of weight-loading were not masked by elevated levels of GUS activity seen in a high expresser.

Based on histochemical analysis of transgenics containing the promoter truncation constructs in section 5.3.2, the expected result would be the presence of a band of GUS expression at the node of plants containing the -569bp construct, whereas weight-loading should have no effect on plants containing the -547bp construct. However, this is not the case. Despite numerous repetitions of this experiment on different plants expressing the transgene to varying levels and weight-loading different aged axillary branches with different weights, a band of GUS expression was unable to be induced by the application of tensile stress in any of the transgenics containing the nine promoter truncation constructs. An example is shown in figure 5.6, where a low-expressing transgenic containing 569bp of the *extA* promoter was weight-loaded. Clearly there is no difference between figure 5.6a and 5.6b showing a nodal cross section of a non-weighted and weighted axillary branch respectively.

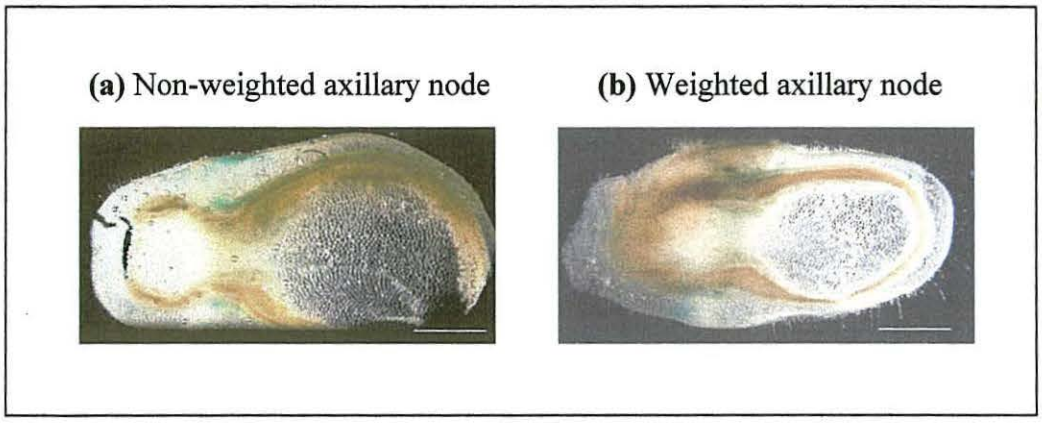


Figure 5.6. The effect of weight-loading the axillary branches of transgenic tobacco plants containing the –569bp *extA* promoter-GUS reporter construct. Bar = 2mm

5.5. Summary

Histochemical analysis of the nine *extA* promoter truncation constructs in transgenic tobacco located a negative regulatory element between -569 and -547bp. When mature internode sections were analysed, transgenics containing -636bp, -619bp, -586bp and -569bp expressed the fusion gene in the phloem whereas transgenics containing -547bp, -524bp, -505bp, -481bp, and -452bp showed non-specific expression in the cortical and pith parenchyma, in addition to the phloem. This expression pattern was repeated in mature petiole sections. As reported previously by Elliott and Shirsat (1998), GUS expression at the node was not affected by the length of the promoter truncation. Here, GUS expression remained non-specific, presumably due to the need for strengthening tissues in this area to support the weight of the developing axillary branch. However, unexpectedly, none of the nine promoter truncation constructs in transgenic tobacco responded to weight-loading.

6. EMSAs of the –569 to –547bp region of the *extA* NRR

6.1. Introduction

EMSAs performed in chapter 3 indicated that three sequences containing putative *cis*-elements (figure 6.1) were able to bind specific nuclear proteins from rape laminar extracts but not from the petiole. Under non-stressed conditions the laminar is the tissue containing the least amount of *extA* transcript (Shirsat *et al.*, 1991). As the gene appears to be under negative control, the fact that only laminar extracts produce a shifted band could indicate the presence of a repressor protein. In addition, one of the probes able to bind rape laminar nuclear proteins (*Vs-2/Enh-2*) contains a sequence showing homology to a negatively regulated element which binds a bZIP protein controlling xylem specific expression in the bean *GRP1.8* gene promoter (Torres-Schumann *et al.*, 1996; Shirsat *et al.*, 2003). However, the binding of a protein *in vitro* is not necessarily an indicator of transcription control *in vivo*. This, together with the fact that binding of a protein with similar mobility occurred to all three sequences tested, prompted further analysis using promoter truncations of the NRR fused to the GUS reporter gene.

In vivo analysis of the NRR from the *extA* promoter (chapter 5) revealed that expression of *extA* is specific to the phloem in mature internode sections from T5 plants containing -569bp of the promoter. Expression becomes non-specific downstream of -547bp in T6 plants. If the previous theory that this gene is negatively regulated is correct (Elliott and Shirsat, 1998), then a negative regulatory element controlling phloem specific expression should be present within the –569 to -547bp region of the promoter (certainly not beyond –569bp). In chapter 6, this region is investigated through sequence analysis and EMSAs.

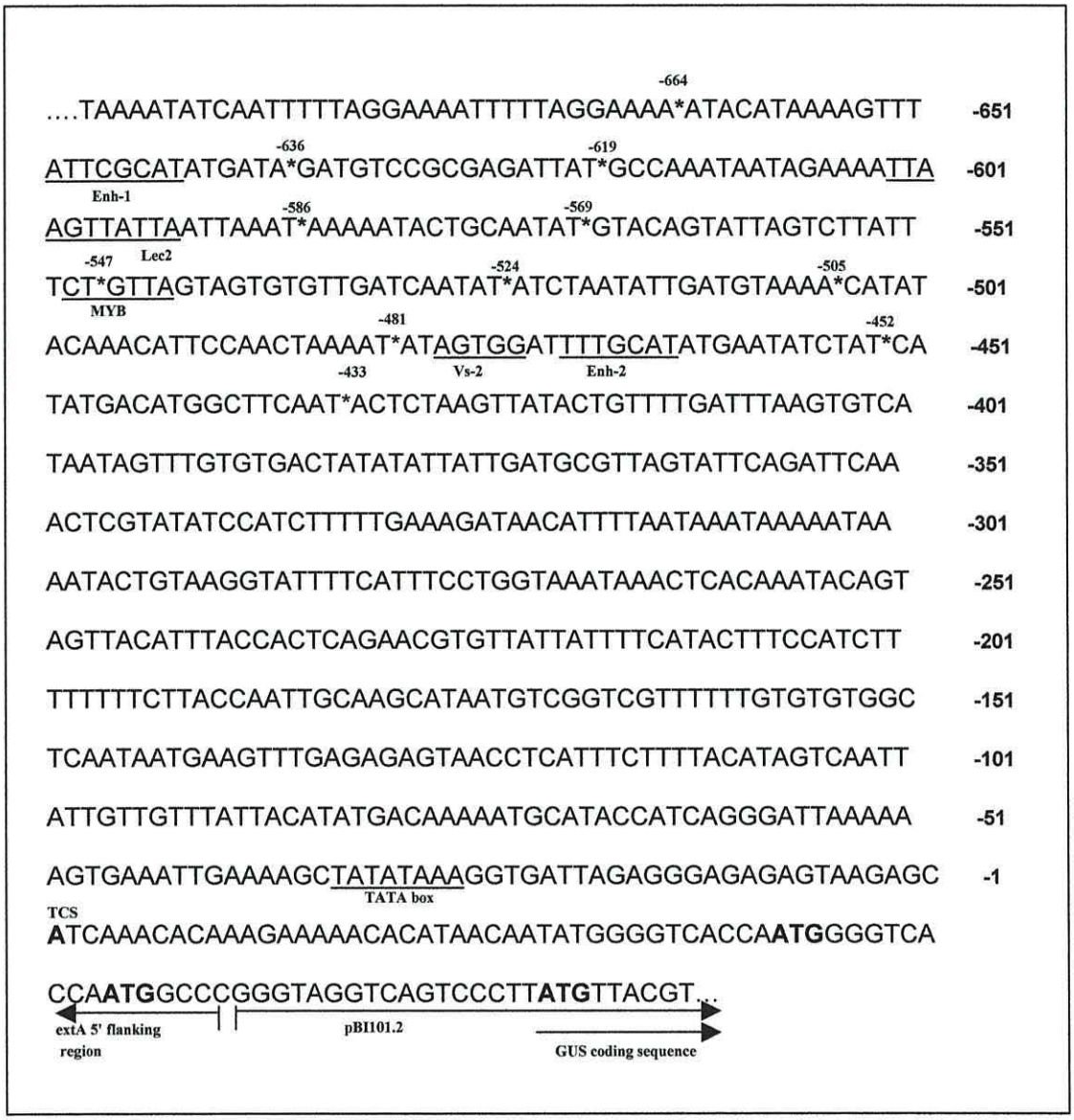


Figure 6.1. The NRR sequence of the *extA* promoter fused to the GUS coding sequence showing the end points of the promoter truncations (*) and the positions of the putative binding sequences encompassed in the oligonucleotide probes used in EMSAs (underlined). Nucleotides are numbered relative to the transcription start site (TCS).

6.2. Sequence analysis of the –569 to –547bp region

DNA sequences can be analysed for potential regulatory elements using a wide variety of programs available on the Internet. Two different methods, described below, were used to analyse the NRR of the *extA* 5′-flanking sequence. The TRANSFAC database (Quandt *et al.*, 1995) is based on a programme that searches

nucleotide distribution matrices and the PLACE database (Higo *et al.*, 1999) is based on an IUPAC consensus search.

6.2.1. The TRANSFAC database

A nucleotide weight distribution matrix is a two dimensional table with the rows of the table corresponding to the four letters of the DNA alphabet and the columns to corresponding consecutive positions of the DNA sequence (Quandt *et al.*, 1995). The MatInd program (http://transfac.gbf.de./TRANSFAC) (Quandt *et al.*, 1995), which is based on the nucleotide weight matrix method, constructs a numerical description for a binding site. The conservation of individual nucleotide positions within the matrix as a numerical value (the *Ci* value) is calculated using the relative frequency each nucleotide appears at the corresponding position. The maximum *Ci* value of 100 is reached by a position with complete conservation of one nucleotide, whereas the minimum value of 0 only occurs at a position with equal distribution of all four nucleotides. MatInd also defines a core region represented by four consecutive nucleotide positions with the highest *Ci* sum i.e. the four most conserved nucleotides.

The nucleotide distribution matrices, the *Ci* value and the core sequence generated by MatInd are used by another programme, MatInspector2.2 (http://transfac.gbf.de./TRANSFAC) (Quandt *et al.*, 1995). This programme scans the imported sequence for matches to each matrix description and utilises the TRANSFAC database to search for potential regulatory elements present in the imported sequence of interest (Heinemeyer *et al.*, 1998). TRANSFAC is a database of eukaryotic *cis*-regulatory elements and *trans*-acting factors from the original literature. The first criterion for a site to be included in TRANSFAC is protein binding and the second is function. There are only a limited number of plant transcription factor binding sites included in the TRANSFAC database.

6.2.2. The plant *cis*-acting regulatory elements database (PLACE) search

The plant *cis*-acting regulatory DNA elements database (PLACE), located at http://www.dna.affrc.go.jp/htdocs/PLACE, was developed in response to the rapid recent progress in genome research in higher plants, such as *Arabidopsis* and rice (Higo *et al.*, 1999). The PLACE database is based on the production of a consensus sequence generated with IUPAC code to represent the occurrence of two or more

nucleotides in a certain position (for example, R codes for A or G). It is a compilation of 380 motifs found in plant *cis*-acting regulatory DNA elements, extracted from previously published reports on the regulatory regions of various plant genes, and their variations in other genes or plant species. All entries are backed up by experimental evidence and the database is updated regularly as new binding sequences are discovered.

6.2.3. The identification of a potential *cis*-regulatory element using TRANSFAC and PLACE databases

The *extA* NRR sequence was analysed using MatInspector and PLACE. Both databases located a MYB core sequence (Biedenkapp *et al.*, 1988) at position –549 to -544bp in the extensin promoter (figure 6.1). This sequence is disrupted by the formation of the –547bp truncation, after which tissue specificity of *extA* is lost (section 5.3.2).

All known animal MYB proteins and at least four plant MYB proteins, ATMYB1 and ATMYB2 from *Arabidopsis* (Lüscher and Eiseman, 1990; Urao *et al.*, 1993), MYB.Ph3 from petunia (Solano *et al.*, 1995) and HvGAMYB from Barley (Gubler *et al.*, 1999), bind to sequences containing the core CNGTT(A/G). The sequence CTGTTA from the *extA* promoter shows 100% homology to the MYB core.

6.2.4. MYB proteins in plants

MYB proteins are related to the product of the avian myloblastosis oncogen (*v-myb*) and its cellular counterpart (*c-myb*) (Lüscher and Eiseman, 1990). They have been identified in many different eukaryotes and are known to act as transcriptional regulators (Grotewold *et al.*, 1994; Slabowski *et al.*, 1994). Structural analysis of MYB proteins has revealed a distinct DNA binding (MYB) domain, *trans*-activation domain and positive and negative regulatory regions (reviewed by Lüscher and Eisenman, 1990). However, although MYB proteins share the homologous MYB domain, differences in their base-contacting residues and in the overall context of their MYB domains produce distinct DNA binding-specificities in different members of the family (Martin and Paz-Ares, 1997).

The CNGTTR sequence is one of two core sequences known to bind plant MYB transcription factors (Solano *et al.*, 1995). The nuclear protein MYB.Ph3 from *Petunia hybrida* recognises both consensus sequences and is able to activate transcription from promoters in other organisms containing these binding sites (Solano *et al.*, 1995). MYB.Ph3 has the general properties of transcriptional activators (Ptashne, 1988) but under certain conditions is able to repress transcription, as in animal c-MYB (Sakura *et al.*, 1989). It is thought to be involved in the regulation of phenylpropanoid biosynthetic genes (Solano *et al.*, 1995). Many other MYB proteins are known to play an important part in the control of phenylpropanoid metabolism (reviewed by Martin and Paz-Ares, 1997). The presence of a MYB core sequence in the *extA* NRR could therefore be significant because a number of phenylpropanoids act as defensive agents against biotic and abiotic stresses. In addition, another branch of phenylpropanoid metabolism produces the precursors for lignin and other soluble phenolics. These soluble phenolics can serve as signalling molecules and antioxidants but also as cell wall cross-linking agents.

Deletion analysis of the eucalyptus cinnamyl alcohol dehydrogenase *EgCAD2* promoter performed in tobacco and poplar allowed the identification of a promoter region essential for vascular cambium/xylem-specific expression (Lauvergeat *et al.*, 2002). This region was shown to contain a sequence showing high homology to a MYB core sequence. Xylogenesis comprises four major steps, including cell division, elongation, cell wall thickening and programmed cell death (Fukuda, 1996). As mentioned previously, lignin biosynthesis involves the general phenylpropanoid pathway leading to common precursors of a wide range of end products involved in various aspects of plant development and defence. MYB proteins may therefore be an important control feature of the transcription of structural protein genes.

Many other functions have been demonstrated for different members of the MYB protein family. A well-established role for plant MYB genes is in the control of cell shape. For example, the MYB gene *MIXTA* from *Antirrhinum* has been shown to be essential for controlling the shape of petal epidermal cells and the *Arabidopsis* MYB gene *GL1* has been shown to be essential for the differentiation of hair cells in some parts of the leaf and stem (Oppenheimer *et al.*, 1991; Noda *et al.*,

1994). One of the main roles of MYB proteins in animals is in cellular proliferation. Such a role for MYB proteins has also been suggested for plants (Chung and Parish, 1995; Ito *et al.*, 1998; Tréhin *et al.*, 1999). In addition, there is a subclass of MYB proteins (including ATMYB1 from *Arabidopsis*) that bear greater structural similarity to vertebrate MYB proteins than to other plant MYB proteins. This structural similarity could reflect similar cellular functions (Martin and Paz-Ares, 1997). The CTGTTA MYB core sequence located in the NRR is located primarily in vertebrate promoters. Finally, a role in response to abiotic stress has been proposed for ATMYB2 from *Arabidopsis*, as the gene coding for this transcription factor, *AtMyb2*, is induced by drought stress, high salt conditions and the hormone abscisic acid (Urao *et al.*, 1993).

At least 40 MYB proteins have been identified in *Petunia hybrida* (Avila *et al.*, 1993) and more than 93 genes encoding MYB transcription factors have been identified in *Arabidopsis* (Meissner *et al.*, 1999). However, only approximately 10% of the plant MYB genes have been attributed a function (Martin and Paz-Ares, 1997), therefore the full extent of the participation of this transcription factor gene family in plant growth and development is only just being realised.

Taken together, the involvement of the MYB transcription factor with genes involved in growth regulation, vascular specific expression and plant defence points to a possible involvement of MYB with extensin regulation, a gene which has been shown to be involved in all three responses. It was therefore decided to investigate this putative binding site further by performing EMSAs on synthetic oligonucleotides designed from this region.

6.3. Detection of DNA-protein interactions in the –584bp to –535bp region of the *extA* promoter

6.3.1. Design of oligonucleotides to be used as probes in EMSAs

Three oligonucleotides were designed to establish whether the MYB core motif represented a binding site and if interactions were detected, to determine whether these interactions were tissue specific. The oligonucleotide designated *Myb* was designed to span the MYB core and to encompass several nucleotides on either side.

½Myb was designed to disrupt the MYB core sequence so that if specific binding occurred to *Myb* but not *½Myb* then the involvement of this sequence in binding a nuclear protein would be confirmed. The *½Myb* sequence also spans the whole region –569 to –547bp, which, according to *in vivo* analysis, should contain an element negatively regulating extensin expression (section 5.3.2). If the *½Myb* probe encompassing this region bound a specific protein then the involvement of the MYB core would be unlikely and involvement of other sequences within the region could be assumed. Both oligonucleotides were 25bp in length. A third oligonucleotide, *LongMyb,* was designed to encompass both sequences, including an additional 10bp towards the 5′ end of the promoter. The purpose of this was to allow for the fact that sequences flanking the core have been found to be important in controlling binding affinity (Williams *et al.*, 1992; Izawa *et al.*, 1993; Urao *et al.*, 1993; Yanagisawa and Schmidt, 1999). In addition, a larger oligonucleotide would present a greater number of bases to possible binding factors. The oligonucleotide sequences can be seen in table 6.1, with the MYB core sequence shown in bold. The positions of the oligonucleotides in the region of the *extA* NRR important for directing vascular specific expression are shown in figure 6.2.

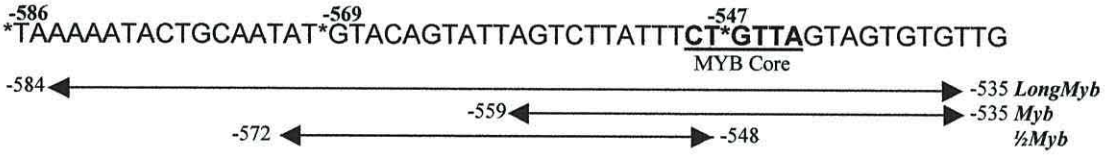


Figure 6.2. Diagram showing the position of bases in a section of the NRR used to synthesise double stranded oligonucleotides for use as probes in EMSAs. The region upstream of –547bp was shown to direct vascular specific expression of the *extA* gene when *in vivo* experiments with *extA* promoter truncations fused to GUS were performed (section 5.3.2). The MYB core sequence, disrupted in T6 plants containing the -547bp truncation, is underlined.

Probe	Oligo-nucleotide	Sequence (Upper strand is shown 5′ to 3′ and complimentary strand is shown 3′ to 5′)	Position in *extA* promoter relative to the TCS
LongMyb	LongMyb+	AAAATACTGCAATATGTACAGTATTAGTCTTATTT**CTGTTA**GTAGTGTGT	-584 to -535
	LongMyb-	TTTTATGACGTTATACATGTCATAATCAGAATAAA**GACAAT**CATCACACA	
½Myb	½Myb+	TATGTACAGTATTAGTCTTATTT**CT**	-572 to -548
	½Myb-	ATACATGTCATAATCAGAATAAA**GA**	
Myb	Myb+	AGTCTTATTT**CTGTTA**GTAGTGTGT	-559 to -535
	Myb-	TCAGAATAAA**GACAAT**CATCACACA	

Table 6.1. Oligonucleotides used as probes for EMSAs with the MYB consensus sequence shown in bold.

6.3.2. The binding reaction

Binding reactions were carried out as before (see section 2.14.5.2 and 3.2.4) with fresh nuclear protein extracts from rape laminar and petiole and tobacco internode, petiole and laminar. Again, the non-specific competitor used in the reaction mixture was an unrelated 25bp sequence from *Stomoxys calcitrans* (see section 3.2.4.), unless stated otherwise.

6.3.2.1. Binding patterns of nuclear protein extracts from oilseed rape

All three oligonucleotides, *LongMyb, ½Myb* and *Myb* bound a rape laminar nuclear protein of similar mobility designated B1 (figure 6.3, lanes 1, 2 and 3 respectively), although the intensity of the shifted bands was not identical. The strongest B1 band was produced when *LongMyb* was used as a probe (lane 1). A second shift of lower mobility designated B2 was also seen when *LongMyb* was used as a probe (figure 6.3, lane 1). This oligonucleotide encompasses the sequences in both *Myb* and *½Myb* plus an additional 10bp in the 5′ direction.

The addition of a 150-fold molar excess of cold homologous oligonucleotide (CHO) competitor caused a slight decrease in intensity of the B1 shifted band with *½Myb* and *Myb* probes, although the decrease in intensity was the same when a 150-fold molar excess of cold non-homologous oligonucleotide (CN-HO) was added to the binding reaction mixture (compare lanes 5 and 8 and 6 and 9, figure 6.3). These interactions are therefore unlikely to be specific. However, the addition of CHO to

the binding reaction mixture before incubation with the *LongMyb* probe greatly reduced the intensity of the main shift, B1, and abolished the B2 complex completely (figure 6.3, lane 4). The addition of CN-HO to the binding reaction mixture had no effect on the formation of B1 or B2, demonstrating the specificity of this interaction (figure 6.3 lane 7).

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Probe	*LM*	*½M*	*M*	*LM*	*½M*	*M*	*LM*	*½M*	*M*
Homologous competitor				+	+	+			
Non-homologous competitor							+	+	+

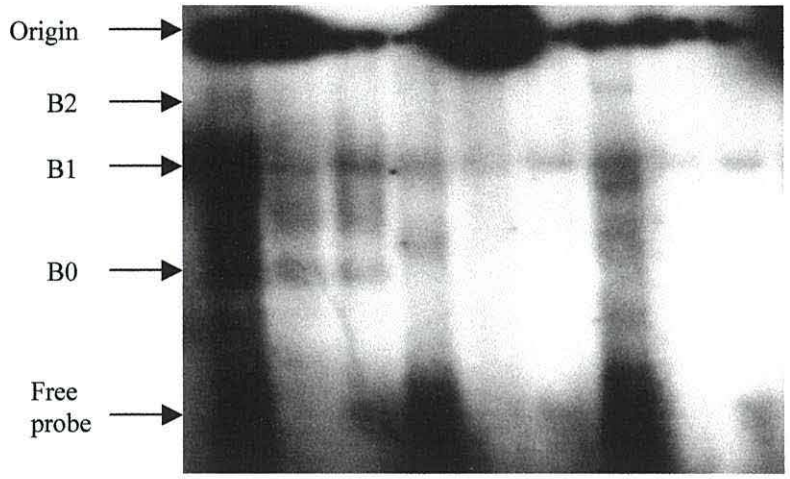


Figure 6.3. EMSA showing the complexes formed with the three Myb probes, *LongMyb, ½ Myb* and *Myb* respectively when rape laminar nuclear protein was used in the binding reaction mixture. The complex B2 formed with the *LongMyb* probe can be out-competed with a 150-fold molar excess of cold homologous oligonucleotide (CHO) competitor (lane 4). This excess also greatly reduces the formation of the *B1-LongMyb* complex (lane 4). Cold non-homologous oligonucleotide (CN-HO) competitor (a 25p sequence from an *S. calcitrans* promoter) does not compete with the *LongMyb* probe for binding (lane 7). The addition of CHO to the binding reaction mix does not cause a reduction in the intensity of the B1 shift when the shorter Myb probes were used. Lanes 1-9 contain 5µg of rape laminar extract. The name of the probe used in each reaction is given in italics. **1.** *LongMyb*. **2.** *½Myb*. **3.** *Myb*. **4.** *LongMyb,* CHO. **5.** *½Myb,* CHO. **6.** *Myb*, CHO. **7.** *LongMyb*, CN-HO. **8.** *½Myb*, CN-HO. **9.** *Myb*, CN-HO.

The strength of binding in the B1 and B2 complexes bound by *LongMyb* was unable to be reduced when a 150-fold molar excess of *½Myb* (figure 6.4, lane 3) was used as non-homologous competitor DNA. This implies that although *½Myb* bound a protein of similar mobility to the B1-*LongMyb* complex, this protein either has different binding properties with different sequences or the interactions involve different proteins. However, when *Myb* was used as competitor DNA, a reduction in intensity of B2 was observed (figure 6.4, lane 2). This implies that the nucleotides present in the *Myb* probe affect binding of the B2 complex, but are unable to compete completely for binding.

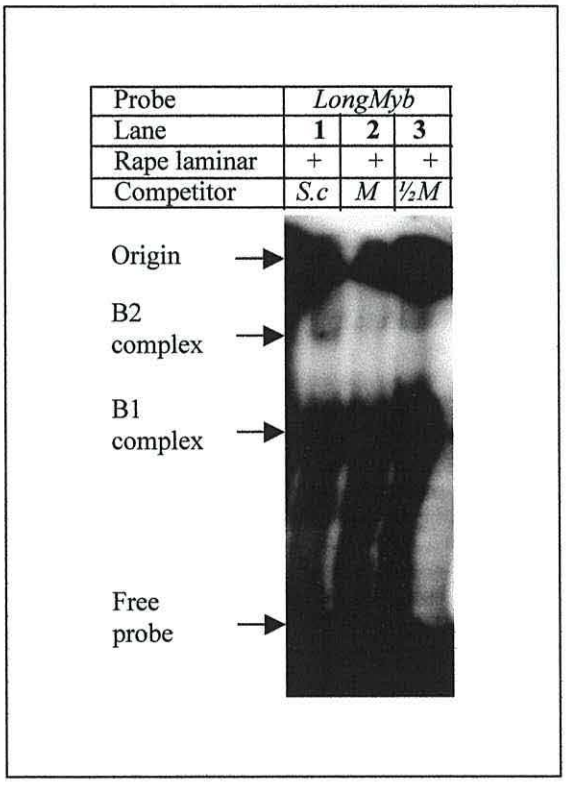


Figure 6.4. EMSA to show that an unlabelled *S. calcitrans* sequence (*S.c*) or an unlabelled *½Myb* (*½M*) oligonucleotide cannot compete for binding with the B1 and B2 complexes to the *LongMyb* fragment, whereas partial competition of B2 occurs when *Myb* (*M*) is used as a competitor. Lanes 1-3 = a 150-fold molar excess of unlabelled **1.** *S. calcitrans* sequence, **2.** *Myb* oligonucleotide, **3.** *½Myb* oligonucleotide. Each binding reaction mixture contained 5µg of rape laminar nuclear protein extract probed with radio-labelled *LongMyb*.

Figure 6.5 shows that the protein interaction is tissue-specific. Binding occurred to all three Myb oligos when rape laminar nuclear extracts were used but not when rape petiole extracts were used. Figure 6.5 also revealed that the mobility of the bound protein in the acrylamide gel matrix to the three Myb oligos was very similar to that of the protein bound to the *Vs-2/Enh-2* oligonucleotide, investigated in chapter 3 (compare lane 2 with 4, 6 and 8). It is therefore possible that the same protein binds to all sequences, although binding to *Myb* and *½Myb* appears to be non-specific.

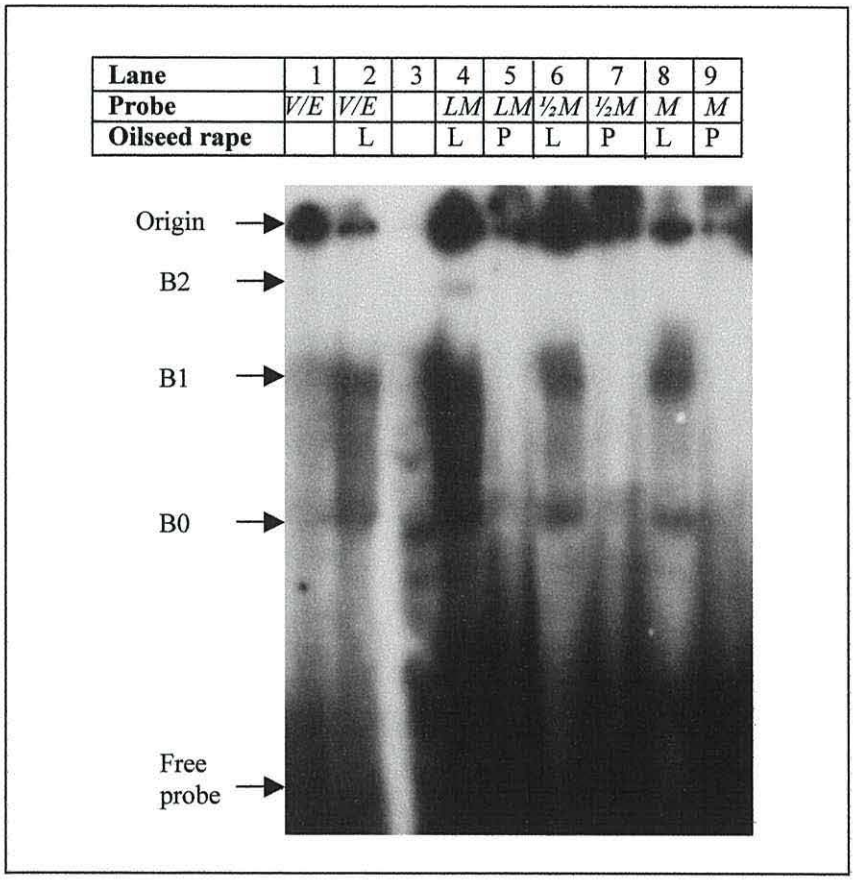


Figure 6.5. EMSA showing that the B1 complex is formed with all probes but only from rape laminar extracts. Each binding reaction mixture (except 3) contained 5µg of either rape laminar (L) or rape petiole (P) nuclear protein extract. **1.** *Vs-2/Enh-2* oligonucleotide, no N.P. **2.** *Vs-2/Enh-2* oligonucleotide (L). **3.** Blank. **4.** *LongMyb oligonucleotide (L).* **5.** *LongMyb oligonucleotide (P).* **6.** *½Myb* oligonucleotide (L). **7.** *½Myb* oligonucleotide (P). **8.** *Myb* oligonucleotide (L). **9.**. *Myb* oligonucleotide (P).

A weaker shift designated B0, seen further down the gel in figure 6.5 (and to a lesser extent in figure 6.3) is visible with all extracts and all probes, including *Vs-2/Enh-2*. The *Vs-2/Enh-2* probe has never been shown to bind a protein of this

mobility before (figure 3.5) and therefore it is likely that the protein is nuclear extract specific. Figure 6.3 (lanes 5 and 6) shows that a 150-fold molar excess of CHO can abolish binding of this protein to *½Myb* and *Myb* probes, although binding was also abolished by the addition of CN-HO (lanes 7, 8 and 9), demonstrating the proteins lack of specificity. However, this shift acts as a useful marker when comparing rape petiole and laminar binding, as it validates the integrity of the petiole extracts.

Figure 6.6 shows that the system with respect to the *LongMyb*-protein complex is saturated at a concentration of 5µg of rape laminar nuclear extract because the addition of further nuclear protein extract has no effect on the intensity of either the main shift B1 or on B2 (lanes 4-13). When half the quantity of extract was used, however, there was a reduction in the intensity of both B1 and B2 (lane 3). Unlike the nuclear proteins binding to the sequences tested in chapter 3, there does not appear to be a sequential decrease in the mobility of either B1 or B2 with the addition of an increasing amount of nuclear extract.

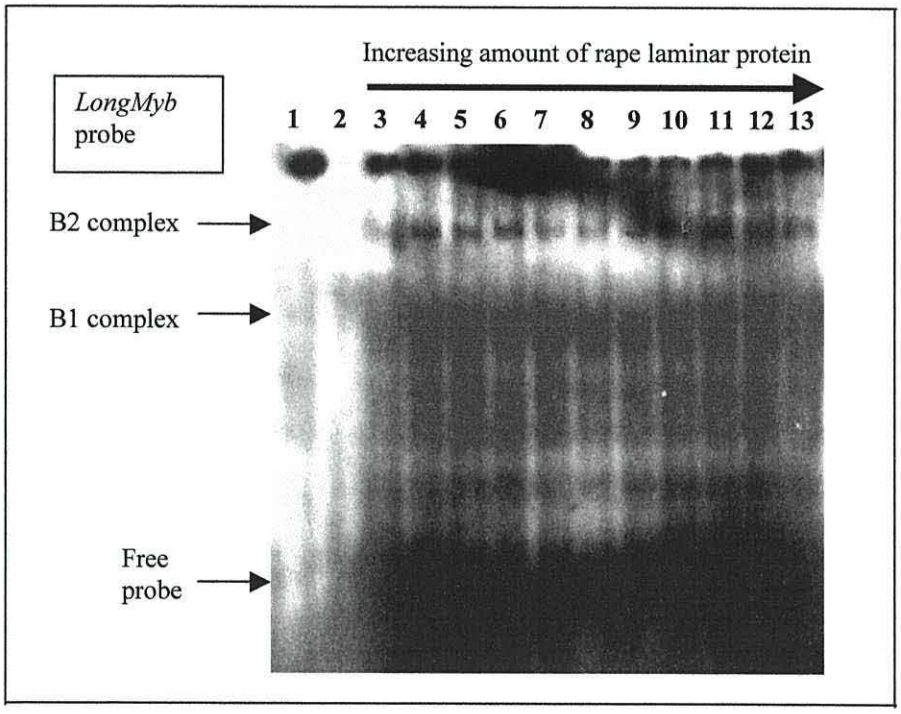


Figure 6.6. EMSA using an increasing amount of rape laminar nuclear protein (NP) extract in the binding reaction mixtures, with *LongMyb* as the probe. **1.** Negative control (no protein). **2.** Blank. **3.** 2.5µg. **4.** 5µg. **5.** 5.5µg. **6.** 6µg. **7.** 7µg. **8.** 7.5µg. **9.** 8µg. **10.** 9µg. **11.**10µg. **12.** 11µg. **13.** 12µg. The position of the two complexes (B1 and B2) is shown.

6.3.3.2. Binding patterns of nuclear protein extracts from tobacco

Previous EMSAs with tobacco nuclear protein extracts failed to reveal any binding to oligos that were able to bind rape laminar extracts (section 3.2.5). However, when the three Myb oligos were used as probes it was found that extracts from tobacco laminar bound to all three and with a similar mobility. Their mobility was slightly different from the *Vs-2/Enh-2* probe bound with a rape laminar nuclear protein (figure 6.7, lanes 5, 6 and 7 compared to lane 3) and therefore also to the Myb oligos bound with rape laminar nuclear protein i.e. the B1 complex. The intensity of the shifted band was identical when all three Myb probes were used (figure 6.7, lanes 5, 6 and 7), in contrast to rape laminar extracts, which bound more strongly to the *LongMyb* probe.

The internode and petiole nuclear extracts from tobacco did not produce a shifted band, in common with what has been found for extracts from similar rape tissues (figure 6.7, lanes 8-13). Unlike rape laminar extracts, however, tobacco laminar extracts were not able to produce a B2 complex with the *LongMyb* probe (figure 6.7, lane 5) and the intensity of binding to the three probes did not decrease when a 150-fold molar excess of cold homologous oligonucleotide was added to the binding reaction mix (figure 6.8, lanes 2, 5 and 8). Moreover, this binding was unable to be out-competed even by a 750-fold molar excess of CHO. Figure 6.9 shows that a sequential increase in CHO does not even cause a reduction in the intensity of the shifted band (lanes 1-5) when *LongMyb* was used as a probe. The bound protein behaved in a similar way to the protein(s) that bound to the *Vs-2/Enh-2, Enh1* and *Lec2* probes in chapter 3, in that it was unable to be out-competed.

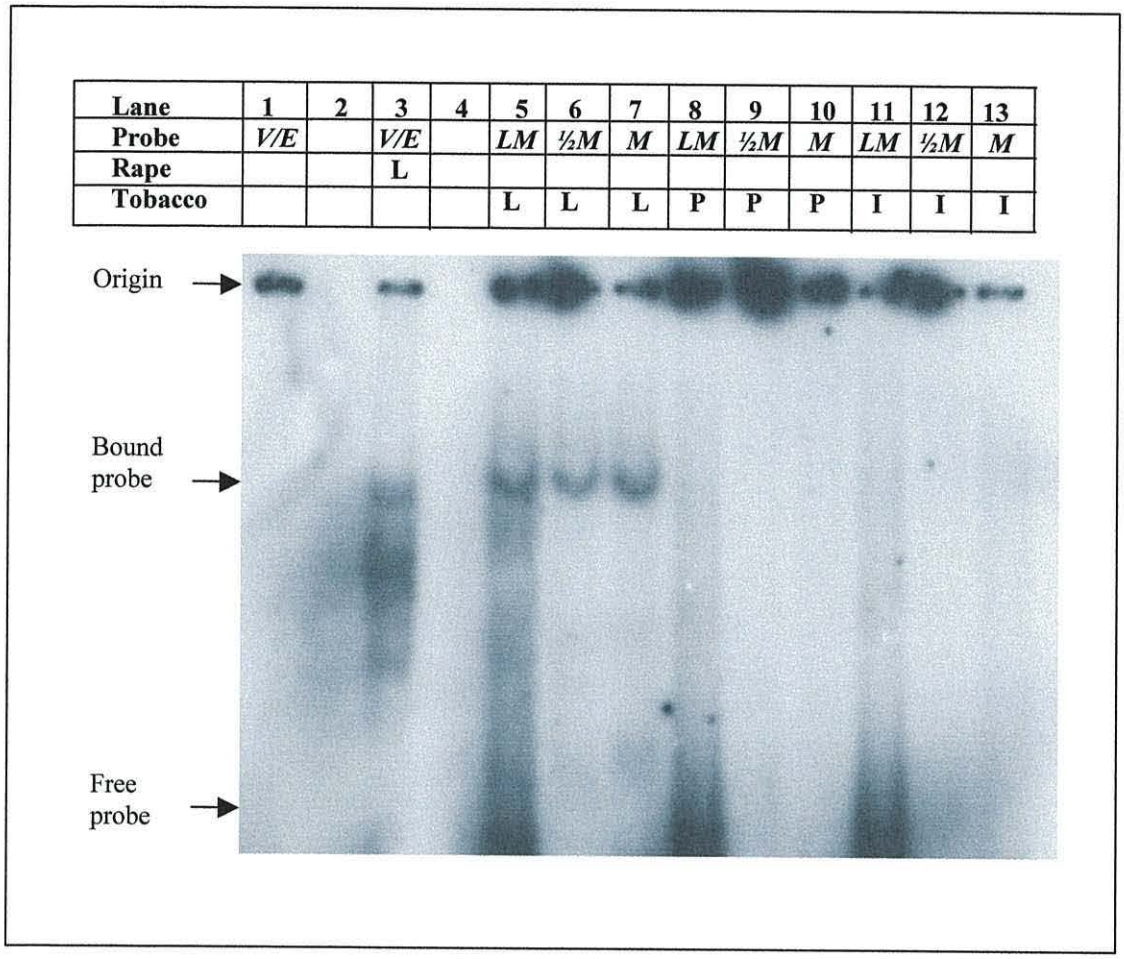


Figure 6.7. EMSA to show that nuclear proteins from tobacco laminar extract bind to the three Myb probes with a similar mobility and affinity and that nuclear proteins from tobacco petiole and internode tissues do not bind to the three Myb probes. Binding to the Myb probes is compared to the *Vs-2/Enh-2* probe bound with a complex from rape laminar nuclear protein extract. 5µg of nuclear protein extract was used in each reaction mixture. **1.** Negative control (no protein). **2.** Blank. **3.** Rape laminar nuclear protein and *Vs-2/Enh-2 (V/E)* as the probe. **4.** Blank. Lanes **5-13** contain tobacco nuclear protein (NP) extract. **5.** Laminar (L) NP extract with *LongMyb (LM)* probe. **6.** Laminar NP extract with *½Myb* (*½M*) probe. **7.** Laminar NP extract with *Myb (M)* probe. **8.** Petiole (P) NP extract with *LongMyb* probe. **9.** Petiole NP extract with *½Myb* probe. **10.** Petiole NP extract with *Myb* probe. **11.** Internode (I) NP extract with *LongMyb* probe. **12.** Internode NP extract with *½Myb* probe. **13.** Internode NP extract with *Myb* probe.

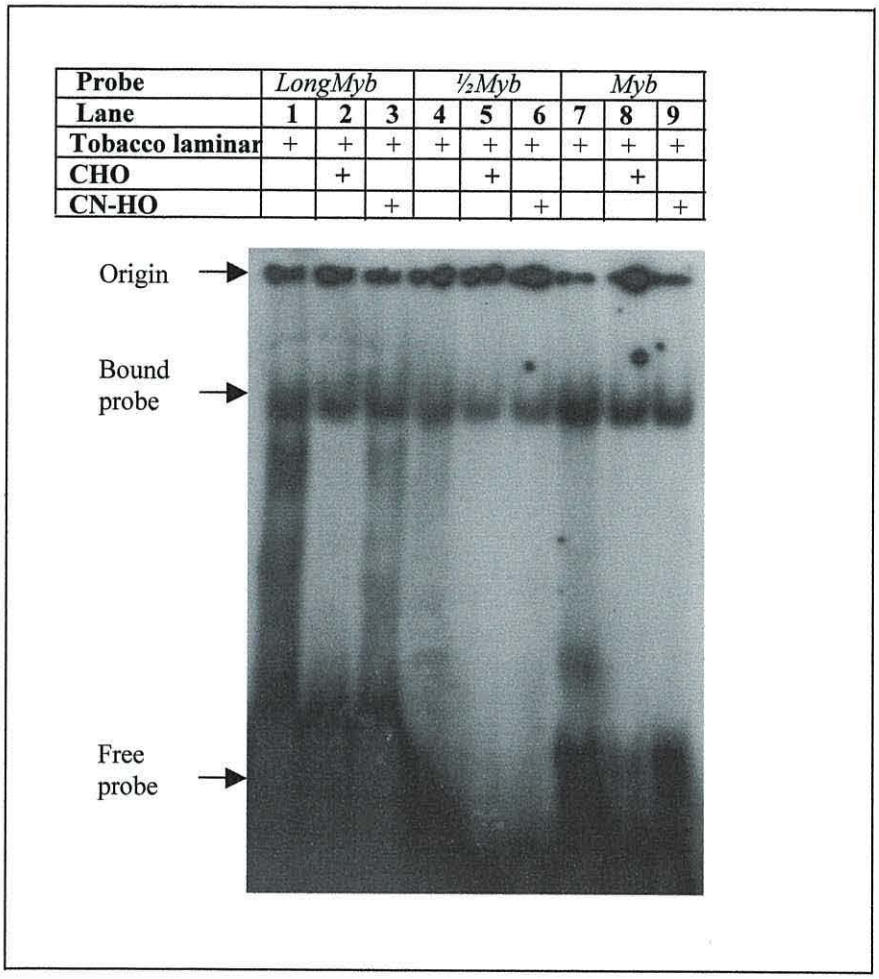


Figure 6.8. EMSA to show the effect of specific and non-specific competition on the binding of nuclear proteins from 5µg of tobacco laminar extract with *LongMyb*, *½Myb* and *Myb* probes respectively. Each probe bound to tobacco laminar nuclear protein has no competition, competition by a 150-fold molar excess of cold homologous oligonucleotide (CHO) and a 150-fold molar excess of cold non-homologous oligonucleotide (CN-HO), a non-specific sequence from *S. calcitrans,* respectively. **1.** *LongMyb* probe, no competitor. **2.** *LongMyb* probe, 150-fold molar excess of CHO. **3.** *LongMyb* probe, 150-fold molar excess CN-HO. **4.** *½Myb* probe, no competitor. **5.** *½Myb* probe, 150-fold molar excess CHO. **6.** *½Myb* probe, 150-fold molar excess CN-HO. **7.** *Myb* probe, no competitor. **8.** *Myb* probe, 150-fold molar excess CHO. **9.** *Myb* probe, 150-fold molar excess CN-HO.

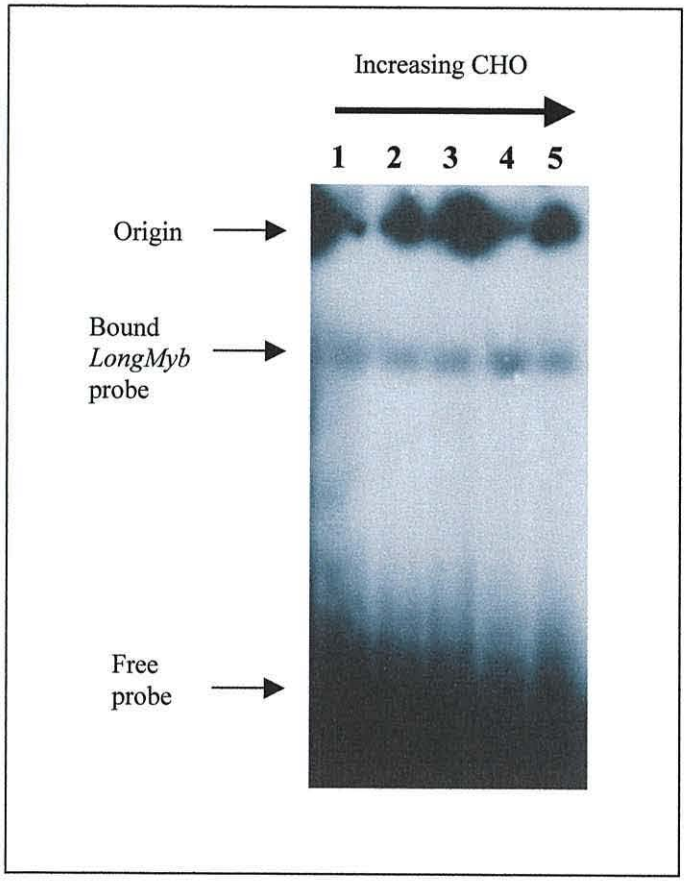


Figure 6.9. EMSA to show that increasing the amount of cold homologous oligonucleotide (CHO) competitor in the binding reaction mixture has no effect on the intensity of binding by nuclear proteins from tobacco laminar extracts to the *LongMyb* probe. 5µg of tobacco laminar nuclear protein extract was used in each binding reaction mixture. Molar excess refers to an excess of competitor DNA in relation to the radioactive probe DNA. **1.** 150-fold molar excess CHO. **2.** 300-fold molar excess CHO. **3.** 450M-fold molar excess CHO. **4.** 600-fold molar excess CHO. **5.** 750-fold molar excess CHO.

6.4. Summary

Analysis of sequences immediately upstream of –547bp in the *extA* promoter using TRANSFAC and the PLACE database revealed the presence of a MYB core sequence. This was disrupted in transgenic tobacco plants containing the –547bp promoter truncation–GUS fusion, therefore three oligonucleotides were designed to investigate the possibility that this sequence bound a nuclear protein from tobacco and rape tissue extracts. EMSAs were used to establish that all three sequences formed a similar protein/DNA complex of reduced mobility, designated B1. The mobility of B1 was similar to the complexes formed with the *Lec-2, Vs-2/Enh-2* and *Enh-1* probes investigated in chapter 3. As with the *Lec-2, Vs-2/Enh-2* and *Enh-1* complexes, the formation of this complex was specific to rape laminar nuclear protein extracts. However, binding to the *½Myb* and *Myb* sequences by rape laminar nuclear proteins was unlikely to be specific because a reduction in binding was seen with the addition of homologous competitor, and was paralleled by a reduction in shift intensity when an unrelated 25bp sequence from *S. calcitrans* was used as a probe. However, when the *LongMyb* probe, encompassing the two shorter probe sequences and an additional 10bp of NRR in the 5′ direction was used, a 150-fold molar excess of non-labelled *LongMyb* competed for binding with the more strongly bound B1 complex. This suggests that this complex is unique to the *LongMyb* sequence, despite the fact that it has a similar mobility to that formed with the other oligonucleotide probes from the NRR. In addition, *LongMyb* formed a highly specific second complex (B2) when rape laminar extracts were used which could be out-competed by the addition of 150-fold molar excess of CHO. Partial competition of B2 was observed by unlabelled *Myb* probe encompassing the MYB core sequence, implying that nucleotides in this sequence affected the binding affinity of proteins to the *LongMyb* sequence.

When tobacco extracts were used in the binding reaction mixture, a similar pattern was observed in that only laminar nuclear protein extracts caused a retarded band. In addition, all three oligonucleotide sequences produced a band of similar mobility. However, the shifted band was of a slightly lower mobility than that seen with rape laminar extracts, shifted band intensity was the same for all three probes and no second complex was produced with the *LongMyb* probe. Specificity of binding to the *½Myb, Myb* and *LongMyb* sequences by tobacco laminar nuclear

proteins could not be established because the addition of homologous competitor DNA did not reduce binding, even in large quantities. These interactions with tobacco extracts were therefore unlikely to be specific.

7. The effect of mechanical stress on expression of the *B. napus extA* extensin gene

7.1. Introduction

The induction of extensin upon the application of mechanical stress has been observed by Neibel *et al* (1993), Shirsat *et al.* (1996a), Elliott and Shirsat (1998), Kozbial *et al* (1998), Merkouropoulos (2000), Salvà and Jamet (2001) and Shirsat *et al.* (2003). Kozbial *et al.* (1998) showed that two transcripts, which hybridise to the *extA* probe, accumulate in the leaves of *B. napus* upon membrane-damaging freezing treatments. However, although freezing is likely to cause mechanical stress, it would also cause wounding, both of which would necessitate cell wall strengthening by extensins. Similarly, in a study by Neibel *et al* (1993), infection of tobacco roots with the root nematode pathogen *Meloidogyne javanica* resulted in extensin transcript accumulation in cortical cells in galls. This was thought to be the result of mechanical pressure exerted by the developing gall, although it may also have been in direct response to the pathogen infection and/or the need for additional structural tissue in actively dividing cells of the gall.

Confirmation of the direct link between mechanical stress and extensin gene expression came from a study by Shirsat *et al.* (1996a). When the *B. napus extA* extensin promoter was fused to the GUS reporter gene and introduced into tobacco by transformation, low expressing transgenic plants produced a distinct band of GUS expression extending through the cortex and pith of the node when 10g weights were hung from axillary flowering stalks. Un-weighted axillary branches did not produce this expression pattern (Shirsat *et al.*, 1996a). Several years later, Merkouropoulos (2000) performed a similar experiment when flower stalks of transgenic tobacco containing the *atExt1* promoter from *Arabidopsis* fused to GUS were weight-loaded. This stress was sufficient to mildly induce expression of the transgene.

Salvà and Jamet (2001) applied mechanical stress to *N. tabacum* plants by changing the light direction with respect to the plant apex i.e. the response to phototropism. This led to an accumulation of *Ext1.4* extensin mRNAs in cells where it does not normally occur (cortical cells of internodes). The same authors also

carried out experiments on transgenic tobacco plants carrying a -1331/+46bp *Ext 1.4*-GUS gene fusion. The response of a change in the direction of stem growth after contact with the top of culture jars (thigmomorphogenesis) was examined in the same work. Roots were mechanically stressed by forcing them to curve on contact with the bottom of culture vessels. In both cases, the mechanical stress induced expression of the *Ext1.4* gene.

The following chapter aims to build on the current knowledge of the plant response to mechanical perturbation by examining the effects on *extA* expression when plants are shaken (seismomorphogenesis). In addition, northern analysis is used to determine whether *extA* mRNA can be induced when petiole from *B. napus* is weight-loaded.

7.2. Identification of developmental stages when the *extA* gene is induced

Under non-stressed conditions *extA* mRNA is absent in the laminae and present in the petioles of mature *B. napus* plants (Shirsat *et al.*, 1996a). However, in younger plants, the opposite is true – *extA* mRNA is absent in the petiole and present in young laminae (Elliott, 1998). The aim of the following set of experiments was to establish the age at which constitutive expression was lost in the laminae and gained in the petiole so that any change in *extA* mRNA levels caused by mechanical perturbations could be observed with greater accuracy.

7.2.1. Extraction of RNA from tissues

Tissues at various developmental stages were taken from *B. napus* seedlings, immediately frozen in liquid nitrogen and stored at –80°C before RNA extraction (section 2.13.2). Figure 7.1(a) and figure 7.2 shows the developmental stages at which lamina tissue was taken, with age of the plant measured as number of days from seed germination. Lamina 1 is the first lamina to emerge and is therefore the oldest. Laminae were designated as "young" if they were not fully expanded. Figure 7.2 also shows the developmental stages when petiole was taken. The most mature petiole tissue was taken for analysis at each time point (day 36, 43, 50 and 67).

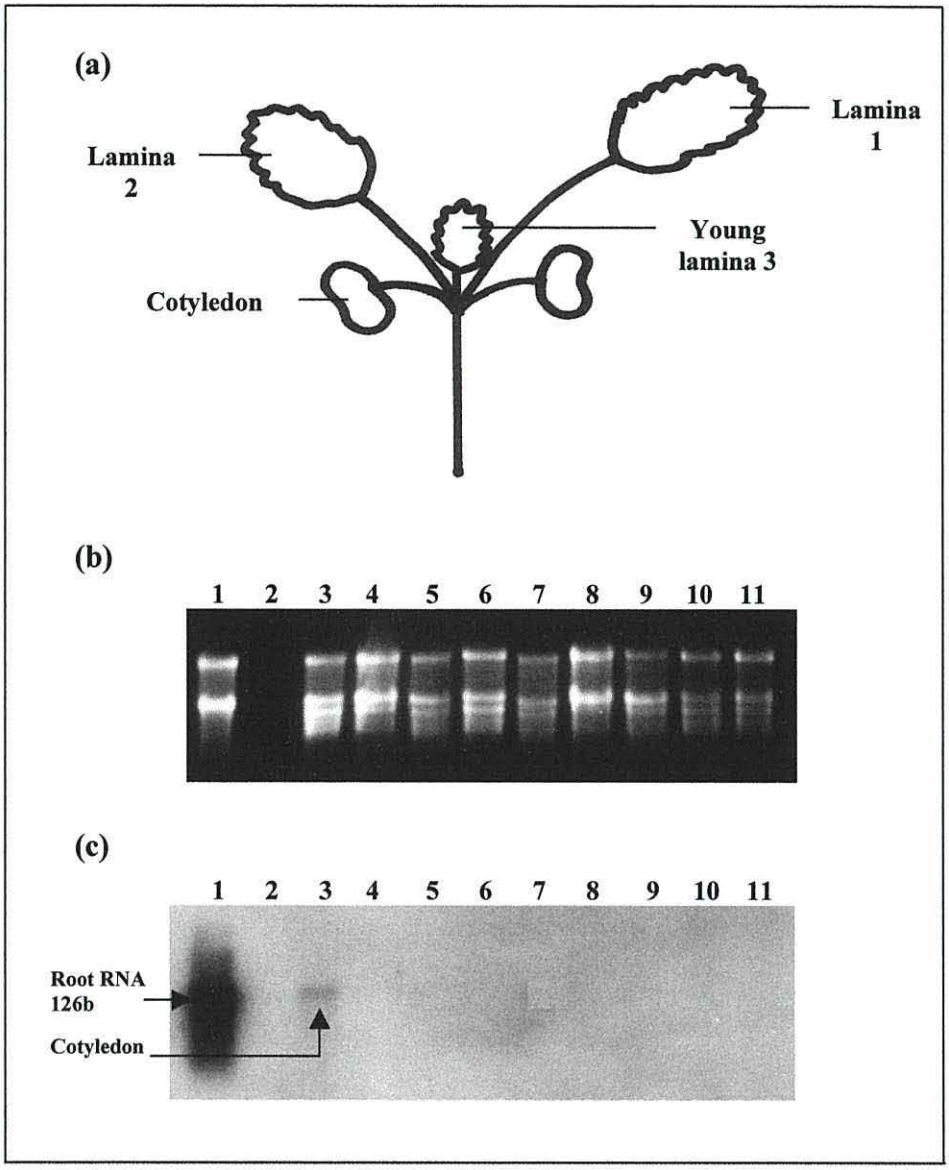


Figure 7.1. Expression of *extA* transcripts in different laminae of *B. napus* seedlings.

(a). Diagram of a *B. napus* seedling. Laminae are numbered according to order of emergence. For example, lamina 1 is the oldest lamina, then lamina 2 and so on. Only fully expanded laminae were taken for analysis, otherwise laminae were referred to as "young lamina" (YL).

(b). Formaldehyde gel of developmental stages from *B. napus* lamina for northern analysis. 10μg of lamina RNA was taken from rape seedlings. L = lamina. YL = young lamina. d refers to the age of the seedling, measured as number of days from germination. **1.** Root positive control. **2.** Blank. **3.** Cotyledon lamina @ d 12. **4.** YL1 @ d12. **5.** L1 @ d 23. **6.** L2 @ d 23. **7.** L3 @ d 23. **8.** YL4 @ d23. **9.** L4 @ d 26. **10.** YL5@ d 36. **11.** L6 @ d 43.

(c). Autoradiograph of Northern blot of the above gel hybridised against the *extA* coding sequence.

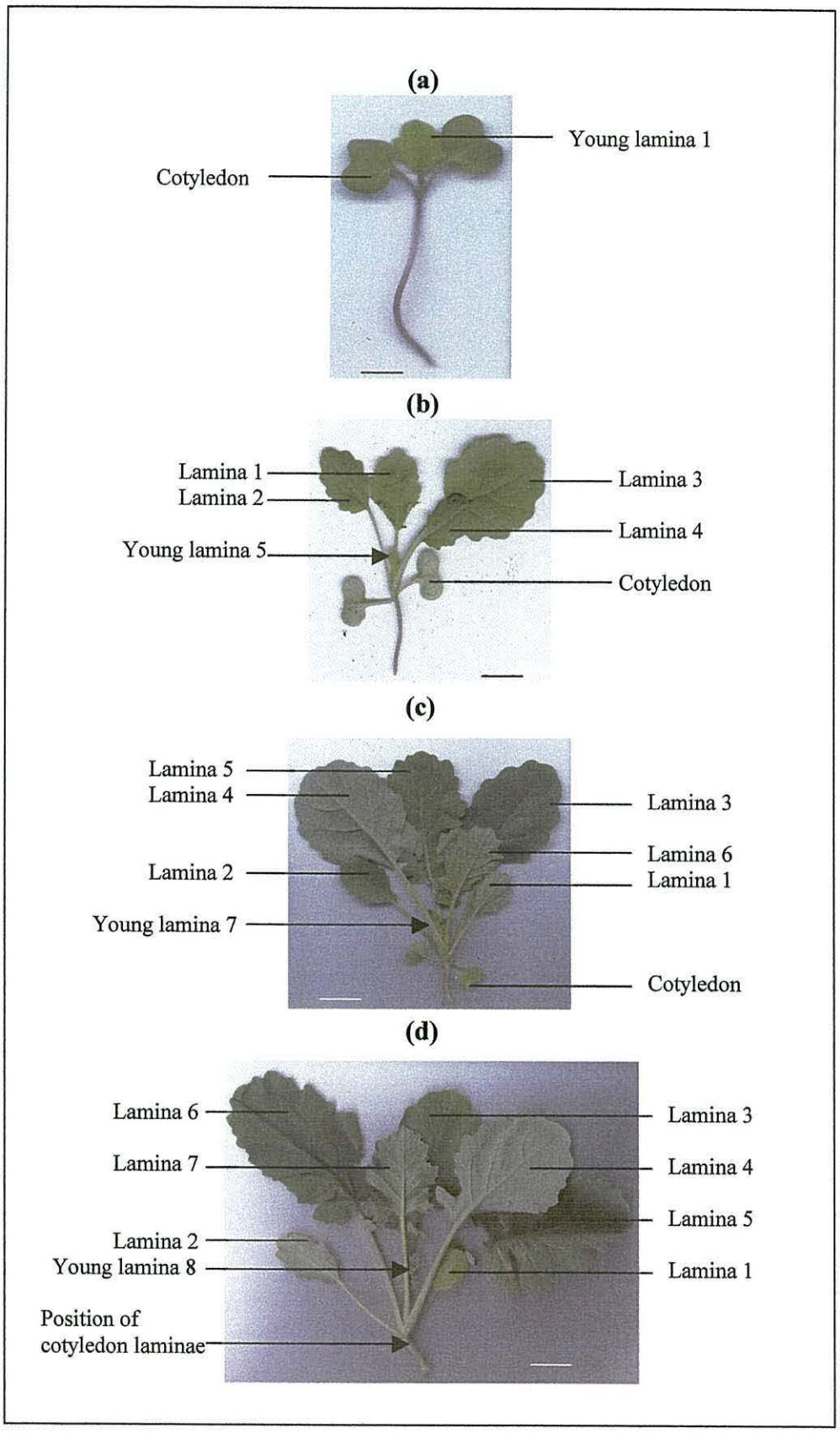


Figure 7.2. Four developmental stages of *B. napus* at which tissue was taken for analysis. **(a)** day 12. Bar = 1cm . **(b)** day 36. Bar = 1.25cm. **(c)** day 43. Bar = 1.5cm **(d)** day 50. Bar = 2cm.

7.2.2. Northern analysis of *extA* expression in lamina and petiole at various developmental stages

10μg of RNA from lamina and petiole tissues was run on formaldehyde gels, with 10μg of *B. napus* root RNA as a positive control (figure 7.1(b) and 7.3(a) respectively) because the extensin *extA* transcript is always expressed to high levels in the root (Chen and Varner, 1985a; Showalter and Varner, 1987; Gatehouse *et al.*, 1990, Tiré *et al.*, 1994; Shirsat *et al.*, 1991; Wycoff *et al.*, 1995; Hirsinger *et al.*, 1997; Merkouropoulos *et al.*, 1999). The gels were northern blotted and the membranes containing RNA were hybridised against the *extA* coding sequence (section 2.13.4).

The plasmid pRλS5 is a sub-clone in pUC18 of an 800bp sequence from the genomic clone lambda B31 (Evans *et al.*, 1990). The 800bp sequence comprises the whole of the *extA* coding region and the 5′ sequence up to and including the TATA box, bounded by *Nsi* I at the 5′ end and *Hinc* II at the 3′ end. The *extA* probe was made by cleavage of the plasmid pRλS5 with the restriction enzymes *Hind* III and *EcoR* I. When washed to high stringency, the *extA* coding sequence hybridises to a single transcript of 1260b, as predicted by S1 nuclease mapping of the *extA* genomic clone (Evans *et al.*, 1990). However, when washed to low stringency, the coding sequence hybridises to a second transcript of 1450b from root extract. The gene responsible for this transcript has not been identified, although it is likely that it is another member of the *B. napus* extensin multigene family, from which only *extA* has been fully characterised (Evans *et al.*, 1990). The position of the 1260b transcript is indicated on all northern blots.

Figure 7.1(c) shows the resulting autoradiograph for different lamina stages. It is clear in the very young seedlings that the cotyledons are the only laminae to produce a detectable signal (lane 3). Even young laminae, not yet fully expanded, do not produce *extA* mRNA (lanes 4, 8 and 10). This demonstrates the tissue specificity of *extA* mRNA distribution in the young seedling.

Figure 7.3(a) shows a formaldehyde gel with petiole RNA taken at different time points and figure 7.3(b) shows the corresponding autoradiograph. *ExtA* mRNA is first detected at day 43, where a very faint signal can be seen. At this time point the plant has six fully expanded laminae and the presence of a seventh young lamina,

cotyledon laminae are beginning to senesce, and the petioles are becoming more solid and waxy (see figure 7.2c). At other developmental stages, the signal becomes stronger, with a large up-regulation occurring in the 7 days between day 43 and 50. The strongest signal is seen in the oldest tissue (day 67), where mRNA levels are comparable to the large quantities seen constitutively in the root positive control.

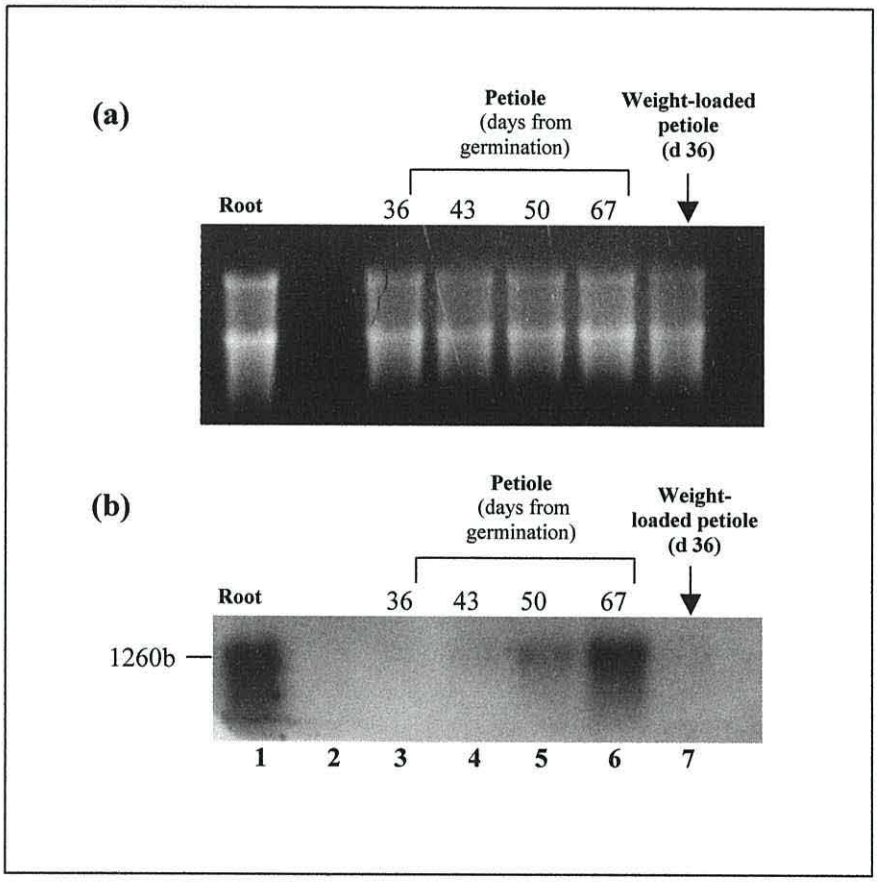


Figure 7.3. Northern analysis of developmental expression of *extA* in non-stressed *B. napus* petiole and the effect of weight-loading on a young *B. napus petiole* (36 days after germination).

(a). Formaldehyde gel with 10μg petiole RNA from each sample. Positive control is RNA from *B. napus* root.

(b). Autoradiograph of northern blot of the above gel when hybridised against the *extA* coding sequence.

7.3. Expression of the *B. napus* extensin gene, *extA*, in response to weight-loading the petiole

Previous experiments with *extA*-GUS promoter-reporter fusions (Shirsat *et al.*, 1996a; Shirsat *et al.*, 2003) and *atExt1*-GUS fusions (Merkouropoulos, 2000) showed that there was an increase in reporter gene activity when external weights were applied to the axillary flowering branch nodes of transgenic tobacco plants. The purpose of the following set of experiments was to establish whether weight-loading of *B. napus* petioles also induced an *extA* response.

7.3.1. The effect of weight-loading the petiole of *B. napus*

B. napus petioles were chosen for weight-loading because the morphology of oilseed rape is different from that of tobacco. A lack of axillary branches in oilseed rape therefore did not permit a direct comparison between rape and tobacco.

Petioles from 36 day old *B. napus* seedlings (figure 7.2b) were weight-loaded for 72 hours using a 1.5g weight and RNA extracted from frozen tissue as before. Petiole of this age was chosen because no *extA* mRNA was detectable by northern analysis, as determined in section 7.2 (figure 7.3b, lane 3). An up-regulation of mRNA caused by weight-loading would be clearly seen. Figure 7.3b, lane 7, clearly shows that weight-loading had no effect on *extA* mRNA production, as no signal is detected. This was unexpected because weight-loading transgenic tobacco plants containing the *extA* promoter fused to GUS had induced expression (Shirsat *et al.*, 1996b; Shirsat *et al.*, 2003).

Previously published work has implied that the regulatory mechanisms involved in the synthesis of extensin may require the presence of an established developmental programme (Ebener *et al.*, 1993). For example, tomato extensin gene expression, which is high in stems and absent in leaves, can only be enhanced in stems and not in leaves by wounding (Showalter and Varner, 1987). In the following experiment, older petiole was taken from plants at day 50 to establish whether developmental control at day 36 was masking the effects of weight-loading. Older petiole was strong and waxy therefore a greater weight (3g) was required to induce bending (figure 7.4a). At this age, the petiole was also large enough to be divided into four equal regions so that if a positive signal were to be detected, it would be

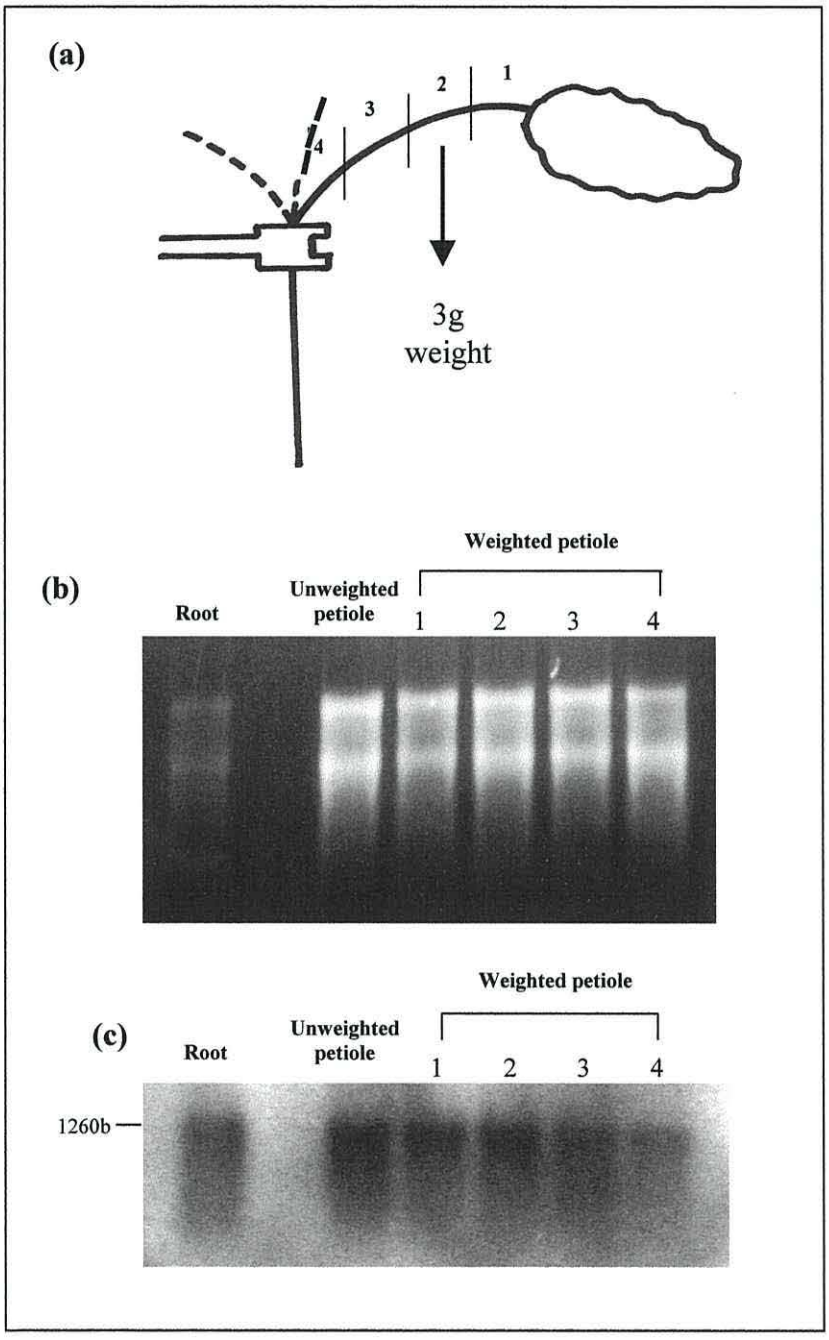


Figure 7.4. Weight-loading of mature *B. napus* petiole

(a). Diagram showing how mature (50 days from germination) *B. napus* petiole was weight-loaded. Numbers 1-4 refer to the petiole segments used in the analysis.

(b). Formaldehyde gel containing 10μg RNA from each section of *B. napus* petiole weight-loaded with a 3g weight for 72 hours. Positive control is *B. napus* root. 1, 2, 3 and 4 weighted petiole refer to the section of the petiole taken for analysis. All weighted petiole sections were compared to RNA from un-weighted petiole of the same age (50 days from germination).

(c). Autoradiograph of a Northern blot of the above gel, hybridised against the *extA* coding sequence.

possible to visualise an increase in that signal with greater proximity to the point of maximum tensile stress (figure 7.4(a)). However, when RNA was extracted from older petiole sections weight-loaded for 72 hours and northern analysis performed, there was no observed up-regulation of *extA* mRNA in response to tensile stress, with the signal remaining the same (figure 7.4(c), tracks 1, 2, 3 and 4).

7.4 Expression of the *B. napus* extensin gene, *extA*, in response to a seismic stress

Touch genes coding for the calcium binding protein calmodulin are up-regulated in response to thigmic stresses in *Arabidopsis thaliana* (Braam and Davis, 1990) (see section 1.1.3). Unpublished preliminary results with *Arabidopsis* (Shirsat, personal communication) have shown that *atExt1* extensin mRNA is detected in the leaves of plants shaken vigorously for 24, 48 and 72 hours, with the signal declining in strength over time (figure 7.5). However, apart from these preliminary results, nothing is known about the response of extensin genes to mechanical stresses of a seismic nature.

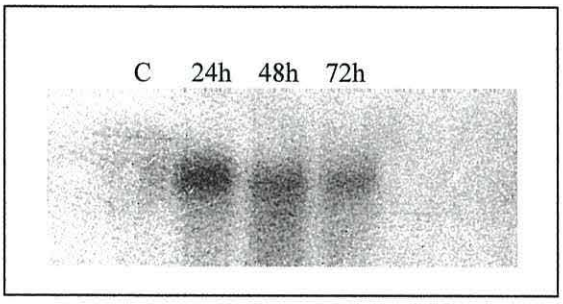


Figure 7.5. Northern blot of *atExt1* mRNA when *Arabidopsis* was shaken vigorously for 24, 48 and 72 hours respectively. C=control (non-shaken). By kind permission of Dr A.H. Shirsat.

7.4.1. Shaking experiments with *B. napus*

Six week old oilseed rape seedlings were chosen for this experiment, based on the previous observations that *extA* mRNA is not detected by northern analysis in either the lamina or petiole at this stage (figure 7.1c, 7.3b and section 7.2.2). Any up-regulation of the genes should have been seen. Seedlings were shaken vigorously on an IKA-VIBRAX-VXR shaking platform rotating at 800rpm under growth room

conditions (section 2.2) for 72 hours. Shaking above this level resulted in a standing wave and therefore a reduction in the amount of stress experienced by the plants. The most vigorously shaken, healthiest petioles and laminae were taken for analysis. Non-shaken tissue of the same age was also taken for comparison. The results of northern analysis can be seen in figure 7.6a and 7.6b, where only the root positive control shows a signal. It therefore appears that seismic stress has no effect on *extA* mRNA production in petiole or lamina tissue from *B. napus*.

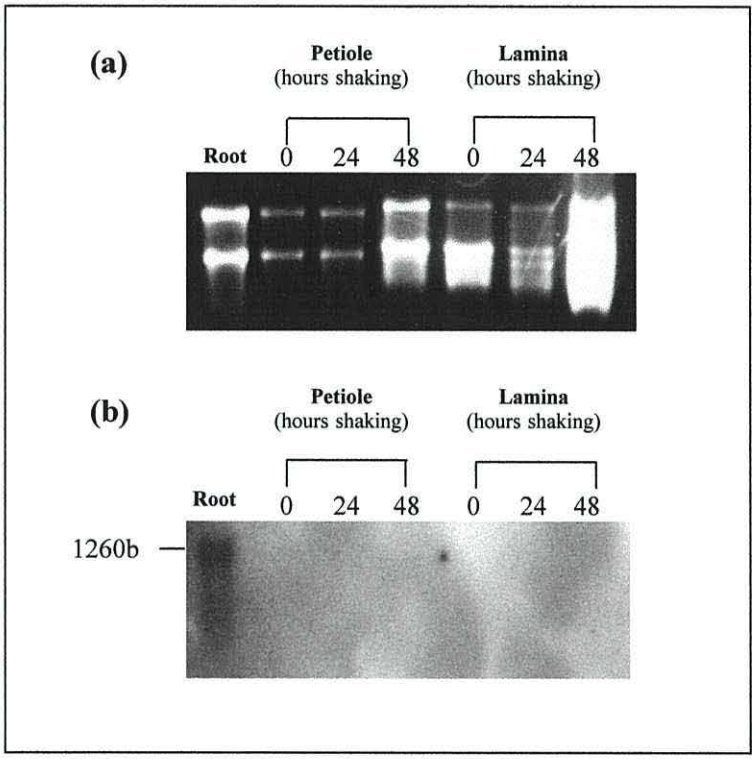


Figure 7.6. Northern analysis of the effect of seismic stress on *B. napus* petiole and lamina tissue when six week old plants were shaken vigorously for 24 and 48 hours.

(a) Northern gel containing 10μg RNA from shaken and non-shaken lamina and petiole at different times after the onset of the shaking stimulus. The lane containing *B. napus* root RNA is a positive control.

(b) Autoradiograph of the northern blot of the above gel, hybridised against the radiolabelled *extA* coding sequence.

7.4.2. Shaking experiments with transgenic *N. tabacum* plants containing 940bp of the *extA* promoter fused to GUS

The GUS enzymatic assay is sufficiently sensitive to pick up subtle changes in *extA* expression that are beyond the limits of detection of northern analysis. Transgenic

tobacco plants containing 940bp of *extA* promoter fused to the GUS reporter gene were therefore studied for their response to seismic stress. Three-month old transgenic tobacco plants were shaken vigorously, as before, on a shaking platform rotating at 800rpm. Cross sections of mature stem and petiole were taken because extensin expression is specific to the vascular tissue at this stage (Elliott and Shirsat, 1998), therefore any increase in GUS activity would not be masked by non-specific expression. Sections were incubated in X-Gluc (see section 2.10) and examined for blue staining.

Figure 7.7b shows representative sections from six transgenic tobacco plants that were shaken for 72 hours. In the tissues that were taken, that there is no up-regulation of the *extA* extensin gene in a heterologous system in response to seismic stress, as sections remained un-stained.

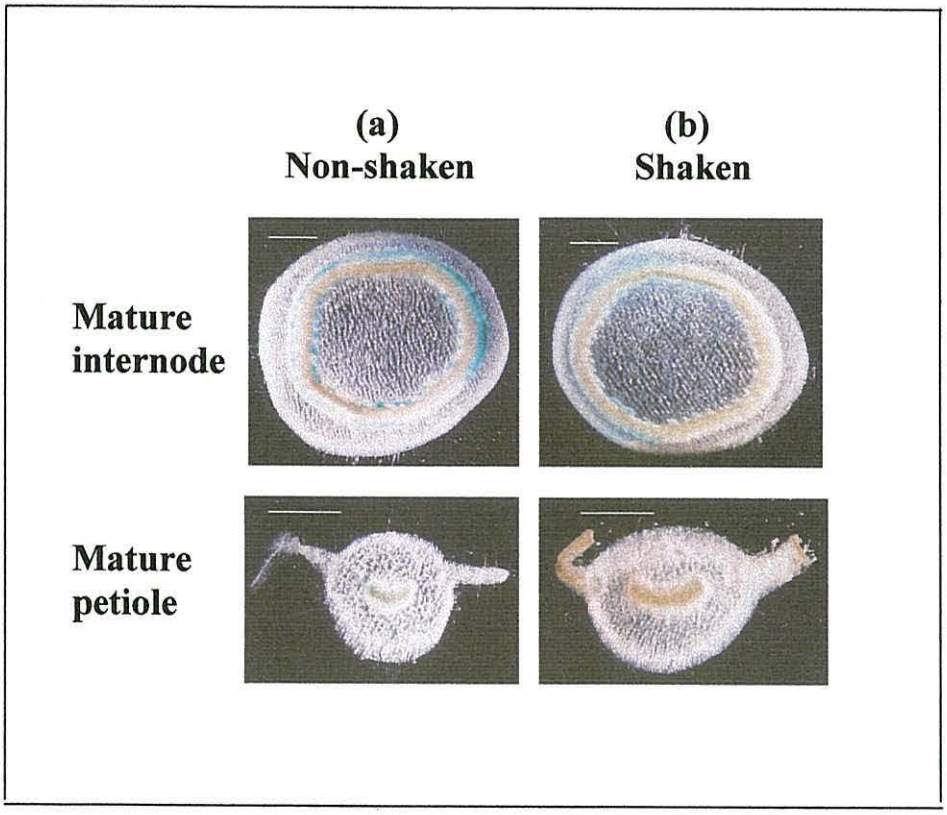


Figure 7.7. Transverse sections taken through mature stem and petiole of non-shaken **(a)** and shaken **(b)** transgenic tobacco plants carrying 940bp of the *extA* promoter fused to GUS. Expression was analysed using the GUS histochemical assay, which was carried out in 1mM cycloheximide. Bar = 2 mm. Plants were shaken vigorously for 72 hours.

7.5. Summary

Developmental patterns of *extA* mRNA accumulation were examined in *B. napus* seedlings. Northern analysis revealed that *extA* mRNA was only detected in *B. napus* cotyledons and was otherwise completely absent in lamina tissue. Conversely, *extA* mRNA was absent in young petioles up to the age of 36 days, after which the transcript gradually increased, reaching very high levels by day 50.

The effect of mechanical stresses on *extA* transcript accumulation was examined in a homologous system for the first time. However, northern analysis was unable to detect an *extA* signal after the application of stress. *ExtA* also failed to be induced by seismic stress when expression was analysed by northern hybridisation and histochemistry in a homologous and heterologous system, respectively.

8. Discussion

8.1. The effect of mechanical stress on the expression of *extA*

8.1.1. Weight-loading rape petioles does not induce *extA* mRNA synthesis

Previous work on the *extA* gene in transgenic tobacco has shown that the application of weights induced expression of the GUS reporter gene when fused to the *extA* promoter (Shirsat *et al.*, 1996a; Shirsat *et al.*, 2003). However, in the experiments detailed in section 7.3, when weights were applied to rape petiole, northern analysis failed to reveal the presence of *extA* mRNA.

When the expression of *extA* was examined in transgenic tobacco and untransformed *B. napus* and *B. juncea* tissues, northern analysis showed that the gene maintained its normal pattern of expression when transferred to tobacco (Shirsat *et al.*, 1996a). Typical expression patterns were also maintained when the *extA* promoter was used to drive the expression of a *gusA* reporter gene in apple (Gittins *et al.*, 2001). Expression was localised to tissues requiring structural support, such as the maturing phloem parenchyma cells and phloem fibres, but in particular at the nodal junctions. This implies that previous mechanical stress responses of *extA* seen in transgenic tobacco are not the result of differential expression patterns of the gene in a heterologous system, but are real, and related to the function of the protein.

However, when comparing the two systems (tobacco and rape), it is important to be aware that different mechanisms of detection were used. Although weight-loading tobacco produced a band of *extA* expression at the point of maximum tensile stress, the intensity of the GUS expression was still low compared to that seen in the high expressing plants in the phloem vascular bundles (Shirsat, unpublished). It is possible that northern analysis was not sensitive enough to detect a slight up-regulation of the gene. The GUS enzymatic assay, conversely, is extremely sensitive to such changes (Jefferson *et al.*, 1987). In addition β-glucuronidase is extremely stable in extracts and cells, with a half-life in living mesophyll protoplasts of approximately 50 hours (Jefferson *et al.*, 1987). Such stability could act to magnify expression levels of the transgene.

Intra- and inter-specific variation in the plant response to mechanical stress has also been noted (Jaffe, 1973; Suge, 1978; Telewski and Jaffe, 1986a; Johjima *et al.*, 1992). Tobacco and rape have a different physiology and therefore it is likely that both respond differently to mechanical stresses. In both cases, the most suitable organs were used in the weight-loading experiments, although these may not have been comparable, as *extA* is developmentally expressed in different tissues. It is known that tissue-specific elements play a role in the well-studied wounding response, since a given extensin gene is not wound inducible in all tissues. For example the tomato extensin gene Tom L-4 is wound inducible in stems but not in roots and leaves (Zhou *et al.*, 1992; Showalter *et al.*, 1992) and the rape *extA* gene is induced in wounded stem, petiole and lamina but repressed in wounded roots (Shirsat *et al.*, 1996a). It is therefore possible that a similar mechanism of differential expression is occurring in the experiments detailed in section 7.3.

Many authors have shown that the conditions surrounding the application of mechanical stress are extremely important when determining the physiological and morphological responses. In an experiment by Knight *et al.* (1992), transgenic tobacco seedlings expressing the calcium sensitive luminescent photoprotein, aequorin, emitted a spike of blue light when touched. (Section 1.1.3 examines the mounting evidence for the role of calcium in the mechanical stress response.) However, statically bent tissues did not bioluminesce. Cytosolic calcium increases apparently occur only while cell wall compression and tension are changing and are therefore related to the time taken for the tissue to bend. Repeated stimulation caused attenuation of luminescence, with restoration of the full response requiring a one-minute rest. If calcium ions are involved in the signalling pathway to extensin gene transcription then it is possible that a similar response is occurring in oilseed rape, and that the experimental application of mechanical stresses should be modified to take this into account.

8.1.2. Seismic stress does not induce *extA* expression in oilseed rape and transgenic tobacco plants

To date there is no published work on the relationship between extensin expression and seismic stress, although there is much evidence to suggest that seismic stress could induce extensin expression, based on the knowledge that extensins are induced

by mechanical stimuli (Tiré *et al.*, 1994; Shirsat *et al.*, 1996a; Hirsinger *et al.*, 1999; Merkouroplolous, 2000; Shirsat *et al.*, 2003). Unpublished preliminary work by Shirsat (figure 7.5) has shown that the *Arabidopsis* extensin gene, *atExt1,* is induced by seismic stress. However, results presented in section 7.4 clearly indicate that there was no response of *extA* to seismic stress, under the conditions used in both a homologous (oilseed rape) and heterologous (tobacco) system, using two different detection methods (northern hybridisation and histochemical analysis of the gene fusion respectively). There may be a number of factors contributing to this unexpected result.

Firstly, it is possible that the shaking conditions experienced by the rape and tobacco plants were not conducive for *extA* expression. In bean, tomato and elm seedlings, the degree of reduction in stem elongation caused by exposure to mechanical perturbation decreased exponentially with increasing dose (Jaffe *et al.*, 1980; Garner and Bjorkman, 1996; Telewski and Pruyn, 1998). The level of seismic stress experienced by a plant is crucial to the physiological and morphological response that it produces. Several studies have shown that low-amplitude mechanical vibration in the frequency range of 50 to 60 Hz actually stimulates rather than inhibits overall plant growth (Takahashi *et al.*, 1991). Such vibration for 30 minutes each hour for 7 days stimulated growth size and mass of tomato seedlings (Akers and Mitchell, 1980). Some Japanese growers apply vibration and even music below benches and position speakers in contact with components of commercial hydroponic culture systems (Tohoku Pioneer Electrical Corp., Tendo, Japan). The level of seismic stress experienced in section 7.4 may therefore have been too low or too high to induce *extA*.

As mentioned in section 8.1.1, statically bent calcium-sensitive tobacco seedlings did not induce a Ca^{2+} flux whereas a short burst of stress did (Knight *et al.*, 1992). Shirsat (unpublished) also found that the duration of stress was important when *Arabidopsis* seedlings were subjected to seismic stress. The signal declined in strength with increasing exposure to the stress (figure 7.5). It is therefore possible that the plants in section 7.4 were shaken for too long so that a signal was not seen.

The plant response to mechanical stress depends not only on the level of stress received, but also on the type of mechanical stress experienced. Thigmic stress applied directly to elongating stem tissue retarded elongation rate more than shaking

the whole plant (Beyl and Mitchell, 1983). A similar explanation could account for the lack of response of *extA* to one type of mechanical stress (seismic) compared to the positive response to another type of mechanical stress when transgenic tobacco plants were weight-loaded (Shirsat *et al.*, 1996a). In addition, the physiological effects of thigmic and seismic stress can be quite different (Heuchert *et al.*, 1983), implying that different signal transduction pathways, leading to differential gene expression, are at work.

Another explanation for the lack of *extA* expression in response to seismic stress could be that growth room conditions were not ideal. For example, periodic shaking enhanced stem elasticity by enhancing the cellulose component of tomato stem fibre in winter but not in summer (Heuchert and Mitchell, 1983). Non-optimal environmental conditions may also alter the threshold of plant responses to mechanical stress. Each species is thought to have its own temperature range for optimum mechanical stress sensitivity. Tomato seedlings grown at 32°C days/28°C nights were much less responsive to periodic shaking than if they were grown at 27°C days/28°C nights (Heuchert and Mitchell, 1983). The daily intensity of sunlight is also an important consideration. When soybean plants were grown at various levels of shading in a controlled environment, sensitivity to mechanical stress was inversely proportional to the level of light at which the plants were grown (Jones *et al.*, 1990). At photon fluxes greater than 300$\mu molm^{-2}s^{-1}$, soybean growth was hardly affected by a vigorous daily shaking regime.

Another important consideration is the developmental stage of the plant. The insolubilisation of structural proteins, including extensins, occurs when cell elongation ceases (Carpita and Gibeaut, 1993). If extensin is involved in a seismic stress response then an up-regulation may only be seen in expanding tissues. Shaking would have no effect when growth was complete as the process is irreversible once the cell wall has been laid down. For example, light manual stroking of the elongating internodes of red kidney bean seedlings drastically limited the final internode length (Jaffe, 1976a). However, once retardation of internode elongation had occurred, cessation of thigmic treatment did not reverse the compression of those internodes, although subsequent developing internodes elongated normally. Following experiments with mechanical stress induced by phototropic responses of plants, Salvà and Jamet (2001) suggested that the role of

Ext1.4, as is the role of other extensins, is to strengthen and stiffen cell walls of the recently expanded curved region, as accumulation of RNAs occurs after curvature is complete. Its deposition would be followed by insolubilisation to prevent further extension of cell walls (curvature is not reversible as a second change in light direction with regard to the apex caused curvature of stems closer to the apex).

The association of extensin with cessation of growth has been well documented (Sadava and Chrispeels, 1973; Klis, 1976; Van Holst *et al.*, 1980; Carpita and Gibeaut, 1993; Ahn *et al.*, 1998), therefore the synthesis of extensins in areas subjected to mechanical stress is not surprising, since part of the specific stress response is a decrease in the length of stems and internodes. Microscopical studies have revealed that retardation of axial stem growth may occur exclusively by inhibition of cell elongation (Jaffe and Biro, 1979) or by inhibiting both cell division and cell elongation (Boyer, 1967). However, the other main physiological response to mechanical stress, stem swelling, involves both promotion of cell division, especially of secondary xylem, and lateral enlargement of inner tissues (Biro *et al.*, 1980). There have been mixed reports about the involvement of extensins in dividing cells. Hirsinger *et al.* (1999) found that expression of *Ext 1.4* did not seem to be related to cell division in adult tobacco plants. However, in some cases, the expression of HRGP genes is correlated with cell division (Ludevid *et al.*, 1990; Smallwood *et al.*, 1994). When Ito *et al.* (1998) applied the method of differential screening of a library prepared from *Catharanthus roseus* cells to isolate plant cell-cycle-dependent genes, two extensin cDNAs were identified. The expression of these genes, *cyc15* and *cyc17*, was regulated through the cell division cycle and it was proposed that it might be related to cell proliferation. However, the highest levels of transcripts were observed in the stationary phase of the growth cycle and therefore gene expression was apparently stimulated when cell proliferation ceased. This implies that at least two distinct mechanisms may regulate gene expression in *C. roseus* cells, one that works in actively dividing cells and another that stimulates expression upon the cessation of cell proliferation. These observations are consistent with the multiple roles that extensins are proposed to play in cell wall formation, and therefore in the mechanical stress response.

Finally, *extA* belongs to a large extensin multigene family, containing at least 7 other members (Evans *et al.*, 1990). As all extensins examined so far are thought

to have a role in maintaining the structural integrity of the cell wall, it is possible that other rape extensin genes are fulfilling this role in response to seismic stress to a greater extent than *extA*.

8.1.3. Future Work

Further experiments should concentrate on optimising conditions for a mechanical stress response, as this is a complex interaction of many factors. The level of seismic stress should be increased gradually and tissue samples taken at each level of stress. Duration of seismic stress is also an important consideration, therefore a similar experiment should be performed with gradual increases in shaking time. Perhaps future experiments should also concentrate on short bursts of stress. A more sensitive detection method, such as RT-PCR, should be used in further investigations, as northern analysis is limiting when working with low levels of mRNA. Taken together, the results of these experiments should build a more complete picture of *extA* gene expression in response to seismic stress.

It is possible that other genes in the extensin gene family, or other structural cell wall proteins are expressed to a greater extent than *extA*, which would explain the inability to induce *extA* in response to mechanical stress of a seismic nature. It would therefore be useful to differentially screen a cDNA library of shaken and non-shaken plants. Genes expressed under both conditions would cancel each other out so that only differentially expressed genes would be identified (Diatchenko *et al.*, 1996). The results of this would enable genes that were up-regulated in response to seismic stress to be identified. It would be interesting to see if these mirrored the "touch" genes found to be up-regulated in response to thigmic stress in *Arabidopsis* (Braam and Davis, 1990).

8.2. The production and analysis of transgenic tobacco plants containing *extA* promoter truncations of the NRR fused to GUS

8.2.1. The production of transgenic tobacco plants containing *extA* promoter truncations of the NRR

Nine promoter truncations of the negative regulatory region (NRR) from the *B. napus extA* promoter were constructed by PCR using the plasmid pAS41 as a template. Truncations were translationally fused to the GUS reporter gene in the binary vector pBI101.2 at the *Hind* III and *Sma* I sites. Each of the pBI101.2-based plasmids containing the *extA* promoter truncations were mobilised from *E. coli* into the disarmed *Agrobacterium tumefaciens* strain LBA4404 in a tri-parental mating and the resulting strains used to transform tobacco plants.

The resulting transgenic tobacco plants were analysed by Southern hybridisation to identify plants containing intact and un-rearranged copies of the gene. Several transgenic plants containing rearranged T-DNA insertions were identified, where several bands of varying sizes were found to hybridise to the GUS probe. This type of rearrangement is caused by breakage of the T-DNA during the transfer process or rearrangements during integration (Herman *et al.*, 1990). However, in general rearrangements proved to be rare, ranging from 0% to 9% in all but the –481bp truncation transformation (T9 plants). In this case, the number of re-arrangements was relatively high at 36%, although still within the range reported for other plant transformations (Herman *et al.*, 1990).

The introduction of T-DNA into the plant genome is dependent on infection by *Agrobacterium*, therefore plants containing a high proportion of rearrangements, such as the T9s, may have been subjected to a higher concentration of *Agrobacterium* cells in the co-cultivation media. This may have increased the likelihood of plant cells being infected. This has been previously demonstrated in tobacco where the transformation efficiency increased from 6% to 67% as the number of *Agrobacterium* cells was increased from 10^7 to 10^{10} (Lin *et al.*, 1995). Increasing the *Agrobacterium* cell concentration is also likely to increase the chance of several copies of T-DNA being taken up by a single cell, resulting in multi-copy

plants. Such plants are likely to express the transgene to higher levels. This would explain why certain transformations produced plants with a greater percentage of high expressers. Indeed, transformations containing the greatest number of rearrangements, such as the T2, T3 and T9s also tended to produce the greatest number of high expressers.

Variation in the amount of GUS expression in the transgenics may also be due to the position effect. The *extA* promoter truncation-GUS fusions are most probably inserted at random locations in the tobacco genome. Each transgene is therefore subject to different chromatin structures, which can influence the transcriptional activity of the inserted gene (Coates *et al.*, 1987). Non-transcribed DNA is usually found in a compact, well ordered nucleosome array, whereas active or potentially active sequences have more 'open' conformations, ranging from a less compact nucleosome structure to nucleosome-free stretches of DNA where transcriptional activity is high. Neighbouring regulatory elements such as enhancers and silencers may also affect the expression of the introduced gene (Breyne *et al.*, 1992). Gittins *et al.* (2001) noted position effects when 940bp of the *extA* promoter fused to GUS was introduced into apple by transformation. Histochemical data from *extA* in tobacco (Elliott and Shirsat, 1998) also showed that there were extremely large variations in expression of the transgene in petiole and stem sections containing the sequential deletions.

8.2.2. An element located between –569 and -547bp of the *extA* promoter negatively regulates phloem-specific expression

In previous work (Elliott and Shirsat, 1998), a region of the *extA* extensin promoter between -664 and -433bp (the NRR) was shown to direct phloem specific *extA*-driven GUS expression in mature tissues of transgenic tobacco. It was postulated that this region contained a *cis*-element (the NRE) that bound a repressor protein, the removal or modification of which resulted in loss of negative control, leading to non-specific expression.

Histochemical analysis of the nine PCR-generated NRR promoter truncations presented in chapter 5 revealed that this element is located between –569 and –547bp of the *extA* promoter, relative to the transcription start site. When present (in the -636bp, -619bp, -586bp, -569bp truncations) the NRE represses expression in non-

phloem cell-types. However, once deleted (in the -547bp, -524bp, -505bp, -481bp, and -452bp truncations), repression no longer occurs and expression is seen in all cell types, including the phloem.

Sequence analysis using the TRANSFAC and PLACE databases revealed the presence of a MYB core sequence (Biedenkapp *et al.*, 1988) between –549bp and –544bp, which was disrupted in the –547bp promoter truncation construct. MYB proteins have been shown to be involved in the regulation of phenylpropanoid plant defence genes in *Petunia hybrida* (Solano *et al.*, 1995) and have been implicated in the vascular specific expression of the cinnamyl alcohol dehydrogenase gene from eucalyptus trees (Lauvergeat *et al.*, 2002). The sequence, therefore, deserved further investigation.

8.2.3. Weight-loading is unable to induce GUS expression in transgenics containing any of the *extA* promoter truncation constructs

Shirsat *et al.* (2003) reported that the NRR is responsive to tensile stress as transgenics containing this region expressed GUS when weight-loaded, whereas transgenics lacking the NRR did not. Section 5.3 detailed the identification of a region between –569 and –547bp of the *extA* 5′ region that appeared to direct phloem specific expression of GUS, as discussed above in section 8.2.2. If the same element that controls phloem specific expression also controls the response to tensile stress, then transgenic tobacco containing -569bp should respond to weight-loading whereas those containing -547bp should not. As an example of a stressed node from a plant containing the -547bp construct shows (figure 5.6), weight-loading does not induce GUS expression in this or any of the transgenics containing the nine constructs.

It is possible that the environmental conditions in the growth room were not conducive for a mechanical stress response to occur. This phenomenon is discussed in section 8.1.2. The experiment was repeated many times on different plants of differing ages and using weights of a different mass, therefore it is unlikely that any of these factors could have caused the unexpected result.

A possible explanation for the lack of induction of *extA*/GUS in any of the construct-containing transgenics in response to weight-loading may be due to the fact that a different mechanism controls the plant response to mechanical stress than the

phloem-specific expression. This may be linked to the complex binding properties of the NRR discussed in detail in the next section.

8.3. EMSAs to determine *in vitro* binding properties of sequences within the NRR

EMSAs presented in chapter 3 using oligonucleotides from the NRR containing putative binding sequences showed that a rape lamina nuclear protein bound to all three sequences with similar mobility in the gel matrix. The properties of this binding interaction were also similar in that a large amount of competitor DNA was required to abolish binding to the sequence, and that the protein in question appeared to bind as a complex, as figure 3.10 showed a sequential decrease in electrophoretic mobility of the bound probe with the addition of more nuclear protein extract. The formation of this complex was, however, relatively sequence specific as binding did not occur to an unrelated DNA sequence from the *S. calcitrans* promoter. Binding was also tissue and species specific because no complex was formed with the addition of nuclear extract from rape petiole or any of the tobacco tissues.

EMSAs in chapter 6 focussed on sequences upstream of -547bp from the *extA* promoter because *in vivo* analysis using internal promoter truncations of the NRR revealed that vascular specific expression was lost downstream of this region (chapter 5). This implied that a sequence between -569bp and -547bp was negatively regulating *extA* extensin gene expression. Oligonucleotides were designed to investigate a putative element identical to a MYB core sequence (Biedenkapp *et al.*, 1988).

Two synthetic double-stranded oligonucleotides, designated *Myb* and *½Myb* were designed to span the MYB core and disrupt the core respectively. A third, *LongMyb* was designed to incorporate both sequences. EMSAs using nuclear protein extracts from rape and tobacco lamina revealed relatively non-specific binding to *Myb* and *½Myb* sequences to form a complex of similar mobility (B1). However, two highly specific complexes (B1 and B2) were formed with the *LongMyb* probe and rape lamina nuclear protein.

8.3.1. The composite nature of the NRR

From the evidence presented in chapter 3 and 6, it would appear that the NRR binds several nuclear proteins *in vitro*. The composite nature of an extensin promoter has been previously documented. Granell *et al* (1992) (see section 3.1.1) reported that two probes, 1 and 2 (figure 8.1), from a carrot wound-induced extensin promoter region bound proteins of similar electrophoretic mobility and that binding to either probe could not be out-competed by competition with the other probe. What is more, there was no sequence similarity between the two sites, apart from the fact that both regions were AT-rich. The interaction was also tissue specific for probe 1, as the complex was absent when nuclear extracts prepared from carrot cells were used and was present when nuclear extracts from carrot protoplasts were used. Binding occurred with extracts from both tissues when probe 2 was used. In addition, a second shift of lower mobility and lower intensity than the main shift was observed when probe 2 was used but only with extracts prepared from carrot cells. This implied that a second complex was formed.

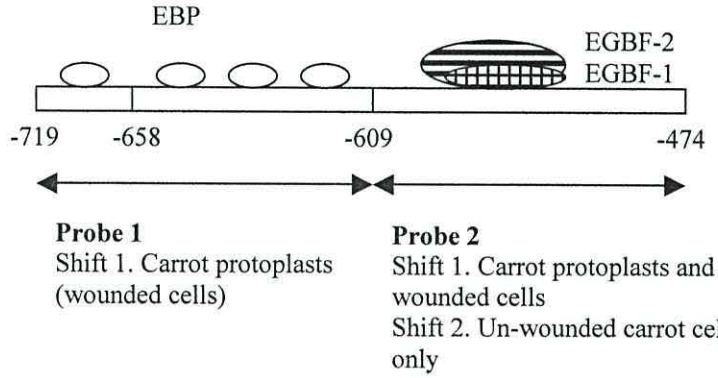


Figure 8.1. Diagram to show the regions of the carrot *pDC5AI* extensin promoter that bind nuclear proteins from wounded (protoplast) and unwounded cells. Two probes from this region were used in EMSAs, probes 1 and 2. EBP = Extensin Binding Protein. EGBF-1 and-2 = Extensin Gene Binding Factor-1 and -2.

The similarity between the nuclear protein binding patterns of the *extA* promoter from oilseed rape and the *pDC5AI* carrot extensin promoter is striking. In common with the carrot extensin promoter, the 231bp region from *extA* between -664bp and -433bp, previously identified by Elliott and Shirsat (1998) as being a negatively regulated region, was also shown to bind nuclear proteins of similar

mobility at different sites showing no common sequence similarity. However, only the *Lec-2* sequence from the *extA* promoter is AT-rich. Despite this, binding of proteins to the different carrot probes cannot simply reflect the AT content of the probes because of the inability of the two promoter fragments to compete for binding (Granell *et al.*, 1992). In the work by Granell *et al* (1992), DNA methylation interference studies established that binding of the same protein (EBP) occurred to four sites within probe 1, consisting of the upstream promoter region from -719 to -609bp (figure 8.1). This fragment contained the distal 61bp, which reduced extensin expression by 48% when removed (section 1.3.6). Only a single protein-DNA complex was observed in the EMSAs with this fragment. From the methylation interference studies, this was seen to correspond to a situation in which all four sites were occupied. Only one of these four sites resided within the 61bp fragment that, when deleted, reduced expression levels. Granell *et al* (1992) hypothesised that all four sites were occupied *in vivo* and contributed to the high level of expression characterised for the full-length extensin promoter. This result is indicative of combinatorial control.

Several examples of combinatorial control in animals have been identified and shown to lead to the formation of complex enhanceosomes (Carey, 1998). For example, a detailed analysis of an area of the IFN$^{\beta}$ (interferon$^{\beta}$) promoter responsible for enhancement in response to viral infection revealed a number of distinct sequences located close to each other to form a compact and complex regulatory region (Thanos and Maniatis, 1995; Kim and Maniatis, 1997). Six distinct proteins were shown to bind to this region.

Combinatorial control has also been observed in plant promoters. The auxin-responsive soybean *GH3* gene promoter is composed of multiple response elements (AuxREs) contributing incrementally to the strong auxin inducibility of the promoter (Ulmasov *et al.*, 1995). At least three independent AuxREs are found in the *GH3* promoter, two of these contain a TGTCTC element and a third lacks this element (Liu *et al.*, 1994). It has been proposed that the organisation of AuxRE modules and the types of composite AuxREs that make up these modules allows for a wide variety of developmental and spatial patterns of auxin-induced gene expression (Ulmasov *et al.*, 1995), a property that would be important in all genes with complex regulation, including those coding for extensin. Yamauchi (1997) found that several sequences

in the upstream region of a seed storage gene from the Japanese jack bean interacted with embryo nuclear proteins. Some proteins interacted with AT-rich sequences whereas others bound to regions containing CA-rich sequences. Heat shock genes from a number of organisms including soybean are regulated by multiple heat shock elements (Baumann *et al.,* 1987). Finally, the activation of the *Nicotiana sylvestris* cyclin B1 promoter has also been shown to involve multiple regulatory elements, one of which contains a MYB binding sequence (Tréhin *et al.*, 1999).

Detailed analysis of the *grp1.8* gene promoter from *Phaseolis vulgaris* showed that a combinatorial interaction of three regulatory elements was responsible for vascular specificity (Keller and Baumgartner, 1991; Keller and Heierli, 1994). One of these elements was a negative regulatory sequence, the deletion of which resulted in lower expression and loss of vascular specificity (Keller and Baumgartner, 1991). A region overlapping with the NRE was later shown to bind a bZIP transcription factor called VSF-1 (vascular specificity factor 1) (Torres-Schumann *et al.*, 1996). A sequence showing homology to this element was also found in the *extA* NRR, and the oligonucleotide incorporating this sequence (*Vs-2/Enh-1*) was found to bind nuclear proteins from rape lamina extract (section 3.2.5 and Shirsat *et al.*, 2003). The composite nature of the *grp 1.8* promoter has been suggested by studies performed by Ringli and Keller (1998) which indicated that more than one factor is involved in the control of gene expression by the *vs-1* element.

In the current work, part of the NRR from *extA* has been mapped by EMSAs. It is possible that all sites tested in the *extA* promoter are also occupied *in vivo* and contribute to the overall mechanical stress response. Regulation of extensin in response to this type of stress has been shown to be extremely varied and complex, which may be reflected in the complexity of promoter organisation and binding properties. Such co-operative binding would account for the lack of induction of extensin upon the application of mechanical stress to any of the transgenics containing the promoter truncation constructs. If all elements were required for the mechanical stress response then extensin expression would not be seen.

Based on analysis of promoter truncation constructs of the NRR in transgenic tobacco (section 5.3.2), the vascular specific expression of *extA* would appear to be controlled by elements downstream of -569bp, or at least elements within this region

are the most important for tissue specific expression. The composite nature of the *extA* NRR can only be hypothesised from these results, as additional information on the exact binding positions is missing. However, what is certain is the importance of the region incorporated in the *LongMyb* probe. In addition to directing tissue specific expression of the gene fusion, this region also forms two specific complexes (B1 and B2) with rape lamina extracts. B1 and B2 complexes formed with *LongMyb* have binding properties that differ from those seen in the *½Myb-*, *Myb-* and *Vs-2/Enh-2-, Lec-2* and *Enh-1*-DNA-protein complexes. A 150-fold molar excess of cold homologous probe is sufficient to abolish the formation of the second *LongMyb* complex (B2) and to significantly reduce binding of the main complex (B1). This, together with the inability of *½Myb* to compete for binding with either complex formed with *LongMyb*, implies that different binding factors are involved, albeit with a similar mobility. Granell *et al* (1992) also found that probes 1 (containing the 61bp sequence necessary for high expression levels) and 2 (containing EGBF-1 and EGBF-2 sites) produced shifts of similar mobility, despite the fact that different factors were involved in binding (figure 8.1). Mobility of a complex in a gel is therefore not necessarily an indicator of binding protein homology. It is more likely that multiple DNA binding proteins exist in plant nuclear extracts and are able to interact specifically with promoter sequences. Holdsworth and Laties (1989a) proposed that EGBF-2 was a different form of EGBF-1, as footprinting revealed different binding properties.

If the protein that bound to the *½Myb* oligonucleotide containing the disrupted MYB core sequence was specific then the MYB core sequence as a putative binding site could be ruled out. However, factors binding to *Myb* and *½Myb* were shown to be non-specific (section 6.3.2). Despite the fact that specific binding did not occur to the *Myb* probe containing an intact MYB core, it is still possible that the MYB core is involved in binding. This is because a 150-fold molar excess of un-labelled *Myb* oligonucleotide partially competed for binding with the B2 complex, whereas an unrelated sequence from *S. calcitrans* and un-labelled *½Myb* probe did not (figure 6.4). The higher-affinity binding site (*LongMyb*) competed more protein away from the labelled site, resulting in a stronger reduction in the intensity of the retarded band, observed in figure 6.3, lane 4. Lower affinity sites, such as *Myb,* are less efficient at competition, leaving more protein for complex formation with

labelled oligonucleotide (Dent *et al.*, 1999) (figure 6.4). The fact that only partial competition was observed implies that the *Myb* probe contains nucleotides important for the formation of the B2 complex but that essential nucleotides are present within the extended *LongMyb* probe. In this case, sequences flanking the core present in the longer probe could be important for facilitating binding. This has been demonstrated many times with other plant gene promoters (Williams *et al.*, 1992; Izawa *et al.*, 1993; Urao *et al.*, 1993; Yanagisawa and Schmidt, 1999). When sequences flanking the VSF-1 binding site on the French bean *grp 1.8* gene (which directs vascular specific expression by negative regulation) were mutated, differences in binding affinity of VSF-1 were observed (Ringli and Keller, 1998).

8.3.2. Nuclear proteins from rape lamina extract bind promiscuously to the NRR

The composite nature of the NRR has been discussed in section 8.3.1 but it is not clear whether the same factor is binding to all sequences non-specifically or whether there are different factors involved. The presence of multiple binding sites for *trans*-acting factors is a common feature of eukaryotic enhancers (Schaffner *et al.*, 1988).

An example of promiscuous binding and negative regulation in animals is found in the glucocorticoid receptor protein (reviewed by Schaaf and Cidlowski, 2002). Sakai *et al.* (1988) mapped seven glucocorticoid receptor-binding sites (GREs) in the upstream region of the prolactin gene and showed that at least three played a role in the negative regulation of prolactin gene expression. These elements were termed nGREs. The sequences of these elements were too heterogeneous to identify a consensus sequence but it is thought that binding of the receptor protein replaces or prevents binding of a transcription factor that would induce transcription (Drouin *et al.*, 1989; Nakai *et al.*, 1991). The glucocorticoid receptor protein can also act as an activator protein and can bind to receptor sites bearing minimal resemblance to the nGREs (Sakai *et al.*, 1988).

Several authors have shown an interaction of plant bZIP proteins with palindromic sequences which lack the consensus ACGT core or even with non-palindromic sequences (dePater *et al.*, 1994; Ulmasov *et al.*, 1995; Dröge-Laser *et al.*, 1997; Ringli and Keller, 1998).

However, a DNA binding protein that exhibits promiscuous interaction specificity *in vitro* may acquire substantially improved specificity when complexed with one or more other proteins *in vivo* (Johnson and McKnight, 1989). An ancillary factor that improves the binding specificity of a gene regulatory protein might operate by contributing additional contact surfaces with the DNA through its own polypeptide structure, or indirectly by prompting an allosteric change in the protein, causing it to bind certain DNA sequences more avidly (Johnson and McKnight, 1989). Yamamoto (1985) suggested that the specificity of an enhancer is generated through the varied combinatorial arrangement of its *cis*-regulatory elements. He used the analogy of a jigsaw to illustrate how a regulatory protein must be compatible with multiple surfaces to fit into an enhancer. These surfaces include both its cognate DNA sequence motif and protein structural motifs presented by adjacently bound proteins. Such an analogy can be applied to the *extA* NRR, where the apparent promiscuous binding of nuclear proteins could represent important pieces in the jigsaw that results in *extA* gene expression.

It is possible that a MYB protein is involved in all these interactions as MYB is known to bind non-specifically to DNA (Lüscher and Eiseman, 1990). Furthermore, it has been suggested that the binding specificity of MYB transcription factors may be affected by interactions with other protein factors (Sainz *et al.*, 1997). If this is the case, then the presence of the B2 complex when *LongMyb* probe was used could have led to the increased specificity of the B1 complex.

8.3.3. A model for the binding of rape lamina nuclear proteins to the NRR

A model for proteins binding to the NRR based on EMSAs with all probes is proposed in figure 8.2. Sequences containing *Enh-1, Lec-2* and *Vs-2/Enh-2* elements bind a nuclear protein with similar properties (figure 8.2, lanes 1, 2 and 7). Figure 8.2b shows this as the same protein (red triangles), although it has not been established whether this is a homologous protein or three different proteins recognising different sequences, albeit with similar binding properties. In the model, all DNA-protein complexes of the same mobility are designated B1. Despite sharing the same mobility, some of these proteins exhibit different binding properties so are either different proteins or different forms of the same protein, as in EGBF-1 and EGBF-2 (Granell *et al.*, 1993). The protein binding non-specifically to *Myb* and

½Myb is shown in green. It is possible that this also binds non-specifically to *LongMyb,* which could explain the residual shift following competition with 150-fold molar excess of non-labelled *LongMyb* (figure 6.3, lane 4 and figure 8.2a, lane 4). The most important interaction is shown in blue. Here, a specific protein (B1) binds to a sequence on the *LongMyb* probe. Figure 8.2b shows this sequence as being the MYB core, although binding to the exact core sequence has not been established. A second highly specific complex (B2) is formed on the *LongMyb* probe (lane 3). A possible mechanism of repression could involve the mediation of DNA secondary structure by B2, allowing B1 to bind specifically to the MYB core. Equally, the binding of B2 could be mediated by the presence of B1. It is also possible that B2 does not bind DNA directly but binds as a heterodimer to B1. For example, early auxin response proteins have been shown to bind as heterodimers to proteins binding to AuxREs, suggesting that they act as transcription factors, despite being unable to bind DNA directly (Ulmasov *et al.*, 1997a; 1997b). These proteins function as active repressors by dimerising to auxin response factors bound to auxin response elements (Tiwari *et al.*, 2001).

Despite the composite nature of the NRR control motifs of the *extA* promoter, it appears that some elements are more important than others. Granell *et al* (1992) found that nuclear factors bound to 4 regions of the carrot extensin promoter fragment but that the first of these elements was the most important in controlling extensin expression levels. In the current work, *in vivo* analysis of the *extA* promoter found that the region between –569 and –547bp was important in directing vascular specific expression. *In vitro* analysis has shown that there is much binding activity in the region upstream of –547bp. However, vascular specific expression was only affected by a deletion of the –547bp region, implying that proteins bound to elements upstream of –569bp, such as rape lamina proteins bound to *Lec-2* and *Enh-1,* are not essential for this expression pattern but may contribute to control in some other way. It is therefore possible that the highly specific B1 and B2 binding proteins are the factors responsible for repression of *extA* in non-phloem tissues because they interacted with the region between –569bp to –535bp. The whole sequence in this region is necessary for binding to occur because neither specific complex was observed when only part of the sequence was present i.e. when *Myb* and *½Myb* probes were used.

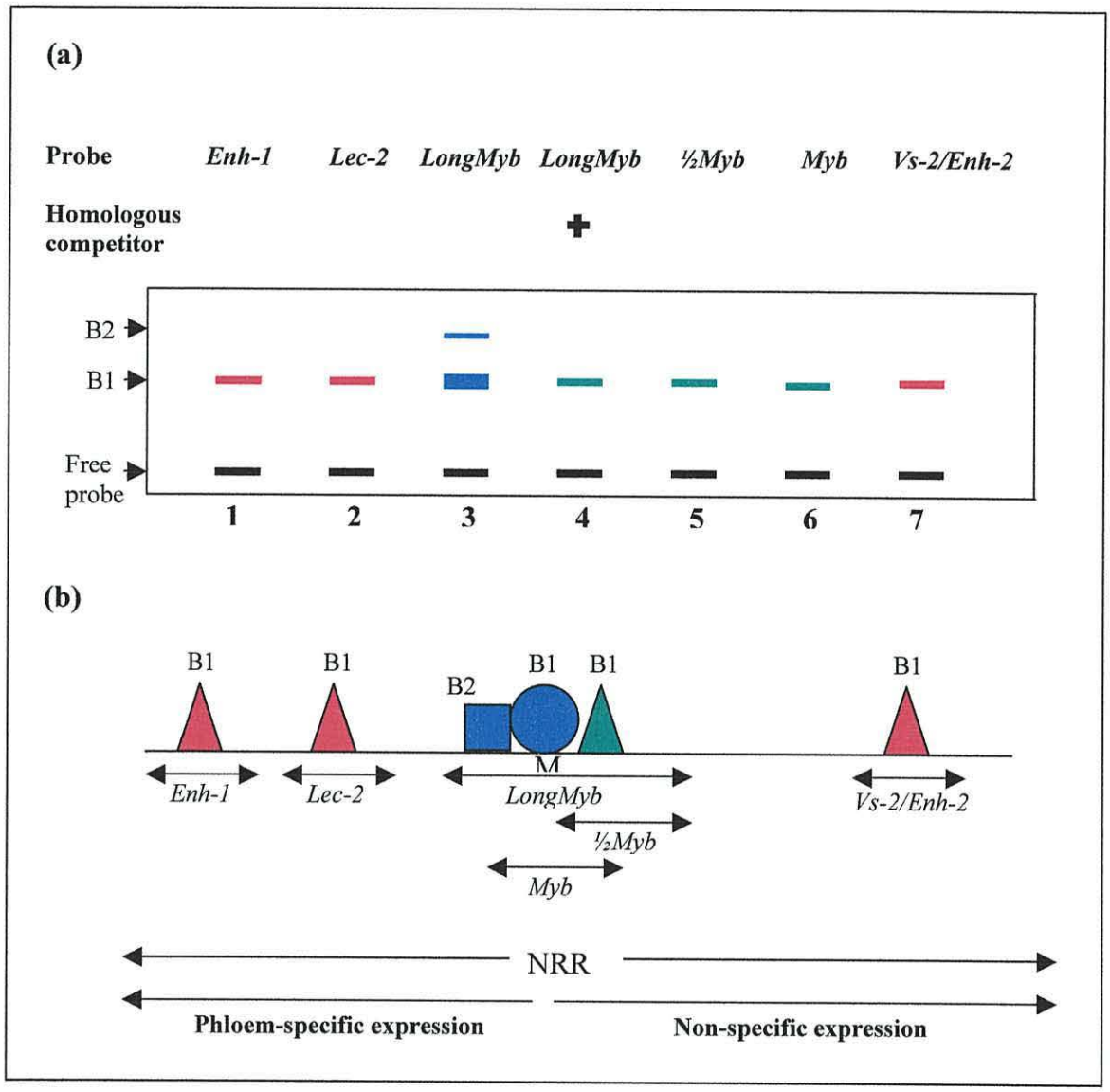


Figure 8.2. Schematic of a possible binding scenario for rape lamina nuclear proteins to the *extA* NRR (not to scale).

(a) Diagrammatic representation of the EMSA results obtained in chapters 3 and 6 and in relation to the model in (b).

(b) Model of possible binding scenarios of the complexes identified in (a) to the NRR.

Binding occurs to all sequences to form a complex of similar mobility, B1. The sequence overlapping between *½Myb* and *Myb* is able to form B1 non-specifically (▲). A second B1complex with the same mobility (●) binds to the MYB core (**M**), but only when B2 (■) is present i.e. only when the longer fragment *LongMyb* is used as a probe. These specific complexes (lane 3) can be out-competed by a 150-fold molar excess of non-radioactive *LongMyb* oligonucleotide leaving a band corresponding to non-specific B1 complex (lane 4) (▲). Another B1 complex is formed with *Enh-1, Lec-2* and *Vs-2/Enh-2* probes (▲).

8.3.4. Repressor proteins are only present in rape lamina extracts, where the *extA* transcript is absent

Extracts from rape lamina contained nuclear proteins able to bind to sequences within the NRR in EMSAs, whereas petiole extracts did not. The tissue specificity of the binding interactions is extremely significant. Northern analysis of *B. napus* has shown that the *extA* transcript is not present in lamina extracts but is present to low levels in the petiole and stem (Shirsat *et al.*, 1991). Work presented in section 7.2 has shown that *extA* mRNA is very abundant in mature petioles and totally absent in mature lamina. Tissues from *B. napus* used for nuclear protein extraction were taken when the plants were very mature. The presence of a repressor protein in lamina extracts, suppressing expression of *extA* would therefore account for the lack of *extA* transcript in lamina tissue. If this hypothesis is correct, then the absence of a repressor protein (or proteins) in petiole tissue would enable transcription to proceed.

The *extA* promoter has been shown to be fully functional in several heterologous systems (Shirsat *et al.*, 1996a; Gittins *et al.*, 2001) including tobacco, therefore it can be assumed that tobacco contains the necessary nuclear factors for gene activation. Despite this, EMSAs with tobacco nuclear extracts presented in chapter 3, failed to reveal any binding to putative elements that were able to bind rape lamina nuclear proteins. However when the *Myb*, *½Myb* and *LongMyb* oligonucleotides were used in the assays, a protein present in tobacco lamina extracts was able to bind to all probes, with a slightly lower mobility to that seen with rape lamina extracts. Internode and petiole extracts did not produce a shifted band.

Unlike rape lamina extracts, tobacco lamina extracts were not able to form a second complex (B2) with the *LongMyb* probe. This could mean that tobacco lamina does not contain all the proteins necessary for the formation of this complex or that B2 does exist but in such small quantities that it was not detectable on the autorad (sometimes B2 was only just visible when oilseed rape lamina extracts were used). Shirsat (personal communication) was unable to detect a signal in northern analysis when the GUS coding sequence was used to probe transgenic tobacco tissue RNA extracts containing *extA* promoter-GUS fusions. This implies that, although the transgene is clearly expressed in tobacco, as visualised by histochemical analysis, this may only be to low levels, i.e. beyond the limits of detection for northern analysis. Conversely, northern analysis clearly detects *extA* mRNA from *B. napus*

tissue extracts. It is therefore possible that the system operates at two different levels in tobacco and oilseed rape. This might explain the lack of binding to probes by nuclear proteins from tobacco extracts. It is also possible that tobacco regulates *extA* expression by a different mechanism. Different nuclear proteins may regulate *extA* expression in tobacco and the binding properties of these proteins may fall outside the limits of the EMSAs in these experiments. Future experiments should alter the binding conditions and increase the concentration of tobacco nuclear protein extracts added to the binding reaction mixture to establish whether the probe is saturated and whether a second complex can be produced.

The *LongMyb* probe complexes formed with factors from tobacco and rape lamina extracts are unlikely to be composed of the same protein because both complexes have different binding properties. In addition to the fact that binding to rape lamina nuclear extract is stronger and involves the presence of a second complex, binding of protein from tobacco extracts can not be out-competed by a 150-fold molar excess of cold homologous probe, or even significantly reduced. The binding pattern of factors from tobacco lamina extracts is more like that of the factors from rape lamina seen to the *Vs-2/Enh-2, Lec-2* and *Enh-1* probes, than to that of the highly specific binding seen to *LongMyb*.

8.3.5. Future work

Further work involving experiments using EMSAs should be undertaken to locate the exact nucleotides involved in binding nuclear proteins. Point mutations within the oligonucleotides should establish which bases are essential for binding. EMSAs based on the developmental expression of *extA* in oilseed rape (section 7.2) may confirm that the absence of a factor binding to petiole tissue is indeed determined by the fact that *extA* mRNA is expressed to high levels in the petiole. Results from section 7.2 have shown that *extA* mRNA is not present in young petioles but is present in cotyledon laminae. EMSAs with these tissues should be informative.

A footprint of the NRR should clarify the role of each site shown to bind nuclear proteins from rape lamina and possibly elucidate more sites of DNA-protein interaction. The most common type of footprinting is deoxyribonuclease I (Dnase I) protection mapping and is based on the fact that bound proteins protect the phosphodiester backbone of DNA from Dnase I catalysed hydrolysis. Binding sites

are visualised by autoradiography of the DNA fragments that result from hydrolysis, following separation by electrophoresis on denaturing DNA sequencing gels (Brenowitz *et al.*, 1989).

Once a binding site for the protein in question has been established then this could be mutated and the *extA* promoter containing the mutated binding site fused to the GUS reporter gene to establish whether specificity of binding is still maintained.

Once the binding properties of the protein in question have been identified, questions about the nature of the protein need to be answered. The size of a DNA-binding protein can be determined by covalently cross-linking the protein to its regulatory sequence using UV light and resolving the protein complexes on an SDS polyacrylamide gel (Buratowski, 1996). The ultimate goal would be to isolate the factors involved in the negative regulation of *extA* expression. The gene responsible for the production of the binding protein could be identified using south-western screening and subsequently cloned. This step could be followed by the radioactive labelling of a multimer of the binding sequence to use as a probe to screen an expression library. The gene coding for the putative transcription factor involved in the regulation of extensin gene expression in response to mechanical stress could then be characterised.

The possibility that a MYB protein is binding to the *LongMyb* probe could be investigated using antibody supershift experiments. Antisera raised against the MYB transcription factor may affect its binding by one of two methods (Dent *et al.*, 1999). First, the antibody may bind to a site on the transcription factor that is essential for DNA binding, therefore blocking the ability of the factor to bind DNA and resulting in the complete absence of a complex from the gel. Secondly, if the antibody binds to a non-essential site on the factor, DNA binding may not be impaired but the mobility of the complex will be altered resulting in a super-shift to a position of lower mobility. Such an experiment could also be performed using the other probes from the NRR to establish whether the protein binding to all sequences is homologous.

8.3.6. Applications for agriculture

Altering the expression of certain transcription factors can greatly influence plant stress tolerance. For example, over-expression of two *Arabidopsis* AP2/ERBP

genes, *CBF1/DREB1B* and *DREB1A* results in enhanced tolerance to drought, salt and freezing (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999). These two transcription factors bound to a cold responsive *cis*-element and activated the expression of target genes. In another example, knockout mutants of the *Arabidopsis AtMyb4* gene were found to produce higher levels of UV light-protective compounds, such as sinapate esters and to be more tolerant to UV-B light, whereas transgenic plants over-expressing *AtMyb4* were found to contain reduced levels of sinapate esters and to be more sensitive to UV-B light (Jin *et al.*, 2000). The expression of *AtMyb4* itself was repressed by UV-B light treatment, leading to the conclusion that *AtMyb4* may function as a negative regulator in controlling genes involved in the synthesis of protective sinapate esters. Such a knockout mechanism could also be used to over-express *extA* to create a stronger, more disease resistant plant.

The use of tissue-specific promoters to limit transgene expression has a huge biotechnological application as constitutive promoters such as CaMV 35S, with a lack of temporal and spatial regulation, have a number of potential drawbacks for use in genetically improved crops of the future (Gittins *et.al.*, 2000). There is the potential to use the *extA* promoter to drive transgenes whose gene products are active in stems to bring about dwarfing or to alter lignin composition, both major goals of tree biotechnology (Gittins *et al.*, 2001). For example, in the future, the use of antisense inhibition technology to control lignin biosynthesis in trees (Hu *et al.*, 1999) may require tissue specific promoters that target wood forming tissues rather than all parts of the tree.

8.4. Concluding remarks

In vivo analysis of NRR promoter truncation constructs fused to GUS revealed that the region between –569bp and –547bp was important for phloem specific expression of *extA*. When investigated further with EMSAs, an oligonucleotide comprising the region between -584 to –535bp was shown to form two highly specific complexes (B1 and B2). Based on their location in the NRR and the fact that these factors are only present in lamina extracts, where *extA* mRNA is absent, it is likely that these proteins are involved in the repression of *extA* expression. The fact that sequences upstream of –569bp were shown to be unimportant in directing

vascular specific expression of *extA in vivo*, implies that the negative regulatory element proposed by Elliott and Shirsat (1998) exists between –569bp and –535bp. A MYB core sequence disrupted by the formation of the –547bp truncation may be important in binding one or both of these proteins. If this is the case then a MYB protein could bind to the sequence. These MYB proteins have been shown to act as transcriptional activators and repressors and are known to be important in plant defence (Solano *et al.*, 1995; Martin and Paz-Ares, 1997), cellular proliferation (Chung and Parish, 1995; Ito *et al.*, 1998; Tréhin *et al.*, 1999) and the maintenance of plant structure (Oppenheimer *et al.*, 1991; Noda *et al.*, 1994), all of which have been proposed by various authors to be functions of extensin (section 1.3.4).

A nuclear protein from rape lamina extracts of similar mobility to B1 bound promiscuously and with similar binding properties to the *Lec-2, Enh-1* and *Vs-2/Enh-2* probes. This protein is thought to be abundant within rape lamina extracts and was unable to bind non-specifically to an unrelated sequence from *S. calcitrans*. The composite nature of an extensin promoter has been shown previously (Holdworth and Laties, 1989a; Granell *et al.*, 1993) therefore this possibility also exists for *extA*. Combinatorial regulation is a powerful mechanism enabling transcription to be tightly controlled. Such a powerful control mechanism would be fitting in a gene with such complex regulation as *extA*.

A binding site *in vitro* may not necessarily represent a binding site *in vivo*. *In planta* much of the chromatin is masked by histones and other DNA binding proteins, rendering it inaccessible to transcription factors (Dent *et al.*, 1999). Although sequence analysis of extensin promoters reveals homologies to already described *cis*-elements (see figure 6.1), only a few of them might be functional. There may be no correlation between their presence in a promoter and the way a gene is regulated and equally, there may be no correlation between binding to sequences containing these elements and their functionality in a promoter. A combination of *in vivo* and *in vitro* approaches are the best way to test the putative regulatory domains or elements. There is therefore much scope for future work.

References

- **Adams, C.A., Nelson, W.S., Nunberg, A.N. & Thomas, T.L.** (1992). A wound-inducible member of the hydroxyproline-rich glycoprotein gene family in sunflower. Plant Physiology **99:** 775-776.

- **Agbuehi, S. & Brandle, J.** (1981). Influence of windbreak shelter on soybean production under rain-fed conditions. Agronomy Journal. **73:** 625-628.

- **Ahn, J.H., Choi, Y., Kwon, Y.M., Kim, S.G., Choi, Y.D. & Lee, J.S.** (1996). A novel extensin gene encoding a hydroxyproline-rich glycoprotein requires sucrose for its wound-inducible expression in transgenic plants. Plant Physiology **99:** 775-776.

- **Ahn, J.H., Choi, Y., Kwon, Y.M., Kim, S.G., Kwon, Y.M., Choi, Y, D. & Lee, J.S.** (1998). Expression of a soybean hydroxyproline-rich glycoprotein gene is correlated with maturation of roots. Plant Physiology **116:** 671-679.

- **Akers, S. & Mitchell, C.A** (1980). Stimulation of plant growth by mechanical vibration. HortScience (suppl.) **14:** 6.

- **Albersheim, P.** (1978). Concerning the structure and biosynthesis of the primary cell walls of plants. In: International Review of Biochemistry. Biochemistry of Carbohydrates II. Vol 16. D. Manners (ed.), pp 127-151. University Park Press, USA.

- **Armstrong, G.A., Weisshaar, B. & Hahlbrock, K.** (1992). Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from parsley recognize diverse promoter elements with ACGT cores. The Plant Cell **4:** 525-537.

- **Arsenijevic-Maksimovic, I., Broughton, W.J., & Krause, A.** (1997). Rhizobia modulate root-hair-specific expression of extensin genes. Molecular Plant-Microbe Interactions **10:** 95-101.

- **Auble, D.T. & Hahn, S.** (1993). An ATP-dependent inhibitor of TATA binding protein binding to DNA. Genes and Development **7:** 844-856.

- **Auble, D.T., Hansen, K.E., Mueller, C.G.F., Lane, W.S., Thorner, J. & Hahn, S.** (1994). MOT1, a global repressor of RNA polymerase II transcription inhibits TBP binding to DNA by an ATP-dependent mechanism. Genes and Development **8:** 1920-1934.

- **Austin, R.B., Flavell, R.B., Henson, I.E. & Low, H.J.B.** (1986). Molecular Biology and Crop Improvement. Cambridge University Press, Cambridge.

- **Avila, J., Nieto, C., Cañas, L., Benito, M.J. & Paz-Ares, J.** (1993). *Petunia hybrida* genes related to the maize regulatory *C1* gene and to animal *myb* proto-oncogenes. The Plant Journal **3:** 553-562.

- **Bagley, W.** (1964). Responses of tomatoes and beans to windbreak shelter. Journal of Soil and Water Conservation **19:** 71-73.

- **Bao, W., O'Malley, D.M. & Sederoff, R.R.** (1992). Wood contains a cell-wall structural protein. Proceedings of the National Academy of Sciences, USA **89:** 6604-6608.

- **Baum, G., Levyadun, Fridmann, S., Arazi, T., Katnelson, H., Zik, M. & Fromm, H.** (1996). Calmodulin binding to a glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. The EMBO Journal **15:** 2988-2996.

- **Baumann, G., Raschke, E., Bevan, M and Schoffl, F.** (1987). Functional analysis of sequences required for transcriptional activation of a soybean heat-shock gene. The EMBO Journal **6:** 1161-1166.

- **Belanger, K. & Quatrano, R.S.** (2000). Polarity: The role of localized secretion. Current Opinions in Plant Biology **3:** 67-72.

- **Berg, J.M.** (1990). Zinc fingers and other metal binding domains. Journal of Biological Chemistry **265:** 6513-6516.

- **Berger, F., Taylor, A. & Brownlee, C.** (1994). Cell fate determination by the cell wall in early *Fucus* development. Science **263:** 1421-1423.

- **Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirkse, W., Van Staveren, M., Stiekema, W., Drost, L., Ridley, P., Hudson, S.A., Patel, K., Murphy, G., Piffanelli, P., Wedler, H., Wedler, E., Wambutt, R., Weitzenegger, T., Pohl, T.M., Terryn, N., Gielen, J., Villarroel, R., De Clerck, R., Van Montagu, M., Lecharny, A., Auborg, S., Gy, I., Kreis, M., Lao, N., Kavanagh, T., Hempel, S., Kotter, P., Entian, K.D., Rieger, M., Schaeffer, M., Funk, B., Mueller-Auer, S., Silvey, M., James, R., Montfort, A., Pons, A., Puigdomenech, P., Douka, A., Voukelatou, E., Milioni, D., Hatzopoulos, P., Piravandi, E., Obermaier, B., Hilbert, H., Dusterhoft, A., Moores, T., Jones, J.D.G., Eneva, T., Palme, K., Benes, V., Rechman, S., Ansorge, W., Cooke, R., Berger, C., Delseny, M., Voet, M., Volckaert, G., Mewes, H.W., Klosterman, S., Schueller, C. & Chalwatzis, N.** (1998). Analysis of 1.9Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. Nature **391:** 485-488.

- **Beyl, C. & Mitchell, C.A.** (1977). Characterization of mechanical stress dwarfing in chrysanthemum. Journal of the American Society of Horticultural Science **102:** 591-594.

- **Beyl, C. & Mitchell, C.A.** (1983). Alteration of growth, exudation rate, and endogenous hormone profiles in mechanically dwarfed sunflower. Journal of the American Society of Horticultural Science **108:** 257-262.

- **Biedenkapp, H., Borgemeyer, U., Sippel, E. & Klempnauer, K-H.** (1988). Viral *myb* oncogene encodes a sequence-specific DNA-binding activity. Nature **335:** 835-837.

- **Biggs, K.J. & Fry, S.C.** (1990). Solubilization of covalently bound extensin from capsicum cell walls. Plant Physiology **92:** 197-204.

- **Biro, R., Hunt, E., Erner, Y. & Jaffe, M.J.** (1980). Thigmomorphogenesis: changes in cell division and elongation in the internodes of mechanically perturbed bean plants. Physiologia Plantarum **62:** 289-296.

- **Biro, R. & Jaffe, M.** (1984). Thigmomorphogenesis: Ethylne evolution and its role in the changes observed in mechanically perturbed bean plants. Physiologia Plantarum **62:** 289-296.

- **Bonilla, I., Mergold-Villasénor, C., Campos, M.E., Sánchez, N., Pérez, H., López, L., Castrejón, L., Sánchez, F. & Cassab, G.I.** (1997). The aberrant cell walls of boron-deficient bean root nodules have no covalently bound hydroxyproline/proline-rich proteins. Plant Physiology **115:** 1329-1340.

- **Botella, J.R. & Arteca, R.N.** (1994). Differential expression of two calmodulin genes in response to physical and chemical stimuli. Plant Molecular Biology **24:** 757-766.

- **Bowles, D.J.** (1990). Defence-related proteins in higher plants. Annual Review of Biochemistry **59:** 873-907.

- **Boyer, N.** (1967). Modifications de la croissance de la tige de Bryone (*Bryonia dioica*) a la suite d'irritations tactiles. Comptes Rendus de l' Academie des Sciences Paris **264:** 2114-2117.

- **Boyer, N., Desbiez, M.-O., Hofinger, M. & Gaspar, T.** (1983). Effect of lithium on thigmomorphogenesis in *Bryonia dioica* ethylene production and sensitivity. Plant Physiology **72:** 522-525.

- **Braam, J.** (1999). If walls could talk. Current Opinions in Plant Biology **2:** 521-524.

- **Braam, J. & Davis, R.W.** (1990). Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell **60:** 357-364.

- **Braam, J., Sistrunk, M.L., Polisensky, D.H., Xu, W., Purugann, M.M., Antosiewicz, D.M., Cambell, P., Johnson, C.A.** (1996). Life in a changing world: TCH gene regulation of expression and response to environmental signals. Physiologia Plantarum **98:** 909-916.

- **Bradford, M.** (1976). A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein dye-binding. Analytical Biochemistry **72:** 248-254.

- **Bradley D.J., Kjellbom, P. & Lamb, C.J.** (1992). Elicitor and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defence response. Cell **70:** 21-30.

- **Brenowitz, M., Senear, D.F. & Kingston, R.E.** (1989). DNase I footprint analysis of protein-DNA binding. Current Protocols in Molecular Biology Suppl. **7.**

- **Breyne, P., Gheysen, G., Jacobs, A., Van Montagu, M. & Depicker, A.** (1992). Effect of T-DNA configuration on transgene expression. Molecular and General Genetics **235:** 389-396.

- **Brindle, P.K., Holland, J.P., Willet, C.E., Innes, M.A. & Holland, M.J.** (1990). Multiple factors bind the upstream activation sites of the yeast enolase genes ENO1 and ENO2: ABF1 protein, like repressor protein RAP1 binds cis-acting sequences which modulate repression or activation of transcription. Molecular Cell Biology **10:** 4872-4885.

- **Brisson, L.F. Tenhaken, R. & Lamb, C.** (1994). Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. The Plant Cell **6:** 1703-1712.

- **Brogan, I.W.** (1986). Control of rapeseed quality for crushing. In: Oilseed Rape. D.H. Scarisbrick and R.W. Daniels (eds.), pp 282-300. Collins Professional and Technical, London.

- **Brown, I., Mansfield, J. & Bonas, U.** (1995). Hrp genes in *Xanthomonas campestris p.v. vesicastitoria* determines ability to suppress papilla deposition in pepper mesophyll cells. Molecular and Plant-Microbe Interactions **8:** 825-836.

- **Buratowski, S.** (1994). The basics of basal transcription by RNA polymerase II. Cell **77:** 1-3.

- **Buratowski, S.** (1996). DNA-protein interactions. Current Protocols in Molecular Biology Suppl. **36.**

- **Burch, J.B.E. & Weintraub, H.** (1983). Temporal-order of chromatin structural-changes associated with activation of the major chicken vitellogenin gene. Cell **33:** 65-76.

- **Burden, D.W. & Whitney, D.B.** (1995). In: Biotechnology: proteins to PCR, a course in strategies and lab techniques. Birkhäuser, Boston.

- **Cairns, B.R.** (1998). Chromatin remodeling machines: similar motors, ulterior motives. Trends in Biochemical Science **23:** 20-25.

- **Carey, J.** (1991). Gel retardation. Methods in Enzymology **208:** 103-117.

- **Carey, M.** (1998). The enhancesome and transcriptional synergy. Cell **92:** 5-8.

- **Carpita, N.C. & Gibeaut, D.M.** (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. The Plant Journal **3:** 1-30.

- **Carthew, R.W., Chodosh, L.A. & Sharp. P.A.** (1985). An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell **43:** 439-448.

- **Cassab, G. I.** (1998). Plant cell wall proteins. Annual Review of Plant Physiology and Plant Molecular Biology **49:** 281-309

- **Cassab, G. I. & Varner, J. E.** (1987). Immunocytolocalisation of extensin in developing soyabean seed coats by immunogold-silver staining and by tissue printing on to nitrocellulose paper. Journal of Cell Biology **105:** 2581-2588.

- **Cassab, G. I. & Varner, J. E.** (1988). Cell wall proteins. Plant Physiology and Plant Molecular Biology **39**: 321-53.

- **Chandra, A., Thungapathra, M, Upadhyaya, K.C.** (1994). Molecular cloning and characterization of a calmodulin gene from *Arabidopsis thaliana*. Journal of Plant Biochemistry and Biotechnology **3:** 31-35.

- **Chaultz, E.** (1973). Ethylene-induced phenylalanine ammonialyase activity in carrot roots. Plant Physiology **51:** 1033-1036.

- **Chen, J. & Varner, J.E.** (1985a). An extracellular matrix protein in plants: characterisation of a genomic clone for carrot extensin. The EMBO Journal **4:** 2145-2151.

- **Chen, J. & Varner, J.E.** (1985b). Isolation and characterisation of cDNA clones for carrot extensin and proline-rich 33kDa protein. Proceedings of the National Academy of Sciences USA. **82:** 4399-4403.

- **Chen, W., Chao, G. & Singh, K.B.** (1996). The promoter of a H_2O_2-inducible, Arabidopsis glutathione S-transferase gene contains closely linked OBF- and OBP1-binding sites. The Plant Journal **10:** 955-966.

- **Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G.Z., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Z.Y., Chen, X., Lam, S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J.L., Wang, X. & Zhu, T.** (2002). Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. The Plant Cell **14:** 559-574.

- **Chen, Y-R, Datta, N & Roux, S.J.** (1987). Purification and partial characterization of a calmodulin-stimulated nucleoside triphosphatase from pea nuclei. Journal of Biological Chemistry **262:** 10689-10694.

- **Chen, Z., Silva, H. & Klessig, D.F.** (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science **262:** 1883-1886.

- **Cheng, S-H., Willmann, M. R., Chen, H-C. & Sheen, J.** (2002). Calcium signalling through protein kinases, the Arabidopsis calcium-dependent protein-kinase gene family. Plant Physiology **129:** 469-485.

- **Cheung, W.Y.** (ed). (1980). Calcium and Cell Function, Vol. 1, Calmodulin. New York Academic Press.

- **Choi, Y.J., Cho, E.K., Lee, S.I., Lim, C.O., Gal, S.W., Cho, M.J. & An, G.H.** (1996). Developmentally regulated expression of the rice calmodulin promoter in transgenic tobacco plants. Molecules and Cells 6: 541-546.

- **Chrispeels, M.J. Sadava, D. & Cho, Y.P.** (1974). Enhancement of extensin biosythesis in ageing disks of carrot storage tissue. Journal of Experimental Botany **25:** 1157-1166.

- **Chung, S.K. & Parish, R.W.** (1995). Studies on the promoter of the *Arabidopsis thaliana cdc2a* gene. FEBS Letters **362:** 215-219.

- **Cipollini, D.F.** (1997). Wind-induced mechanical stimulation increases pest resistance in common bean. Oecologia **111:** 84-90.

- **Cipollini, D.F.** (1998). The induction of peroxidase activity in leaves of bean plants by wind-induced mechanical perturbation. American Journal of Botany **85:** 1586-1591.

- **Cipollini, D.F.** (1999). Costs to flowering of a mechanically hardened phenotype in *Brassica napus* L. International Journal of Plant Science **160:** 735-741.

- **Cipollini, D.F. & Schultz, J.C.** (1999). Exploring cost constraints on stem elongation in plants using phenotypic manipulation. American Naturalist **153:** 236-242.

- **Cleland, R. & Karlsnes, A.M.** (1967). A possible role of hydroxyproline-containing proteins in the cessation of cell elongation. Plant Physiology **42:** 669-671.

- **Clifford, S.C., Arndt, S.K., Corlett, J.E., Joshi, S., Sankhla, N., Popp, M. & Jones, H.G.** (1998). The role of solute accumulation, osmotic adjustment and changes in cell wall elasticity in drought tolerance in *Ziziphus mauritiana* (Lamk.). Journal of Experimental Botany **49:** 967-977.

- **Coates, D., Taliercio, E.W. & Ryan, C.A.** (1987). Chromatin structure of integrated T-DNA in crown gall tumours. Plant Molecular Biology **8:** 199-207.

- **Cooper, J.B. Chen, J.A., Van Holst, G. & Varner, J.E.** (1987). Hydroxyproline-rich glycoproteins of plant cell walls. Trends in Biochemical Science. **12:** 24-27.

- **Cooper, J.B. Chen, J.A. & Varner, J.E.** (1984). In: Structure, Function and Biosynthesis of Plant Cell Walls. W.M. Dougger & S. Bartnickigarcia (eds.), p 75. American Society of Plant Physiologists, Rockville.

- **Cooper, J.B., Heuser, J.E. & Varner, J.E.** (1994). 3,4-Dehydroproline inhibits cell wall assembly and cell division in tobacco protoplasts. Plant Physiology **104:** 747-752.

- **Cooper, J.B. & Varner, J.E.** (1983). Insolubilization of hydroxyproline-rich cell wall glycoprotein in aerated carrot root slices. Biochemical and Biophysical Research Communications **112:** 161-167.

- **Cooper, J.B. & Varner, J.E.** (1984). Cross-linking of soluble extensin in isolated cell-walls. Plant Physiology **76:** 414-417.

- **Corbin, D.R., Sauer, N. & Lamb, C.J.** (1987). A hydroxyproline-rich glycoprotein gene family in bean is differentially regulated by infection and wounding. Molecular Cell Biology **7:** 4337-4344.

- **Corneliussen, B., Holm, M., Waltersson, Y., Onions, J., Thornell, A. & Grundstrom, T.** (1994). Calcium/calmodulin inhibition of basic helix-loop-helix transcription factor domains. Nature **368:** 760-764.

- **Cosgrove, D.J.** (1993). How do cell walls extend? Plant Physiology **102:** 1-6.

- **Cosgrove, D.J.** (1996). Plant cell enlargement and the action of expansins. Bioassays **18:** 533-540.

- **Coutland, C. & Moulia, B.** (2000). Biomechanical study of the effect of a controlled bending on tomato stem elongation: local strain sensing and spatial integration of the signal. Journal of Experimental Botany **51:** 1825-1842.

- **Creelman, R.A., Tierney, M.L. & Mullet, J.E.** (1992). Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proceedings of the National Academy of Sciences, USA **89:** 4938-4941.

- **Crivici, A. & Ikura, M.** (1995). Molecular and structural basis of target recognition by calmodulin. Annual Review of Biophysics and Biomolecular Structure **24:** 85-116.

- **Crook, M.J. & Ennos, A.R.** (1996). Mechanical differences between freestanding and supported wheat plants *Triticum aestivum* L. Annals of Botany **77:** 197-202.

- **Crouch, T.H. & Klee, C.B.** (1980). Positive cooperative binding of calcium to bovine brain calmodulin. Biochemisrty **19:** 3692-3698.

- **Darwin, C.** (1881). The power of movement in plants. D. Appleton and Co., New York.

- **De Jaegher, C., Boyer, N., & Gaspar, T.** (1985). Thigmomorphogenesis in *Bryonia dioica*: changes in soluble and wall peroxidases, phenylalanine ammonia lyase activity, cellulose, lignin content and monomeric constituents. Plant Growth Regulation **3:** 133-148.

- **De Loose, M., Gheysen, G., Tiré, C., Gielen, J., Villarroel, R., Genetello, C., Van Montagu, M., Depicker, A. & Inzé, D.** (1991). The extensin signal peptide allows secretion of a heterologous protein from protoplasts. Gene **99:** 95-100.

- **Dent, C.L., Smith, M.D. & Latchman, D.S.** (1999). The DNA mobility shift assay. In: Transcription factors, a practical approach, 2nd Edition. Latchman (ed), pp 1-25. Oxford University Press, Oxford.

- **De Pater, S., Katagiri, F., Kijne, J. & Chau, N-H.** (1994). bZIP proteins bind to a palindromic sequence without an ACGT core located in a seed-specific element of the pea lectin promoter. The Plant Journal **6:** 133-140.

- **Depège, N., Thonat, C., Coutland, C., Julien, J-L. & Boyer, N.** (1997). Morphological responses and molecular modifications in tomato plants after mechanical stimulation. Plant and Cell Physiology **38:** 1127-1134.

- **Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M. & Darnell, J.E.** (1981). Transcriptional control in the production of liver-specific mRNAs. Cell **23:** 731-739.

- **Derykere, F. & Gannon, F.** (1994). A one-hour minipreparation technique for extraction of DNA binding proteins from animal tissues. Biotechniques **16:** 405.

- **Despres, C., Subramanium, R., Matton, D.P & Brisson, N.** (1995). The activation of the potato *PR-10a* gene requires phosphorylation of the nuclear factor PBF-1. The Plant Cell **7:** 589-598.

- **Diatchenko, L., Lau, Y.F.C., Campbell, A.P, Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D. & Siebert, P.D.** (1996). Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proceedings of the National Academy of Sciences, USA **93**: 6025-6030.

- **Ditta, G., Stanfield, S., Corbin, D. & Helinski, D.R.** (1980). Broad host range DNA cloning system for Gram negative bacteria. Construction of a gene bank of *Rhizobium melitoti*. Proceedings of the National Academy of Sciences, USA **77:** 7347-7351.

- **Donald, R.G.K. & Cashmore, A.R.** (1990). Mutation of either G box or I box sequences profoundly affects expression from the *Arabidopsis rbcS-1A* promoter. The EMBO Journal **9:** 1717-1726.

- **Doyle, J.J. & Doyle, J.L.** (1987). A rapid DNA isolation procedure for small quantities of fresh green tissue. Phytochemistry Bulletin **19:** 11-15.

- **Draper, J. & Scott, R.** (1991). Gene transfer to plants. In: Plant Genetic Engineering. D. Grierson, D (ed), pp 38-81. Blackie & Professional, Glasgow.

- **Dröge-Laser, W., Kaiser, A., Lindsay, W.P., Halkier, B.A., Loake, G.J., Doerner, P., Dixon, R.A. & Lamb, C.J.** (1997). Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZip DNA binding protein, G/hbf-1, during the induction of early transcription-dependent defences. The EMBO Journal **16:** 726-738.

- **Drouin, J., Trifiro, M.A., Plante, R.K., Nemer, M., Eriksson, P. & Wrange, O.** (1989). Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of pro-opiomelanocortin gene transcription. Molecular and Cellular Biology **9:** 5305-5314.

- **Dupree, P.** (1996). Cell division forms a pattern. Current Biology **6:** 683-685.

- **Ebener, W., Fowler, T.J., Suzuki, H., Shaver, J. & Tierney, M.L.** (1993). Expression of DcPRP1 is linked to carrot storage root-formation and is induced by wounding and auxin treatment. Plant Physiology **101:** 259-265.

- **Ecker, J.R. & Davis, R.W.** (1987). Plant defence genes are regulated by ethylene. Proceedings of the National Academy of Sciences, USA. **84:** 5202-5206.

- **Edwards, K. & Pickard, B.** (1987). Detection and transduction of physical stimuli in plants. In: The cell surface in signal transduction. H. Greppin, B. Millet and E. Wagner (eds), pp 41-66. Springer-Verlag, Berlin.

- **Eisinger, W.** (1983). Regulation of pea internode expansion by ethylene. Annual review of Plant Physiology **34:** 225-240.

- **Elliott, K.A.** (1998). The regulation of *extA*; An extensin gene from *Brassica napus*. PhD. Thesis, University of Wales, Bangor, UK.

- **Elliott, K. & Shirsat, A. H.** (1998). Promoter regions of the *extA* extensin gene from *Brassica napus* control activation in response to tensile stress. Plant Molecular Biology **37**: 675-687.

- **Ellis, J.G., Tokuhisa, J.G., Llewellyn, D.J., Bouchez, D., Singh, K., Dennis, E. S & Peacock, W.J.** (1987). Does the ocs-element occur as a functional component of the promoter of plant genes? The Plant Journal **4:** 433-443.

- **Ellis, J.R., Shirsat, A.H., Hepher, A., Yarwood, J.N., Gatehouse, J.A., Croy, R.R.D. & Boulter, D.** (1988). Tissue-specific expression of a pea legumin gene in seeds of *Nicotiana plumbaginifolia*. Plant Molecular Biology **10:** 203-214.

- **Ennos, A.R.** (1997). Wind as an ecological factor. Trends in Ecology and Evolution **12:** 108-111.

- **Epstein, L. & Lamport, D.T.A.** (1984). An intramolecular linkage involving isodityrosine in extensin. Phytochemistry **23:** 1241-1246.

- **Erner, Y., Biro, R. & Jaffe** (1980). Thigmomorphogenesis: evidence for a translocatable thigmomorphogenic factor induced by mechanical perturbation of beans. Physiologia Plantarum **50:** 21-25.

- **Erner, Y. & Jaffe, M.** (1982). Thigmomorphogenesis: the involvement of auxin and abscisic acid in growth retardation due to mechanical perturbation. Plant and Cell Physiology **23:** 935-941.

- **Esquerré-Tugayé, M.T., Lafitte, C., Mazau, D., Toppan, A., & Touzé, A.** (1979). Cell surfaces in plant-microorgansim interactions. II. Evidence for

the accumulation of hydroxyproline-rich glycoproteins in the cell wall of diseased plants as a defence mechanism. Plant Physiology **64:** 320-326.

- **Esquerré-Tugayé, M.T. & Lamport, D.T.A.** (1979). Cell surfaces in plant-microorgansim interactions. I. A structural investigation of cell wall hydroxyproline-rich glycoproteins which accumulate in fungus-infected plants. Plant Physiology **64:** 314-319.

- **Evans, I. M., Gatehouse, L. N., Gatehouse, J. A., Yarwood, J. N., Boulter, D. & Croy, R. R. D.** (1990). The extensin gene family in oilseed rape (*Brassica napus* L.): Characterisation of sequences of representative members of the family. Molecular and General Genetics **223**: 273-287.

- **Fedorcsak, I. & Ehrenberg, L.** (1966). Effects of diethyl pyrocarbonate and methyl methanesulphonate on nucleic acids and nucleases. Acta Chemica Scandinavica **20:** 107-112.

- **Feinburg, A.P. & Volgelstein, B**. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to a high specific activity. Analytical Biochemistry **132:** 6-13.

- **Ferris, P.J., Woessner, J.P., Waffenschmidt, S., Kilz, S., Drees, J. & Goodenough, U.W.** (2001). Glycosylated polyproline II rods with kinks as a structural motif in plant hydroxyproline-rich glycoproteins. Biochemistry **40:** 2978-2987.

- **Flaman, J.M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C., Ishioka, C., Friend, S.H., Iggo, R.** (1994). A rapid fidelity assay. Nucleic Acids Research **22:** 3259-3260.

- **Fleurat-Lessard, P., Roblin, G., Bonmort, J. & Besse, C.** (1988). Effects of colchicines, vinablastine, cytochalasin B and phalloidin on the seismic movement of *Mimosa pudica* leaf and on motor cell infrastructure. Journal of Experimental Botany **39:** 209-221.

- **Fourney, R.M., Miyakoshi, J., Day, R.S. & Paterson, M.C.** (1988). Northern blotting: Efficient RNA staining and transfer. Bethesda Research Laboratory Focus **10:** 5-7.

- **Frank, A., Harris, D. & Willis, W.** (1974). Windbreak influence on water relations, growth and yield of soybeans. Crop Science **14:** 761-765.

- **Fried, M.G. & Crothers, D.M.** (1981). Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Research **9:** 6505-6524.

- **Fry, S.C.** (1982). Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. Biochemical Journal **204:** 449-455.

- **Fry, S.C.** (1986). Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annual Review of Plant Physiology **37:** 165-186.

- **Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K. & Matthews, K.J.** (1992). Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochemical Journal **282:** 821-828.

- **Fukuda, H.** (1996). Xylogenesis: initiation, progression, and cell death. Annual Review of Plant Physiology and Plant Molecular Biology **47:** 299-325.

- **Galaud, J.P., Lareyre, J.J. & Boyer, N.** (1993). Isolation, sequencing and analysis of the expression of *Bryonia dioica* calmodulin after a mechanical perturbation. Plant Molecular Biology **23:** 839-846.

- **Garcia-Mas, J., Messeguer, R., Arús, P.& Puigdomènech, P.** (1992). The extensin from *Prunus amygdalus*. Plant Physiology **100:** 1603-1604.

- **Garner, L.C. & Bjorkman, T.** (1996). Mechanical conditioning for controlling excessive elongation in tomato transplants: sensitivity to dose, frequency, and timing of brushing. Journal of the American Society of Horticultural Science **121:** 894-900.

- **Garner, M.M. & Revzin, A.** (1981). A gel electrophoresis method for quantifying binding of proteins to specific DNA regions: Applications to components of the *Escherichia coli* lactose operon regulatory system. Nucleic Acids Research **9:** 3047-3060.

- **Gartner, B.L.** (1994). Root biomechanics and whole plant allocation patterns: responses of tomato to simulated wind. Journal of Experimental Botany **45:** 1647-1654.

- **Gatehouse, L.N., Evans, I.M., Gatehouse, J.A. & Croy, R.R.D.** (1990). Characterisation of a rape (*Brassica napus L.*) extensin gene encoding a polypeptide relatively rich in tyrosine. Plant Science **71:** 223-231.

- **Gehring, W.J., Qian, Y.Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A.F., Resendez-Perez, D., Affolter, M. & Otting, G.** (1994). Homeodomain-DNA recognition. Cell **78:** 211-223.

- **Gilmartin, P.M., Sarokin, L., Memelink, J. & Chua, N.H.** (1990). Molecular light switches for plant genes. The Plant Cell **2:** 369-378.

- **Giridhar, G. & Jaffe, M.** (1988). Thigmomorphogenesis. XXIII. Promotion of foliar senescence by mechanical perturbation of *Avena sativa* and four other species. Physiologia Plantarum **74:** 473-480.

- **Gittins, J. R., Hiles, E. R., Pellny, T. K., Biricolti, S. & James, D. J.** (2001). The *Brassica napus extA* promoter: a novel alternative promoter to CaMV 35S for directing transgenic expression to young stem tissues and load bearing regions of transgenic apple trees (*Malus pumila* Mill.). Molecular Breeding **7:** 51-62.

- **Gittins, J. R., Pellny, T. K., Hiles, E. R., Rosa, C., Biricolti, S. & James, D. J.** (2000). Transgene expression driven by heterologous ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit gene promoters in the vegetative tissues of apple (*Malus pumila Mill.*). Planta **210:** 232-240.

- **Goeschl, R., Rappaport, L., & Pratt, H.** (1966). Ethylene as a factor regulating the growth of pea epicotyls subjected to physical stress. Plant Physiology **41:** 877-884.

- **Gomez, L.D, Casano, L.M. & Trippi, V.S.** (1995). Effect of hydrogen peroxide on degradation of cell wall associated proteins in growing bean hypocotyls. Plant and Cell Physiology **36:** 1259-1264.

- **Goodman, A.M. & Ennos, A.R.** (1996). A comparative study of the response of roots and shoots of sunflower and maize to mechanical stimulation. Journal of Experimental Botany **45:** 1647-1654.

- **Goodman, A.M. & Ennos, A.R.** (1998). Responses of the root systems of sunflower and maize to unidirectional stem flexure. Annals of Botany **82:** 347-358.

- **Grace, J. & Russell, G.** (1977). The effect of wind on grasses. Journal of Experimental Botany **28:** 268-278.

- **Grannell, A., Peretó, J. G., Schindler, U. & Cashmore, A. R.** (1992). Nuclear factors binding to the extensin promoter exhibit differential activity in carrot protoplasts and cells. Plant Molecular Biology **18**: 739-748.

- **Greenblatt, J.** (1997) RNA polymerase II holoenzyme and transcriptional regulation. Current Opinions in Cell Biology **9:** 310-319.

- **Grierson, C., Du, J.S., de Torres Zabala, M., Beggs, K., Smith, C., Holdsworth, M. & Bevan, M.** (1994). Separate cis-sequences and trans-acting factors direct metabolic and developmental regulation of a potato tuber storage protein gene. The Plant Journal **5:** 815-826.

- **Griffiths, S.A., Good, V.M., Gordon, L.A., Hudson, E.A., Barrett, M.C., Munks, R.J.L. & Manson, M.M.** (1995). Characterization of a promoter for λ-glutamyl transpeptidase activated in rat liver in response to aflotoxin, B1 and ethoxyquin. Molecular Carcinogenesis **14:** 251-262.

- **Grobe, K., Becker, W-M., Schlaak, M. & Petersen, A.** (1999). Grass group I allergens (β-expansins) are novel papain-related proteinases. European Journal of Biochemistry **263:** 33-40.

- **Grotewold, E., Drummond, B.J., Bowen, B. & Peterson, T.** (1994). The *myb*-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavanoid biosynthetic gene subset. Cell **76:** 543-553.

- **Gubler, F., Raventos, D., Keys, M., Watts, R., Mundy, J. & Jacobsen, J.V.** (1999). Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. The Plant Journal **17:** 1-9.

- **Guiltinan, M.J., & Miller, L.** (1994). Molecular characterization of the DNA-binding and dimerization domain of the bZIP transcription factor, EmBP-1. Plant Molecular Biology **26:** 1041-1053.

- **Gurley, W.B., Czarnecka, E. & Barros, M.D.C.** (1993). Anatomy of a soybean heat shock promoter. In: Control of Plant Gene Expression. Desh Pal S. Verma (ed.), pp 103-123. CRC Press, Inc., Florida

- **Guyaray, F. & Sachs, F.** (1984). Stretch-activated single ion channel currents in tissue cultured embryonic chick skeletal muscle. Journal of Physiology-London **352:** 685-701.

- **Halliwell, B.** (1978). Lignin synthesis: the generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenols. Planta **140:** 81-88.

- **Hammerschmidt, R., Lamport, D.T.A. & Muldoon, E.P.** (1984). Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerinum.* Physiological Plant Pathology **24:** 43-47.

- **Hardtke, C.S. & Berleth, T.** (1998) The Arabidopsis gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. The EMBO Journal **17:** 1405-1411.

- **Harding, S.A., Oh, S-H. & Roberts, D.M.** (1997). Transgenic tobacco expressing a foreign calmodulin gene shows an enhanced production of active oxygen species. The EMBO Journal **16:** 1137-1144.

- **Hanna-Rose, W. & Hansen, U.** (1996). Active repression mechanisms of eukaryotic transcription repressors. Trends in Genetics **12:** 229-234.

- **Harmon, A.C, Gribskov, M., Gubrium, E. & Harper, J.F.** (2001). The CDPK superfamily of protein kinases. New Phytologist **151:** 175-183.

- **Harrington, R.E. & Winicov, I.** (1994). New concepts in protein-DNA recognition: sequence-directed DNA bending and flexibility. Progress in Nucleic Acid Research and Molecular Biology **47:** 195-270.

- **Harrison, S. C.** (1991). A structural taxonomy of DNA-binding domains. Nature **353**: 715-719

- **Hasenstein, K.H., Evans, M.L.** (1988). Effects of cations on hormone transport in primary roots of *Zea mays*. Plant Physiology **86:** 890-894.

- **Hedrich, R. & Neher, E.** (1987). Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. Nature **329:** 833-836.

- **Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel., A.E., Ignatieva, E.V., Ananko, E.A., Podkolodnaya, O.A., Koplakov, F.A., Podkolodny, N.L. & Kolchanov, N.A.** (1998). Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Research **26:** 362-367.

- **Hellermann, G.R. & Solomonson, L.P.** (1997). Calmodulin promotes dimerization of the oxygenase domain of human endothelial nitric oxide synthase. Journal of Biological Chemistry **272:** 12030-12034.

- **Hemm, M.R., Herrmann, K.M. & Chapple, C.** (2001). AtMYB4: a transcription factor general in the battle against UV. Trends in Plant Science **6:** 135-136.

- **Hepworth, D.G. & Vincent, J.F.V.** (1999). The growth response of the stems of genetically modified tobacco plants (*Nicotiana tabacam 'Samsun'*) to flexural stimulation. Annals of Botany **83:** 39-43.

- **Herman, L., Jacobs, A., Van Montagu, M. & Depicker, A.** (1990). Plant chromsosome / marker gene fusion assay for study of normal and truncated T-DNA integration events. Molecular and General Genetics **224:** 248-256.

- **Heuchert, J.C., Marks, J.S. & Mitchell, C.A.** (1983). Strengthening of tomato shoots by gyratory shaking. Journal of the American Society of Horticultural Science **108:** 801-805.

- **Heuchert, J.C. & Mitchell, C.A.** (1983). Inhibition of shoot growth in greenhouse-grown tomato by periodic gyratory shaking. Journal of the American Society of Horticultural Science **108:** 795-800.

- **Higo, K., Ugawa, Y., Iwamoto, M. & Korenaga, T.** (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Research **27:** 297-300.

- **Hirsinger, C., Parmentier, Y., Durr, A., Fleck, J. & Jamet, E.** (1997). Characterization of a tobacco extensin gene and regulation of its gene family in healthy plants and under various stress conditions. Plant Molecular Biology **33:** 279-289.

- **Hirsinger, C., Salvà, I., Marbach, J., Durr, A., Fleck, J. & Jamet, E.** (1999). The tobacco extensin gene *Ext 1.4* is expressed in cells submitted to mechanical constraints and in cells proliferating under hormonal control. Journal of Experimental Biology **50**: (332) 343-355.

- **Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. & Schilperoot, R.A.** (1983). A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303:** 179-180.

- **Holdsworth, M. J. & Laties, G.** (1989a). Site specific binding of a nuclear factor to the carrot extensin gene is influenced by both ethylene and wounding. Planta **179**: 17-23.

- **Holdsworth, M. J. & Laties, G.** (1989b). Identification of a wound-induced inhibitor of a nuclear factor that binds the carrot extensin gene. Planta **180**: 74-81.

- **Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T.** (1985). A simple and general method for transferring genes into plants. Science **227:** 1229-1231.

- **Hu, W.-J., Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.-J. & Chiang, V.L.** (1999). Repression of lignin biosynthesispromotes cellulose accumulation and growth in transgenic trees. Nature Biotechnology **17:** 808-812.

- **Hunter, T. & Karin, M.** (1992). The regulation of transcription by phosphorylation. Cell **70:** 375-387.

- **Hurst, H.C.** (1995) Transcription factors. 1. bZIP proteins. Protein Profile **2:** 105-168.

- **Ingram, J. & Bartels, D.** (1996). The molecular basis of dehydration tolerance in plants. Annual Review of Plant Physiology and Plant Molecular Biology **47:** 377-403.

- **Islam, N. & Evans, E.J.** (1994). Influence of lodging and nitrogen rate on the yield and yield attributes of oilseed rape (*Brassica napus* L.). Theoretical and Applied Genetics **88:** 530-534.

- **Ito, M., Kodama, H., Komamine, A. & Watanabe, A.** (1998). Expression of extensin genes is dependent on the stage of the cell cycle and cell

proliferation in suspension-cultured *Catharanthus roseus* cells. Plant Molecular Biology **36:** 343-351.

- **Ito, T., Masanori, H., Akama, K., Shimura, Y. & Okada, K.** (1995). Touch-inducible genes for calmodulin and a calmodulin-related protein are located in tandem on a chromsosome of *Arabidopsis thaliana*. Plant and Cell Physiology **36:** 1369-1373.

- **Izawa, T., Foster, R., & Chua, N.-H.** (1993). Plant bZiP protein DNA binding specificity. Journal of Molecular Biology **230:** 1131-1144.

- **Jaffe, M.J** (1973). Thigmomorphogenesis: The response of plant growth and development to mechanical stimulation. Planta **114:** 143-157.

- **Jaffe, M.J.** (1976a). Thigmomorphogenesis: A detailed characterization of the response of beans (*Phaseolus vulgaris* L.) to mechanical stimulation. Zeitschrift fur Pflanzenphysiologie **77S:** 437-453.

- **Jaffe, M.J** (1976b). Thigmomorphogenesis: electrical resistance and mechanical correlates of the early events of growth retardation due to mecanical stimulation in beans. Zeitschrift fur Pflanzenphysiologie **78S:** 24-32.

- **Jaffe, M.J.** (1984). The involvement of callose and elicitors in ethylene production caused by mechanical perturbation. In: Ethylene: biochemical, physiological and applied aspects. Y.Fuschs and E.Chalutz (eds), pp 199-215. Nijhoff/Junk, The Hague, The Netherlands.

- **Jaffe, M.J. & Biro, R.** (1979). Thigmomorphogenesis: the effect of mechanical perturbation on the growth of plants with special reference to anatomical changes, the role of ethylene, and interaction with other environmental stresses. Stress physiology in crop plants. H. Mussell and R. Staples (eds.), pp 25-29. Wiley, New York.

- **Jaffe, M.J, Biro, R. & Bridle, K.** (1980). Thigmomorphogenesis: calibration of the parameters of the sensory function in beans. Physiologia Plantarum **49:** 410-416.

- **Jaffe, M.J. & Forbes, S.** (1993). Thigmomorphogenesis: the effect of mechanical perturbation on plants. Plant Growth Regulation **12:** 313-324.

- **Jaffe, M.J., Huberman, M., Johnson, J. & Telewski, F.W.** (1985). Thigmomorphogenesis: the induction of callose formation and ethylene evolution by mechanical perturbation in bean stems. Physiologia Plantarum **64:** 271-279.

- **Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G. Schabenberger, O. & Thomashow, M.F.** (1998). *Arabidopsis* CBF-1 overexpression induces COR genes and enhances freezing tolerance. Science **280:** 104-106.

- **Jamet, E., Guzzardi, P. & Salvá, I.** (2000). What do transgenic plants tell us about the regulation and function of cell wall structural proteins like extensins? Russian Journal of Plant Physiology **47:** 318-326.

- **Jarvis, M.C.** (1984). Structure and properties of pectin gels in plant cell walls. Plant, Cell and Environment **7:** 153-164.

- **Jefferson, R.A.** (1987). Assaying chimeric genes in plants. The GUS gene fusion system. Plant Molecular Biology Reporter **5:** 387-405.

- **Jefferson, R.A., Burgess, S.M. & Hirsh, D.** (1986). β-glucuronidase from *Escherichia coli* as a gene fusion marker. Proceedings of the National Academy of Sciences, USA **83:** 8447-8451.

- **Jefferson, R.A., Kavanagh, T.A. & Bevan, M.W.** (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal **6:** 3901-3907.

- **Jenkins, E.S., Paul, W., Coupe, S.A., Bell, S.J., Davies, E.C. & Roberts, J.A.** (1996). Characterization of an mRNA encoding a polygalacturonase expressed during pod development in oilseed rape (*Brassica napus* L.). Journal of Experimental Botany **47:** 111-115.

- **Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., Tonelli, C., Weisshaar, B. & Martin, C.** (2000). Transcriptional repression by atMYB4 controls production of UV-protecting sun-screens in *Arabidopsis*. The EMBO Journal **19:** 6150-6161.

- **Jin, H. & Martin, C.** (1999). Multifunctionality and diversity within the plant MYB-gene family. Plant Molecular Biology **41:** 577-585.

- **Jofuku, K. D., Okamuro, J. K. & Goldberg, R. B.** (1987). Interaction of an embryo DNA binding protein with a soybean lectin gene upstream region. Nature **328**: 734-73.

- **Johjima, T., Latimer, J. & Wakita, H.** (1992). The effect of mechanical stress on transplant growth and subsequent yield of four cultivars of tomato and their hybrid lines. Journal of the American Society of Horticultural Science **117:** 384-388.

- **Johnson, P.F. & McKnight, S.L.** (1989). Eukaryotic transcriptional regulatory proteins. Annual Review of Biochemistry **58:** 799-839.

- **Jones, J. & Mullet, J.E.** (1995). A salt- and dehydration-inducible pea gene, Cyp15a, encodes a cell-wall protein with sequence similarity to cysteine proteases. Plant Molecular Biology **28:** 1055-1065.

- **Jones, O.W. & Berg, P.** (1966). Studies on the binding of RNA polymerase to polynucleotides. Journal of Molecular Biology **22:** 199-209.

- **Jones, R., Coe, L., Montgomery, L. & Mitchell, C.A.** (1990). Seismic stress responses of soybean to different photosynthetic photon flux. Annals of Botany **66:** 617-622.

- **Jones, R. & Mitchell, C.A.** (1989). Calcium ion involvement in growth inhibition of mechanically stressed soybean seedlings. Physiologia Plantarum **76:** 598-602.

- **Jones, R. & Mitchell, C.A.** (1992). Effects of physical agitation on yield of greenhouse-grown soybean. Crop Science **32:** 404-408.

- **José, M and Puigdomenech, P.** (1993). Structure and expression of genes coding for structural proteins of the plant cell wall. New Phytologist **125:** 259-282.

- **Josefsson, E.** (1968). Investigations into shattering resistance of cruciferous oil crops. Zeitschrift fur Pflanzenzuchtung **59:** 384-396.

- **Kang, H.G. & Singh, K.** (2000). Characterization of salicylic acid-responsive *Arabidopsis* Dof domain proteins: overexpression of OBP3 leads to growth defects. The Plant Journal **21:** 329-339.

- **Karin, M. & Hunter, T.** (1995). Transcriptional control by protein phosphorylation: signal transduction from the cell surface to the nucleus. Current Biology **5:** 747-757.

- **Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Schinozaki, K. & Shinozaki, K.** (1999). Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology. **17:** 287-291.

- **Kaulin, H., Schell, J. & Kreuzaler, F.** (1986). Light-induced expression of the chimeric chalcone synthase NPTII gene in tobacco cells. The EMBO Journal **5:** 1-8.

- **Kauss, H. & Glaser, C.** (1974). Carbohydrate-binding proteins from plant cell walls and their possible involvement in extension growth. FEBS Letters **45:** 304-307.

- **Kawalleck, P., Schmelzer, E., Hahlbrock, K. & Somssich, E. E.** (1995). Two pathogen-responsive genes in parsley encode a tyrosine-rich

glycoprotein (hrgp) and an anionic peroxidase. Molecular and General Genetics **247:** 444-452.

- **Kawaoka, A., Sato, S., Nakahara, K., Matsushima, N., Okada, N., Sekine, M., Shinmyo, A. & Takano, M.** (1992). Expression and promoter activity of genes for isozymes of horseradish-peroxidase. Plant and Cell Physiology **33:** 1143-1150.

- **Kawasaki, H. & Kretsinger, R.H.** (1994). Calcium-binding proteins 1: EF-hands. Protein Profile **1:** 343-517.

- **Kay, R., Chan. A., Daly. M. & McPherson. J.** (1987). Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science **236:**1299-1302.

- **Keegan, L., Gill, G. & Ptashne, M.** (1986). Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science **231:** 699-704.

- **Keith, C.H., DiPaola, M., Maxfield, F.R. & Shelanski, M.L.** (1983). Microinjection of Ca^{2+}-calmodulin causes a localized depolymerization of microtubules. Journal of Cell Biology **97:** 1918-1924.

- **Keller, B. & Baumgartner, C.** (1991). Vascular-specific expression of the bean *grp1.8* gene is negatively regulated. The Plant Cell **3:** 1052-1061.

- **Keller, B. & Heierli, D.** (1994). Vascular specific expression of the *grp1.8* promoter is controlled by three specific regulatory elements and one unspecific activation sequence. Plant Molecular Biology **26:** 747-756.

- **Keller, B. & Lamb, C.J.** (1989). Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. Genes and Development **3:** 1639-1646.

- **Keller, E. & Steffen, K.L.** (1995). Increased chilling tolerance and altered carbon metabolism in tomato leaves following application of mechanical stress. Physiologia Plantarum **93:** 519-525.

- **Kieliszewski, M.J. & Lamport, D.T.A.** (1994). Extensin: Repetitive motifs, functional sites, post-translational codes and phylogeny. The Plant Journal **5:** 157-172.

- **Kieliszewski, M.J., O'Neill, M., Leykam, J.F. & Orlando, R.** (1995). Tandem mass spectrometry and structural elucidation of glycopeptides from a hydroxyproline-rich plant cell wall glycoprotein indicate that contiguous hydroxyproline residues are the major sites of hydroxyproline O-arabinosylation. Journal of Biological Chemistry **270:** 2541-2549.

- **Kim, T.K. & Maniatis, T.** (1997). The mechanism of transcriptional synergy of an in vitro assembled interferon-beta enhanceosome. Molecular Cell **1:** 119-129.

- **Klis, F.M.** (1976). Glycosylated seryl residues in wall protein of elongating pea stems. Plant Physiology **57:** 224-226.

- **Knight, M.R., Campbell, A.K., Smith, S.M. & Trewavas, A.J.** (1991). Transgenic plant aequorin reports the effect of touch and cold-shock and elicitors on cytoplasmic calcium. Nature **352:** 524-526.

- **Knight, M.R., Smith, S.M. & Trewavas, A.J.** (1992). Wind-induced plant motion immediately increases cytosolic calcium . Proceedings of the National Academy of Science, USA **89:** 4967-4971.

- **Kornberg, R.D.** (1974). Chromatin structure is based on a repeating unit of eight histine molecules and about 200 DNA base pairs. Science **184:** 868-871.

- **Kozbial, P.Z., Jerzmanowski, A., Shirsat, A.H. & Kacperska, A.** (1998). Transient freezing regulatetes expression of extensin-type genes in winter oilseed rape. Physiologia Plantarum **103:** 264-270.

- **Kustu, S., North, A. K. & Weiss, D. S.** (1991). Prokaryotic transriptional enhancers and enhancer-binding proteins. Trends in Biochemistry **16:** 397-402.

- **Lamb, C.J.** (1994). Plant disease resistance genes in signal perception and transduction. Cell **76:** 419-422.

- **Lamb, C.J. & Dixon, R.A.** (1994). Molecular mechanisms underlying induction of plant defence gene transcription. Biochemical Society Symposium **60:** 241-248. In: Molecular Botany: Signals and the Environment, DD. Bowles, P.M. Gilmartin, J.P. Knox & G.G. Lunt (eds.). Portland Press, London.

- **Lamb, C.J., Lawton, M.A., Dron, M. & Dixon, R.A.** (1989). Signals and transduction mechanisms for activation of plant defences against microbial attack. Cell **56:** 215-224.

- **Lamb, P. & McKnight, S.L.** (1991). Diversity and specificity in transcriptional regulation: the benefit of heterotypic dimerization. Trends in Biochemical Science **16:** 417-422.

- **Lamport , D.T.A.** (1965). The protein component of primary cell walls. Advances in Botanical Research **2:** 151-218.

- **Lamport, D.T.A.** (2001). Life behind cell walls: paradigm lost, paradigm regained. Cellular and Molecular Life Sciences **58:** 1363-1385.

- **Lamport, D.T.A. & Catt, J.W.** (1981). Glycoproteins and enzymes of the cell wall. In: Plant Carbohydrates. Tanner, W. *et al.* (eds), pp 133-165.

- **Lane, D., Prentki, P. & Chandler, M.** (1992). Use of gel retardation to analyse protein-nucleic acid interactions. Microbiology Review **56:** 509-528.

- **Latimer, J.G** (1990). Drought or mechanical stress affects broccoli transplant growth and establishment but not yield. HortScience **25:** 1233-1235.

- **Latimer, J.G. & Oetting, R.D.** (1994). Brushing reduces thrips and aphid populations on some greenhouse-grown vegetable transplants. HortScience **29:** 1279-1281.

- **Latimer, J.G, Pappas, T. & Mitchell, C.A.** (1986). Growth responses of eggplant and soybean seedlings to mechanical stress in greenhouse and outdoor environments. Journal of the American Society of Horticultural Science **111:** 694-698.

- **Lau, O.L. & Yang, S.F.** (1974). Synergistic effect of calcium and kinetin on ethylene production by mungbean hypocotyls. Planta **118:** 1-6.

- **Laursen, N.B., Larsen, K., Knudsen, J.Y., Hoffmann, H.J., Poulsen, C., Marcker, K.A. & Jensen, E.O.** (1994). A protein binding AT-rich sequence in the soybean leghemoglobin c3 promoter is a general *cis* element that requires proximal DNA elements to stimulate transcription. The Plant Cell **6:** 659-668.

- **Lauvergeat, V., Rech, P., Jauneau, A., Guez, C., Coutos-Thevenot, P. & Grima-Pettenati, J.** (2002). The vascular expression pattern directed by the *Eucalyptus gunnii* cinnamyl alcohol dehydrogenase EgCAD2 promoter is conserved among woody and herbaceous plant species. Plant Molecular Biology **50:** 497-509.

- **Leach, J.E., Cantrell, M.A., Sequeira, L.** (1982). Hydroxyproline-rich bacterial agglutinin from potato. Plant Physiology **70:** 1353-1358.

- **Legué, V., Blancaflor, E., Wymer, C., Perbal, G., Fantin, D. & Gilroy, S.** (1997). Cytosolic free calcium in Arabidopsis roots changes in response to touch but not to gravity. Plant Physiology **114:** 789-800.

- **Levine, A., Tenhaken, R., Dixon, R. & Lamb, C.** (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:** 583-593.

- **Lieberman, M. & Wang, S.Y.** (1982). Influence of calcium and magnesium on ethylene production by apple tissue slices. Plant Physiology **69:** 1150-1155.

- **Lin, J.-J., Assad-Garcia, N. & Kuo, J.** (1995). Effects of *Agrobacterium* cell concentration on the transformation efficiency of tobacco and *Arabidopsis thaliana*. Bethesda Research Laboratory Focus **16:** 70-75.

- **Ling, V., Snedden, W.A., Shelp, B.J. & Assmann, S.M.** (1994). Analysis of soluble calmodulin-binding protein from fava bean roots: identification of glutamate decarboxylase as a calmodulin-activated enzyme. The plant Cell **6:** 1135-1143.

- **Liu, Z-B., Ulmasov, T., Shi, X., Hagen, G. & Guilfoyle, T.J.** (1994). Soybean GH3 promoter contains multiple auxin-inducible elements. The Plant Cell **6:** 645-657.

- **Low, P.S. & Merida, J.R.** (1996). The oxidative burst in plant defence: function and signal transduction. Physiologia Plantarum **96:** 533-542.

- **Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S. & Gruissem, W.** (2002). CaMs and CBLs: calcium sensors for specific signal-response coupling in plants. Plant Cell (suppl) **14:** S389-S400.

- **Ludevid, M. D., Ruiz-Avila, L., Vallés, M. P., Stiefel, V., Torrent, M., Torné, J. M. & Puigdomènech, P.** (1990). Expression of genes for cell-wall proteins in dividing and wounded tissues of *Zea mays* L. Planta **180:** 524-529.

- **Lüscher, B. & Eisenman, R.N.** (1990). New light on Myc and Myb. Part II. Myb. Genes and Development **4:** 2235-2241.

- **MacLeod, J.** (1981). Harvesting. In: Oilseed Rape Book: A manual for growers, farmers and advisors, pp 107-120. Cambridge Agricultural Publishing, Cambridge.

- **Maniatis, T., Goodbourn, S. & Fischer, J.A.** (1987). Regulation of inducible and tissue-specific gene expression. Science **236:** 1237-1245.

- **Marcum, J.M., Dedman, J.R., Brinkley, B.R. & Means, A.R.** (1978). Control of microtubule assembly-disassembly by calcium-dependent regulator proteins. Proceedings of the National Academy of Sciences, USA **75:** 3771-3775.

- **Marshall, J.G., & Dumbroff, E.B.** (1999a). Turgor regulation via cell wall adjustment in white spruce. Plant Physiology **119:** 313-319.

- **Marshall, J.G., Dumbroff, E.B., Thatcher, B.J., Marin, B., Rutledge, R.C. & Blumwald, E.** (1999b). Synthesis and oxidative insolubilization of cell wall proteins during osmotic stress. Planta **208:** 401-408.

- **Martin, C. & Paz-Ares, J.** (1997). MYB transcription factors in plants. Trends in Genetics **13:** 67-73.

- **Martin, E.V. & Clements, F.E.** (1935). Studies on the effect of artificial wind on the growth and transpiration of *Helianthus annuus*. Plant Physiology **10:** 613-636.

- **Martin, K.J.** (1991). The interaction of transcription factors and their adaptors, coactivators and accessory proteins. Bioessays **13:** 499-503.

- **Martin, K.J., Lillie, W.J. & Green, M.R.** (1990). Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. Nature **346:** 147-152.

- **Massa, G.D. & Gilroy, S.** (2003). Touch modulates gravity sensing to regulate the growth of primary roots of *Arabidopsis thaliana.* The Plant Journal **33:** 435-445.

- **Mattheck, C.** (1991). Trees: the mechanical design. Springer Verlag, Berlin.

- **Mattoo, A., Achilea, D., Fuchs, Y. & Chalutz, E.** (1982). Membrane association and some characteristics of ethylene forming enzyme from etiolated pea seedlings. Biochemical and Biophysical Research Communications **105:** 271-278.

- **Mazau, D. & Esquerré-Tugayé, M.T.** (1986). Hydroxyproline-rich glycoprotein accumulation in the cell walls of plants infected by various pathogens. Physiological and Molecular Plant Pathology **29:** 147-157.

- **McCabe, P.F., Valentine, T.A., Forsberg, S. & Pennell, R.I.** (1997). Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. The Plant Cell **9:** 2225-2241.

- **McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M. & Vasil, I.K.** (1991) The *Viviparous1* developmental gene of maize encodes a novel transcriptional activator. Cell **66:** 895-905.

- **McDonell, M.W., Simon, M.N. & Studier, F.W.** (1977). Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. Journal of Molecular Biology. **110:** 119-146.

- **McNeil, M., Darvill, A.G., Fry, S.C. & Albersheim, P.** (1984). Structure and function of the primary cell walls of plants. Annual Review of Biochemistry **53:** 625-663.

- **Meissner RC, Jin HL, Cominelli E, Denekamp M, Fuertes A, Greco R, Kranz HD, Penfield S, Petroni K, Urzainqui A, Martin C, Paz-Ares J, Smeekens S, Tonelli C, Weisshaar B, Baumann E, Klimyuk V, Marillonnet S, Patel K, Speulman E, Tissier AF, Bouchez D, Jones JJD, Pereira A, Wisman E, & Bevan M.** (1999). Function search in a large transcription factor gene family in *Arabidopsis*: Assessing the potential of reverse genetics to identify insertional mutations in R2R3 MYB genes. The Plant Cell **11:** 1827-1840.

- **Melford, J.I., Callos, J.D., Behringer, F.J. & Link, B.M.** (1994). Development of the vegetative shoot apical meristem. In: *Arabididopsis.* E.M. Meyerowitz & C.R. Somerville (eds.). Cold Spring Harbour Laboratory Press, New York.

- **Mellon, J.E. & Helgeson, J.P.** (1982). Interaction of hydroxyproline-rich glycoprotein from tobacco callus with potential pathogens. Plant Physiology **70:** 401-405.

- **Memelink, J., Hoge, J.H.C., Schilperoort, R.A.** (1987). Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. The EMBO Journal **6:** 3579-3583.

- **Menkens, A.E., Schindler, U. & Cashmore, A.R.** (1995). The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. Trends in Biochemical Science **20:** 506-510.

- **Menossi, M., Martínez-Izquierdo, J.A. & Puigdomènech, P.** (1997). Promoter tissue specific activity and ethylene control of the gene coding for the maize hydroxyproline-rich glycoprotein in maize cells transformed by particle bombardment. Plant Science **125:** 189-200.

- **Merkouropoulos, G.** (2000). Regulation and analysis of *atExt1*, an extensin gene from Arabidopsis. PhD. Thesis, University of Wales, Bangor, UK.

- **Merkouropoulos, G., Barnett, D. C. & Shirsat, A. H.** (1999). The *Arabidopsis* extensin gene is developmentally regulated, is induced by wounding, methyl jasmonate, abscisic acid and salicylic, and codes for a protein with unusual motifs. Planta **208**: 212-219.

- **Merkouropoulos, G. & Shirsat, A.H.** (2003). The unusual *Arabidopsis* extensin gene *atExt1* is expressed throughout plant development and is induced by a variety of biotic and abiotic stresses. Planta **217:** 356-366.

- **Meshi, T. & Iwabuchi, M.** (1995). Plant transcription factors. Plant and Cell Physiology **36:** 1405-1420.

- **Messdaghi, D. & Dietz, K.J.** (2000). Characterization of an extracellular chymostatin-sensitive serine protease preferentially expressed in young plant tissues. Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology (2000) **1480:** 107-116.

- **Mitchell, C.A.** (1992). Modification of plant growth and development by acceleration and vibration: concerns and opportunities for plant experimentation in orbiting spacecraft. Advances in Space Research **12:** 219-225.

- **Mitchell, C.A.** (1996). Recent advances in plant response to mechanical stress: theory and application. HortScience **3:** 31-35.

- **Mitchell, C.A. & Myers, P.N.** (1995). Mechanical stress regulation of plant growth and development. Horticultural Reviews **17:** 1-42.

- **Mitchell, C.A., Severson, C., Wott, J. & Hammer.** (1975). Seismomorphogenic regulation of plant growth. Journal of the American Society of Horticultural Sciences **100:** 161-165.
- **Mitchell, P.J. & Tijan, R.** (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245:** 371-378.

- **Mol, J., Grotewold, E. & Koes, R.** (1998). How genes paint flowers and seeds. Trends in Plant Science **3:** 212-217.

- **Mol, J., Jenkins, G., Schafer, E. & Weiss, D.** (1996). Signal perception, transduction, and gene expression involved in anthocyanin biosynthesis. Critical Reviews in Plant Sciences **15:** 525-557.

- **Monro, J.A., Bailey, R.W. & Penny, D.** (1974). Cell wall hydroxyproline-polysaccharide associations in *Lupinus* hypocotyls. Phytochemistry **13:** 375-382.

- **Moore, R.** (1983). Marketing oilseed rape. In: Yield of Oilseed Rape: Course Papers, 1983. pp 9-12. (National Agricultural Centre, Cereal Unit, Stoneleigh, Warwicks.).

- **Mouly, A., Rumeau, D. & Esquerré-Tugayé, M.T.** (1992). Differential accumulation of hydroxyproline-rich glycoprotein transcripts in sunflower plants infected with *Schlerotina sclerotiorum* or treated with oxalic acid. Plant Science **85:** 51-59.

- **Nakai, Y., Usi, T., Tsukada, T., Takahashi, H., Fukata, J., Fukushima, M., Senoo, K. & Imura, H.** (1991). Molecular mechanisms of

glucocorticoid inhibition of human proopiomelanocortin gene transcription. The Journal of Steroid Biochemistry and Molecular Biology **40:** 301-306.

- **Neel, P. & Harris, R.** (1971). Motion-induced inhibition of elongation and induction of dormancy in Liqidambar. Science **173:** 58-59.

- **Ni, M., Dehesh, K., Tepperman, J.M. & Quail, P.H.** (1996). GT-2: *In vivo* transcriptional activation activity and definition of novel twin DNA binding domains with reciprocal target sequence selectivity. The Plant Cell **8:** 1041-1059.

- **Nicol, F. & Höfte, H.** (1998). Plant cell expansion: Scaling the wall. Current Opinions in Cell Biology **1:** 12-17.

- **Nicolas, R.H., Hynes, G. & Goodwin, G.H.** (1999). In: Transcription factors, a practical approach, 2nd Edition. Latchman (ed), pp 97-122. Oxford University Press, Oxford.

- **Niebel, A., Engler, de Almeida Engler, J., Tiré, C., Engler, G., Van Montagu, M. & Gheysen, G.** (1993). Induction patterns of an extensin gene in tobacco upon nematode infection. The Plant Cell **5:** 1697-1710.

- **Niklas, K.J.** (1996). Differences between *Acer saccharum* leaves from open and wind-protected sites. Annals of Botany **78:** 61-66.

- **Ninomiya, H., Fromental, C., Xiao, J.H. & Chambon, P.** (1987). Cell-specific activity of the constituent elements of the simian virus 40 enhancer. Proceedings of the National Academy of Science, USA **84:** 7881-7885.

- **Noda, K.I., Glover, B.J., Lindstead, P. & Martin, C.** (1994). Flower colour intensity depends on specialized cell-shape controlled by a MYB-related transcription factor. Nature **369:** 661-664.

- **Oh, S.A., Kwak, J.M., Kwun, I.C. & Nam, H.G.** (1996). Rapid and transient induction of calmodulin-encoding gene(s) of *Brassica napus* by touch stimulus. Plant Cell Reporter **15:** 586-590.

- **Okamuro, J.K., Caster, B., Villarroel, R., Van Montagu, M. & Jofuku, K.D.** (1997). The AP2 domain of *APETALA2* defines a large new family of DNA binding proteins in *Arabidopsis*. Proceedings of the National Academy of Science, USA **94:** 7076-7081.

- **O'Neil, K.T. & DeGrado, W.F.** (1990). How calmodulin binds its targets: sequence independent recognition of amphiphilic α-helices. Trends in Biochemical Science **15:** 59-64.

- **Oppenheimer, D.G., Herman, P.L., Sivakumaran, S., Esch, J. & Marks, M.D.** (1991). A *myb* gene required for leaf trichome differentiation in *Arabidopsis* is expressed in stipules. Cell **67:** 483-493.

- **Osawa, A.** (1993). Effect of mechanical stresses and photosynthetic production on stem form development of *Populus maximowiczii*. Annals of Botany **71:** 489-494.

- **Parmentier, Y., Durr, A., Marbach, J., Hirsinger, C., Criqui, M.-C., Fleck, J. & Jamet, E.** (1995). A novel wound-inducible extensin gene is expressed early in newly isolated protoplasts of *Nicotiana sylvestris*. Plant Molecular Biology **29:** 279-292.

- **Pennell, R.** (1998). Cell walls: Structure and signals. Current Opinions in Plant Biology **1:** 504-510.

- **Perera, I.Y., Zielinski, R.E.** (1991). Structure and expression of the *Arabidopsis* CaM-3 calmodulin gene. Plant Molecular Biology **19:** 49-64.

- **Perera, I.Y. & Zielinski, R.E.** (1992). Synthesis and accumulation of calmodulin in suspension cultures of carrot (*Daucus carota* L.). Plant Physiology **100:** 812-819.

- **Pickard, B.G.** (1971). Action potentials resulting from mechanical stimulation of pea epicotyls. Planta **97:** 106-115.

- **Pomerantz, J.L., Sharp, P.A. & Co, P.** (1995). Structure-based design of transcription factors. Science **267:** 93-96.

- **Pont-Lezica, R.F., McNally, J.G. & Pickard, B.G.** (1993). Wall-to-membrane linkers in onion epidermis: some hypotheses. Plant, Cell and Environment **16:** 111-123.

- **Price, A.H., Taylor, A., Ripley, S.J., Griffiths, A., Trewavas, A.J. & Knight, M.R.** (1994). Oxidative signals in tobacco increase cytosolic calcium. The Plant Cell **6:** 1301-1310.

- **Ptashne, M.A.** (1988). How eukaryotic transcriptional activators work. Nature **335:** 683-689.

- **Ptashne, M.A.** (1992). Genetic switch, 2nd Edition. Cell Press and Blackwell Scientific Publications.

- **Quandt, K., Frech, K., Karas, H., Wingender, E. & Werner, T.** (1995). MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Research **23:** 4878-4884.

- **Quatrano, R.S., Marcotte Jr, W.R. & Guiltinan** (1993). Regulation of gene expression by abscisic acid. In: Control of Plant Gene Expression. Desh Pal S. Verma (ed), pp 69-90. CRC Press, Inc., Florida.

- **Radke, J. & Burrows, W.** (1970). Soybean plant response to temporary field windbreaks. Agronomy Journal **62:** 424-429.

- **Radke, J. & Hagstrom, R.** (1973). Plant-water measurements on soybeans sheltered by temporary corn windbreaks. Crop Science **13:** 543-548.

- **Rasmussen, C. & Garen, C.** (1993). Activation of calmodulin-dependent enzymes can be selectively inhibited by histone H1. Journal of Biological Chemistry **268:** 23788-23791.

- **Reddy, A.S.N.** (2001). Calcium: silver bullet in signalling. Plant Science **160:** 381-404.

- **Reddy, A.S.N., Day, I.S., Narasimhulu, S.B., Safadi, F., Reddy, V.S., Golovkin, M. & Harnly, M.J.** (2002). Genes encoding calmodulin-binding proteins in the *Arabidopsis* genome. Journal of Biological Chemistry **277:** 9840-9852.

- **Reinhardt, D., Whittwer, F., Mandel, T. & Kuhlemeier, C.** (1998). Localized upregulation of a new expansin gene predicts the size of leaf formation in the tomato meristem. The Plant Cell **10:** 1427-1437.

- **Ridge, I., & Osbourne, D.J.** (1970). Hydroxyproline and oeroxidases in cell walls of *Pisum sativum* - regulation by ethylene. Journal of Experimental Botany **21:** 843-856.

- **Riechmann, J.L., Heard, J., Martin, G. Reuber, L., Jiang, C, Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K. & Yu, C.L.** (2000). Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science **290:** 2105-2110.

- **Ringli, C. & Keller, B.** (1998). Specific interaction of the tomato bZIP transcription factor VSF-1 with a non-palindromic DNA sequence that controls vascular gene expression. Plant Molecular Biology **37:** 977-988.

- **Rodriguez, J.G. & Cardemil, L.** (1995). Tissue specific expression of cell wall proteins of seedlings of *Prosopis chilensis* during development and wound stress. Physiologia Plantarum **93:** 457-463.

- **Roeder, R.G.** (1996). The role of general initiation factors in transcriptional activation by recruitment. Nature **386:** 569-577.

- **Rood, S.B., Williams, P.H., Pearce, D., Murofushi, N., Mander, L.N. & Pharis, R.P.** (1990). A mutant gene that increases gibberellin production level in *Brassica.* Plant Physiology **93:** 1168-1174.

- **Rosenberg, N., Leacher, D. & Neild, R.** (1966). Responses of irrigated snap beans to wind shelter. Journal of the American Society of Horticultural Science **90:** 169-179.

- **Rudd, J.J. & Franklin-Tong, V.E.** (2001). Unravelling response-specificity in Ca^{2+} signalling pathways in plant cells. New Phytologist **151:** 7-33.

- **Rushton, P.J. & Somssich, I.E.** (1998). Transcriptional control of plant genes responsive to pathogens. Current Opinions in Plant Biology **1:** 311-315.

- **Ryan, C.A.** (1990). Proteinase inhibitors in plants: Genes for improving defences against insects and pathogens. Annual Review of Phytopathology **28:** 425-449.

- **Sadava, D. & Chrispeels, M.J.** (1973). Hydroxyproline-rich cell wall protein (extensin): role in the cessation of cell elongation in excised pea epicotyls. Developmental Biology **30:** 49-55.

- **Sainz, M.B., Goff, S.A. & Chandler, V.L.** (1997). Extensive mutagenesis of a transcriptional activation domain identifies single hydrophobic and acidic amino acids important for activation in vivo. Molecular and Cellular Biology **17:** 115-122.

- **Sakai, D.D., Helms, S., Carlstedt-Duke, J., Gustafsson, J.A., Rottman, F.M. & Yamamoto, K.R.** (1988). Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes and Development **2:** 1144-1154.

- **Sakura, H., Kanei-Ishi, C., Nagase, T., Nakagoshi, H. & Gonda, T.J.** (1989). Delineation of three functional domains of the transcriptional activator encoded by the c-myb proto-oncogene. Proceedings of the National Academy of Sciences, USA **86:** 5758-5762.

- **Sakurai, H., Ohishi, T., Amakasu, H. & Fakasawa, T.** (1994). Yeast GAL11 protein stimulates basal transcription in a gene specific manner by a mechanism distinct from that by DNA-bound activators. FEBS Letters **351:** 176-180.

- **Salvà, I. & Jamet, É.** (2001). Expression of the tobacco *Ext 1.4* extensin gene upon mechanical constraint and localization of regulatory regions. Plant Biology **3:** 32-41.

- **Sambrook, J., Fritsch, E.F. & Maniatis, T.** (1989). Molecular Cloning: A Lab Manual. 2nd Edition. Cold Spring Harbor Laboratory Press.

- **Sanders, D. Pelloux, J., Brownlee, C. & Harper, J.F.** (2002). Calcium at the crossroads of signalling. Plant Cell **14 (Suppl):** S401-S417.

- **Sauer, N., Corbin, D.R., Keller, B. & Lamb, C.J.** (1990). Cloning and characterisation of a wound-specific hydroxyproline-rich glycoprotein in *Phaseolus vulgaris*. Plant, Cell and Environment **13:** 257-266.

- **Schaaf, M.J.M. & Cidlowski, J.A.** (2002). Molecular mechanisms of glucocorticoid action and resistance. The Journal of Steroid Biochemistry and Molecular Biology **83:** 37-48.

- **Schaffner, G., Schirms, S., Muller, M., Baden, B., Weber, F. & Schaffner, W.** (1988). Redundancy of information in enhancers as a principle of mammalian transcriptional control. Journal of Molecular Biology **201:** 81-90.

- **Schindler, U., Terzaghi, W., Beckmann, H., Kadesch, T. & Cashmore, A.R.** (1992). DNA binding site preferences and transcriptional activation properties of the *Arabidopsis* transcription factor GBF-1. The EMBO Journal **11:** 1275-1289.

- **Schnabelrauch, L.S., Kieliszewski, M., Upham, B., Alizedeh, H. & Lamport, D.T.A.** (1996). Isolation of pI 4.6 extensin peroxidase from tomato cell suspension cultures and identification of val-Tyr-Lys as putative intermolecular cross-link site. The Plant Journal **9:** 477-489.

- **Schwechheimer, C., Zourelidou, M. & Bevan, M. W.** (1998). Plant transcription factor studies. Annual Review of Plant Physiology and Plant Molecular Biology **49:** 127-150.

- **Sheng, J., D'Ovidio, R. & Mehdy, M.C.** (1991). Negative and positive regulation of a novel proline-rich protein mRNA by fungal elicitor and wounding. The Plant Journal **1:** 345-354.

- **Shinozaki, K. & Yamaguchi-Shinozaki, K.** (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Current Opinions in Plant Biology **3:** 217-223.

- **Shirsat, A. H., Bell, A., Spence, J. & Harris, J. N.** (1996a). The *Brassica napus extA* extensin gene is expressed in regions of the plant subject to tensile stresses. Planta **199**: 618-624.

- **Shirsat, A.H., Thomson, H.E.C. & Elliott, K.A.** (2003). The *Brassica napus extA* extensin gene negative regulatory region controls expression in response to mechanical stress. Plant, Cell and Environment **26:** 1647-1655.

- **Shirsat, A. H., Wieczorek, D. & Kozbial, P.** (1996b). A gene for *Brassica napus* extensin is differentially expressed on wounding. Plant Molecular Biology **30**: 1291-1300.

- **Shirsat, A.H., Wilford., N, & Croy, R.R.D.** (1989). Gene copy number and levels of expression in transgenic plants of a seed specific gene. Plant Science **61:** 75-80.

- **Shirsat, A.H., Wilford, N.W., Evans, I.M., Gatehouse, L.N. & Croy, R. R. D.** (1991). Expression of a Brassica napus extensin gene in the vascular system of transgenic tobacco and rape plants. Plant Molecular Biology **17:** 701-709.

- **Showalter, A.M.** (1993). Structure and function of plant cell wall proteins. The Plant Cell **5:** 9-23.

- **Showalter, A.M., Bell, J.N., Cramer, C.L., Bailey, J.A., Varner, J.E. & Lamb, C.J.** (1985). Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection. Proceedings of the Natural Academy of Sciences, USA **82:**6551-6555.

- **Showalter, A.M., Butt, A.D. & Kim, S.** (1992). Molecular details of tomato extensin and glycine-rich protein gene expression. Plant Molecular Biology **19:** 205-215.

- **Showalter, A. M. & Rumeau, D.** (1990). In: Organisation and assembly of plant and animal extracellular matrix. W.S. Adair & R.P. Mecham (eds.), p. 247. Academic Press, New York.

- **Showalter, A.M., & Varner, J.E.** (1987). Molecular details of plant cell wall hydroxyproline-rich glycoprotein expression during wounding and infection. In: Molecular Strategies for Crop Protection. Arntzen, C. and Ryan, C. (eds.), pp 375-392. Liss, New York.

- **Showalter, A.M., & Varner, J.E.** (1989). In: The Biochemistry of Plants: a comprehensive treatise, Vol 15. A. Marcus (ed.), pp 485-516. Hancourt Brace Jovanovich, San Diego.

- **Showalter, A.M., Zhou, J., Rumeau, D., Worst, S.G. & Varner, J.E.** (1991). Tomato extensin and extensin-like cDNAs: structure and expression in response to wounding. Plant Molecular Biology **16:** 547-565.

- **Shpak, E., Barbar, E., Leykam, J.F. & Kieliszewski, M.J.** (2001). Contiguous hydroxyproline residues direct hydroxyproline arabinosylation in *Nicotiana tabacam*. Journal of Biological Chemistry **276:** 11272-11278.

- **Shpak, E., Leykam, J.F. & Kieliszewski, M.J.** (1999). Synthetic genes for glycoprotein design and the eliucidation of hydroxyproline-O-glycosylation codes. Proceedings of the National Academy of Sciences, USA **96:** 14736-14741.

- **Sidik, N.M.** (1998). Isolation of extC, an extensin gene from Brassica napus. PhD Thesis. University of Wales, Bangor.

- **Singh, H., Sen, R., Baltimore, D. & Sharp, P.A.** (1986). A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature **319:** 154-158.

- **Singh, K.B.** (1998). Transcriptional regulation in plants: the importance of combinatorial control. Plant Physiology **118:** 1111-1120.

- **Singh, K.B., Foley, R.C. & Oñate-Sánchez.** (2002). Transcription factors in plant defence and stress responses. Current Opinions in Plant Biology **5:** 430-436.

- **Sistrunk, M.L., Antosiewicz, D.M., Purugganan, M.M. & Braam, J.** (1994). Arabidopsis TCH3 encodes a novel Ca^{2+} binding protein and shows environmentally induced and tissue-specific regulation. The Plant Cell **6:** 1553-1565.

- **Sive, H.L. & Roeder, R.G.** (1986). Interaction of a common factor with conserved promoter and enhancer sequences in histone H2B, immunoglobulin and U2 small nuclear RNA (snRNA) genes. Proceedings of the National Academy of Science, USA **83:** 6382-6386.

- **Slabowski, R.W.M., Moyano, E., Culianez-Marcia, A., Schuch, W., Martin, C. & Bevan, M.** (1994). A flower specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. The EMBO Journal **13:** 128-137.

- **Smallwood, M., Bevan, A., Donovan, N., Neill, S. J., Peart, J., Roberts, K. & Knox, J.P.** (1994). Localization of cell wall proteins in relation to the developmental anatomy of the carrot root apex. The Plant Journal **5:** 237-246.

- **Smith, J.J., Muldoon, E.P. & Lamport, D.T.A.** (1984). Isolation of extensin precursors by direct elution of intact tomato cell suspension cultures. Phytochemistry. **23:** 1233-1239.

- **Smith, J.J., Muldoon, E.P., Willard, J.J. & Lamport, D.T.A.** (1986). Tomato extensin precursors P1 and P2 are highly periodic structures. Phytochemistry **25:** 1021-1030.

- **Smith, L.G.** (2001). Plant cell division: Building walls in the right places. Nature Reviews Molecular Cell Biology **2:** 33-39.

- **Smith, V.C. & Ennos, A.R.** (2003). The effects of air flow and stem flexure on the mechanical and hydraulic properties of the stems of sunflowers *Helianthus annuus* L. Journal of Experimental Botany **54:** 845-849.

- **Snedden, W.A. & Fromm, H.** (2001). Calmodulin as a versatile calcium signal transducer in plants. New Phytologist **151:** 35-66.

- **Solano, R., Nieto, C., Avila, J., Diaz, I & Paz-Ares, J.** (1995). Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (MYB.Ph3) from *Petunia hybrida*. The EMBO Journal **14:** 1773-1784.

- **Sommer-Knudsen, J., Lush, W.M., Bacic, A. & Clarke, A.E.** (1998). Re-evaluation of the role of a transmitting tract-specific glycoprotein on pollen tube growth. The Plant Journal **13:** 529-535.

- **Southern, E.M.** (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology **98:** 503-517.

- **Spence, J., Vercher, Y., Gates, P., & Harris, N.** (1996). 'Pod shatter' in *Arabidopsis thaliana*, *Brassica napus* and *B. juncea*. Journal of Microscopy-Oxford **181:** 195-203.

- **Stafstrom, J.P. & Staehelin, L.A.** (1988). Antibody localization of extensin in cell walls of carrot storage roots. Planta **174:** 321-332.

- **Stano, J., Kovacs, P., Safarik, I., Kakoniova, D. & Safarikova, M.** (1998). A simple procedure for the detection of plant extracellular proteolytic enzymes. Biologia Plantarum **40:** 475-477.

- **Stracke, R., Werber, M. & Weisshaar, B.** (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. Current Opinions in Plant Biology **4:** 447-456.

- **Stull, J.T., Manning, D.R., High, C.W. & Blumenthal, D.K.** (1980). Phosphorylation of contractile proteins in heart and skeletal muscle. Federation Proceedings of the American Society for Experimental Biology **39:** 1552-1557.
- **Suge, H.** (1978). Growth and gibberellin production in *Phaseolus vulgaris* is affected by mechanical stress. Plant and Cell Physiology **19:** 1557-1560.

- **Suge, H.** (1980). Dehydration and drought resistance in *Phaseolus vulgaris* as affected by mechanical stress. Reports of the Institute of Agricultural Research, Tohoko University 31: 1-10.

- **Swords, K.M.M. & Staehelin, L.A.** (1993). Complementary immunolocalization patterns of cell wall hydroxyproline-rich glycoproteins studied with the use of antibodies directed against different carbohydrate epitopes. Plant Physiology **102:** 891-901.

- **Szymanski, D.B., Liao, B. & Zielinski, R.E.** (1996). Calmodulin isoforms differentially enhance the binding of cauliflower nuclear proteins and recombinant TGA3 to a region derived from the *Arabidopsis* CaM-3 promoter. The plant Cell **8:** 1266-1273.

- **Tabata, T., Nakayama, T., Mikami, K. & Iwabuchi, M.** (1991). HBP-1a and HBP-1b: leucine zipper-type transcription factors of wheat. The EMBO Journal **10:** 1459-1467.

- **Tagu, D., Walker, N., Ruiz-Avila, L., Burgess, S., Martìnez-Izquierdo, J.A., Leguay, J-J., Netter, P. & Puigdomènech, P.** (1992). Regulation of the maize HRGP gene expression by ethylene and wounding. mRNA accumulation and qualitative expression analysis of the promoter by microprojectile bombardment. Plant Molecular Biology **20:** 529-538.

- **Takahashi, H. & Jaffe, M.** (1982). The interaction of callose and ethylene in thigmomorphogenesis. Phytologist **25:** S139-S140.

- **Takahashi, H., Suge, H. & Kato, T.** (1991). Growth promotion by vibration at 50 Hz in rice and cucumber seedlings. Plant and Cell Physiology **32:** 729-732.

- **Takezawa, D., Liu, Z.H., An, G. & Poovaiah, B.W.** (1995). Calmodulin gene family in potato: developmental and touch-induced expression of the mRNA encoding a novel isoform. Plant Molecular Biology **27:** 693-703.

- **Telewski, F.W.** (1995). Wind-induced physiological and developmental responses in trees. In: Wind and Trees. M.P. Coutts and J. Grace (eds). Cambridge University Press, Cambridge.

- **Telewski, F.W & Jaffe, M.J** (1986a). Thigmomorphogenesis: anatomical, morphological and mechanical analysis of genetically different sibs of *Pinus taeda* in response to mechanical perturbation. Physiologia Plantarum **66:** 219-226.

- **Telewski, F.W & Jaffe, M.J** (1986b). Thigmomorphogenesis: field and laboratory studies of Abies fraseri in response to wind or mechanical perturbation. Physiologia Plantarum **66:** 211-218.

- **Telewski, F.W. & Pruyn, M.L.** (1998). Thigmomorphogenesis: a dose response to flexing in *Ulmus Americana* L. seedlings. Tree Physiology **18:** 65-68.

- **Thanos, D. & Maniatis, T.** (1995). Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. Cell **83:** 1091-1100.

- **Thompson, K.F. & Hughes, W.G.** (1986). Breeding and varieties. In oilseed Rape. D.H. Scarisbrick and R.W. Daniels (eds.), pp 32-82.Collins Professional and Technical, London.

- **Thonat, C. Mathieu, C., Crevecouer, M., Penel, C., Gaspar, T., Boyer, N.** (1997). Effects of a mechanical stimulation on localization of annexin-like proteins in *Bryonia dioica* internodes. Plant Physiology **114:** 981-988.

- **Tierney, M.L., Wiechert, J. & Pluymers, D.** (1988). Analysis of the expression of extensin and p33 related cell wall proteins in carrot and soybean. Molecular and General Genetics **211**: 393-399.

- **Tiré, C., de Ryke, R., De Loose, M., Inze, D., Van Montagu, M. & Engler, G.** (1994). Extensin gene expression is induced by mechanical stimuli leading to local cell wall strengthening in *Nicotiana plumbaginifolia*. Planta **195:** 175-181.

- **Tiwari, S.B., Wang, X-J., Hagen, G. & Guilfoyle, T.J.** (2001). AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. The Plant Cell **13:** 2809-2822.

- **Torres-Schumann, S., Ringli, C., Heierli, D., Amrhein, N. & Keller, B.** (1996). *In vitro* binding of the tomato bZiP transcriptional activator VSF-1 to a regulatory element that controls xylem-specific gene expression. The Plant Journal **9:** 283-296.

- **Tréhin, C., Glab, N., Perennes, C. Planchais, S. & Bergounioux, C.** (1999). M phase-specific activation of the *Nitotiana sylvestris* Cyclin B1 promoter involves multiple regulatory elements. The Plant Journal **17:** 263-273.

- **Trewavas, A.** (1986). Resource allocation under poor growth conditions: a major role for growth substances in developmental plasticity. In: Symposia of the society for experimental biology. XXX. Plasticity in Plants. D. Jennings, A. Trewavas (eds.), pp 31-76. Company of Biologists, Cambridge.

- **Trewavas, A. & Knight, M.** (1994). Mechanical signalling, calcium and plant form. Plant Molecular Biology **26:** 1329-1341.

- **Ulmasov, T., Hagen, G. & Guilfoyle, T.J.** (1997a). ARF1, a transcription factor that binds to auxin response elements. Science **276:** 1865-1868.

- **Ulmasov, T., Murfett, J., Hagen, G. & Guilfoyle, T.J.** (1997b). Aux/IAA proteins repress expression of reporter genes containing natural highly active synthetic auxin response elements. The Plant Cell **9:** 1963-1971.

- **Ulmasov, T., Liu, Z-B., Hagen, G. & Guilfoyle, T.J.** (1995). Composite structure of auxin response elements. The Plant Cell **7:** 1611-1623.

- **Urao, T., Yamaguchi-Shinozaki, K., Urao, S. & Shinozaki, K.** (1993). An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. The Plant Cell **5:** 1529-1539.

- **Van Etten, C.H., Miller, R.W., Earle, F.R. & Wolff, I.A.** (1961). Hydroxyproline content of seed meals and distribution of the amino acid in kernel, seed coat, and pericarp. Journal of Agricultural and Food Chemistry **9:** 433-435.

- **Van Holst, G.J., Klis, F.M., Bouman, F. & Stegwee, D.** (1980). Changing cell wall compositions in hypocotyls of dark-grown bean seedlings. Planta **149:** 209-212.

- **Van Holst, G.J. & Varner, J.E.** (1984). Reinforced polyproline II conformation in a hydroxyproline-rich cell wall glycoprotein from carrot root. Plant Physiology **74:** 247-251.

- **Varner, J.E. & Lin, L.S.** (1989). Plant cell wall architecture. Cell **56:** 231-239.

- **Vera, P., Lamb., C. & Davin L.B.** (1994). Cell-cycle regulation of hydroxyproline-rich glycoprotein HRGPnt3 gene expression during the initiation of lateral root meristems. The Plant Journal **6:** 717-727.

- **Waffenschmidt, S., Woessner, J.P., Beer, K. & Goodenough, U.W.** (1993). Isodityrosine cross-linking mediates insolubilization of cell walls in *Chlamydomonas*. The Plant Cell **5:** 809-820.

- **Wallace, W., Secor, J. & Schrader, L.E.** (1984). Rapid accumulation of aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to a lower temperature, darkness or mechanical manipulation. Plant Physiology **75:** 170-175.

- **Wang, L.-W., Showalter, A.M. & Ungar, I.A.** (1997). Effect of salinity on growth, ion content and cell wall chemistry in *Atriplex prostrata*. American Journal of Botany **84:** 1247-1255.

- **Wang, N., Butler, J.P. & Ingber, D.E.** (1993). Mechanotransduction across the cell-surface and through the cytoskeleton. Science **260:** 1124-1127.

- **Wang, N., Maruse, K., Stamenović, D., Fredberg, J.J., Mijailovich, S.M., Tolić-Nørrelykke, I.M., Polte, T., Mannix, R. & Ingber, D.E.** (2001). Mechanical behaviour in living cells consistent with the tensegrity model. Proceedings of the National Academy of Sciences, USA **98:** 7765-7770.

- **Watillon, B., Kettmann, R., Boxus, P. & Burny, A.** (1992). Cloning and characterization of an apple (*Malus domestica* (L) Borkh) calmodulin gene. Plant Science **82:** 201-212.

- **Whitmore, F.W.** (1978). Lignin-protein complex catalysed by peroxidase. Plant Science Letters 13: 241-245

- **Williams, M.E., Foster, R. & Chua, N.H.** (1992). Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. The Plant Cell **4:** 485-496.

- **Wojtaszek, P., Trethowan, J. & Bolwell, G.P.** (1995). Specificity in the immobilisation of cell wall proteins in response to different elicitor molecules in suspension-cultured cells of French bean (*Phaseolus vulgaris* L.). Plant Molecular Biology **28:** 1075-1087.

- **Wolff, D.J., Poirier, P.G., Brostrom, C.O. & Brostrom, M.A.** (1977). Divalent cation binding properties of bovine brain Ca^{2+}-dependent regulator protein. Journal of Biological Chemistry **252:** 4108-4117.

- **Workman, J.L. & Buchman, A.R.** (1992). Trends in Biochemical Science **18:** 90-95.

- **Wu, L., Joshi, C.P. & Chiang, V.L.** (2000). A xylem-specific cellulose synthase gene from aspen (*Populus tremuloides*) is responsive to mechanical stress. The Plant Journal **22:** 495-502.

- **Wyatt, S.E. & Carpita, N.C.** (1993). The plant cytoskeleton-cell-wall continuum. Trends in Cell Biology **3:** 413-417.

- **Wycoff, K.L., Powell, P.A., Gonzales, R.A., Corbin, D.R., Lamb, C. & Dixon, R.A.** (1995). Stress activation of a bean hydroxyproline-rich glycoprotein promoter is superimposed on a pattern of tissue-specific developmental expression. Plant Physiology **109:** 41-52.

- **Xu, W., Purugganan, M., Polisensky, D., Antosiewicz, D., Fry, S. & Braam, J.** (1995). *Arabidopsis* TCH4, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. The Plant Cell **7:** 1555-1567.

- **Yamamoto, K.R.** (1985). Steroid-receptor regulated transcription of specific genes and gene networks. Annual Review of Genetics **19:** 209-252.

- **Yamauchi, D.** (1997). Interaction of seed embryo factors with the 5′-upstream region of seed storage 7S globulin gene from *Canavalia gladiata*.

- **Yanagisawa, S.** (1996). Dof DNA-binding proteins contain a novel zinc finger motif. Trends in Plant Science **1:** 213-214.

- **Yanagisawa, S.** (1998). Transcription factors in plants: physiological functions and regulation of expression. Journal of Plant Research **111:** 363-371.

- **Yanagisawa, S. & Schmidt, R.** (1999). Diversity and similarity among recognition sequences of Dof transcription factors. The Plant Journal **17:** 209-214.

- **Yang, P., Chen, C., Wang, Z., Fan, B. & Chen, Z.** (1999). A pathogen- and salicylic acid-induced WRKY DNA-binding activity recognizes the elicitor response element of the tobacco class I chitinase gene promoter. The Plant Journal **18:** 141-149.

- **Yang, T.B., Segal, G., Abbo, S., Feldman, M. & Fromm, H.** (1996). Characterization of the calmodulin gene family in wheat: structure, chromosomal location, and evolutionary aspects. Molecular and General Genetics **252:** 684-694.

- **Yanissch-Perron, C., Vieira, J. & Messing, J.** (1985). Improved M13 phage cloning vectors and host strains - nucleotide sequences of the M13MP18 and PUC18 vectors. Gene **33:** 103-119.

- **Ye, Z-H., Song, Y.R., Marcus, A. & Varner, J.E.** (1991). Comparative localisation of three classes of cell wall proteins. The Plant Journal **1:** 175-183.

- **Ye, Z.-H & Varner, J.E.** (1991). Tissue specific expression of cell wall proteins in developing soybean tissues. The Plant Cell **3:** 23-37.

- **Yu, L.M., Lamb, C.J. & Dixon, R.A.** (1993). Purification and biochemical characterisation of proteins which bind to the H-box *cis*-element implicated in transcriptional activation of plant defence genes. The Plant Journal **3:** 805-816.

- **Zhang, L. & Lu, Y-T.** (2003). Calmodulin-binding protein kinases in plants. Trends in Plant Science **8:** 123-127.

- **Zhou, J., Rumeau, D. & Showalter, A. M.** (1992). Isolation and characterization of two wound-regulated tomato extensin genes. Plant Molecular Biology **20:** 5-17.

- **Zielinski, R.E.** (1998). Calmodulin and calmodulin-binding proteins in plants. Annual Review of Plant Physiology and Plant Molecular Biology **49:** 697-725.

Appendix I. The sequence of pHT4 plasmid containing –586bp of the *extA* promoter fused to the GUS coding sequence.

This sequence was obtained using the capillary electrophoresis method (see section 2.12 for details) and shows that the construct is in frame. All constructs were sequenced using this method.

TTGAAGANTAACCTCATTTCTTTTNTCATAGTCAATTATTGTTGTTTATTAC

ATATGACAAAAATGCATACCATCAGGGATTAAAAAAGTGAAATTGAAAA

GCTATATAAAGGTGATTAGAGGGAGAGAGTAAGAGCATCAAACACAAAG

TCS

TATA box

Sma I site

AAAAACACATAACAATATGGGGTCACCAATGGCCCGGGTAGGTCAGTCCC

extA coding sequence

pBI101.2

TTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTCGAC

GUS coding sequence

GGCCTGTGGGCATTCAGTCTGGATCGCGAAAACTGTGGAATTGATCAGCG

TTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCAATTGCTGTGCCAGGCA

GTTTTAACGATCAGTTCGCCGATGCAGATATTCGTAATTATGCGGGCAAC

GTCTGGTATCAGCGCGAAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCG

TATCGTGCTGCGTTTCGATGCGGTCACTCATTACGGCAAAGTGTGGGTCAA

TAATCAGGAAGTGATGGAGCATCAGGGCGGCTATACGCCATTTGAAGCCG

ATGTCACGCCGTATGTNATTGCCGGGAAAAGTGTACGTATCACCGTTTGT

GTGAACAACGAACTGAACTGGCAGACTATCCCGCCGGGGAATGGTGATN

ACCGACGANNANCGGCAAGAAAAAGCAGTCNNNCTTNCATGATNTCNTA

ANCTATNCCGG

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The *Brassica napus extA* extensin gene negative regulatory region controls expression in response to mechanical stresses

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ABSTRACT

In the present study the hypothesis that the –433 to –664 bp negative regulatory region (NRR) of the *Brassica napus extA* extensin promoter controls *extA* activation in response to externally applied weight loads was tested. When weight loads were applied to the nodal regions of transgenic tobacco plants containing *extA* promoter deletions fused to GUS, repression controlled by the NRR was overcome and GUS expression was induced only in the transgenics carrying the NRR. This proves that extensin expression in nodal regions is not developmentally controlled, but is induced in response to mechanical stresses, and is controlled by the NRR. It was also shown that the activation of the *extA* promoter during the development of lateral roots is a stress-related response that is also under the control of the NRR but that the constitutive expression of extensin mRNA in the phloem of roots is not due to the mechanical forces the root experiences as it forces it way through the soil. Electrophoretic mobility shift assays using a 25 bp oligonucleotide have been used to show that an 8 bp consensus sequence from the NRR binds nuclear proteins. Wound-induced signals regulating extensin gene expression are shown to travel bi-directionally through the plant, from root to leaf and vice versa.

Key-words: *Brassica napus*; extensin; mechanical stresses; negative regulation; promoter analysis, signal transduction; transcription factors; wounding.

INTRODUCTION

It has long been appreciated that plant growth and development is affected by mechanical stresses. For instance, it is known that wind-loaded trees and shrubs become physiologically dwarfed, and that the shortest stalks in a maize field are found on the windward edge of the stand, suggesting that wind-imposed stresses have contributed to the reduction in size. The term 'thigmomorphogenesis' describes the morphogenetic changes that occur when plants are lightly touched (Jaffe & Biro 1979), and 'seismomorphogenesis' describes plant responses to more severe stresses such as wind, shaking and vibration (Mitchell *et al.* 1975). Numerous reports (reviewed in Mitchell & Myers 1995) have demonstrated that mechanical stresses inhibit internode elongation, result in radial stem swelling, retard leaf expansion, delay flowering, and reduce potato tuber size. The positive effects of such stresses include the strengthening of etiolated tomato stems and an increase in the cellulose fraction of stem fibres – both of which are part of the natural hardening process that plants undergo when transferred from protected greenhouse environments to the field.

Touch (TCH) genes that are up-regulated in response to thigmic stresses in *Arabidopsis thaliana* have been identified (Braam & Davis 1990). These genes code for the calcium binding protein calmodulin, suggesting that calcium flux is involved in the signal transduction pathway leading to the activation of genes contributing to the observed phenotypic differences. In confirmation, experiments using transgenics expressing the calcium-sensitive bioluminescent protein aequorin have shown that rapid and transient calcium fluxes are an early feature of the plant response to mechanical stress (Knight, Smith & Trewavas 1992). However, very few genes which are at the end point of this gene activation pathway, and which directly contribute to the phenotypic changes observed, have so far been identified.

The proteins of the plant cell wall are currently receiving much attention and two recent reviews (Cassab 1998; Lamport 2001) have examined cell wall proteins with regard to their structure and their functional properties. We have previously shown that genes from *Brassica napus* and *Arabidopsis* that code for the structural cell wall hydroxyproline-rich glycoprotein, extensin, are expressed in regions of the plant that are subject to mechanical stresses (Shirsat *et al.* 1996a; Elliott & Shirsat 1998; Merkouropoulos, Barnett & Shirsat 1999). In particular, promoter–reporter fusions of the *B. napus extA* gene fused to GUS, demonstrated that expression of the reporter was seen in regions of the plant such as the nodes which experience load-bearing stresses due to the weight of the developing axillary branch (Shirsat *et al.* 1996a). Further, we were able to show that this expression pattern appeared to be controlled by a pro-

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moter region that we (Elliott & Shirsat 1998) termed the negative regulatory region (NRR). The hypothesis was put forward that the NRR acted as a negative regulator repressing expression, and that this repression was overcome at nodal stress bearing regions, resulting in extensin gene activation.

In the present study,the hypothesis that the artificial application of mechanical stress overrides the repression controlled by the *extA* NRR element was tested by subjecting transgenic plants containing various *extA* promoter deletions to mechanical loads. In addition, we tested the hypothesis (using electrophoretic mobility shift assays) that a specific consensus sequence within the NRR was responsible for this regulation. Further, we tested the commonly held assumption that constitutive expression of extensin mRNA in the root was due to the mechanical constraints the root encounters as it forces its way through the soil.

MATERIALS AND METHODS

Construction of truncated extA promoter-GUS reporter fusion genes

Construction of the truncated *extA* promoter-GUS reporter gene fusions has already been described (Elliott & Shirsat 1998). Briefly, the plasmid pAS44 containing 940 bp of *extA* 5′ flanking sequence linked to the coding sequence of the *gus* reporter gene in the pBI101.2 vector (Jefferson 1987) was used to construct the promoter truncations using the Pharmacia (Amersham Pharmacia, Little Chalfont, Bucks., UK) double-stranded nested deletion kit. Plasmid minipreps of the resulting transformants were analysed by agarose gel electrophoresis to identify clones that appeared to have the desired truncations. The exact end-point of each deletion was determined by DNA sequencing.

Transformation of *Nicotiana tabacum*

Leaf discs of *Nicotiana tabacum* were transformed with *Agrobacterium tumefaciens* LBA4404 containing the truncated *extA* 5′ flanking sequence constructs as described in Ellis *et al.* (1988). The transformants were selected on shoot-inducing media containing 400 μg mL^{-1} of augmentin and 100 μg mL^{-1} of kanamycin sulphate. Regenerated shoots were transferred to a medium containing half strength MS salts (Murashige & Skoog 1962), 100 μg mL^{-1} augmentin and 100 μg mL^{-1} kanamycin sulphate. When the shootlets had developed an extensive root system, they were potted into compost and grown to maturity.

Histochemical localization of GUS expression

Histochemical assays were performed as described by Jefferson (1987). Transverse hand sections of stem internodes, nodes and petioles were incubated in a 50 mM NaH_2PO_4, ph.7.0 buffer containing 1 mM X-Gluc, 1.8 mM cyclohexamide for 14 h at 37 °C. Whole roots were also incubated in the same solution. Sections were fixed in 3% gluteraldehyde at 4 °C overnight, cleared in 100% ethanol, and photographed on a Leica Wild 8 microscope (Leica, Knowhill, Milton Keynes, Beds., UK) using Agfa Ultra 50 colour film (Agfa-Gevaert, Brentford, Middlesex, UK).

Application of mechanical stress to wild-type seedling roots and to nodal regions of mature transgenic plants

In order to subject *B. napus* roots to mechanical stresses, the seeds were first germinated on moist blotting paper and then transferred to Beatson jars filled with sterile autoclaved 1 mm glass beads that were kept thoroughly moistened with Hoagland's nutrient solution. Seedlings were allowed to grow on this material under growth room conditions, and roots were seen to penetrate into the glass bead substratum. Seedlings were harvested 25 d after first sowing, separated into root and leaf tissues, and used for RNA extraction. As a control, seedlings were germinated directly in hydroponic solution. All plants were grown at 20 °C with a 16 h light/8 h dark cycle.

Mechanical loads were applied to the nodal regions of mature plants as described in Shirsat *et al.* (1996a). Three independent transgenic plants from each promoter truncation, which had previously been identified as low expressers in fluorimetric assays, were used. The main stem of each plant was cut to allow two lateral stems of the same age and size to develop; after 3 weeks; both lateral stems had produced a number of axillary branches of equivalent sizes. To apply the load, a 10 g weight was suspended from the youngest axillary branch at the apex of one of the lateral stems. The control axillary branch on the other stem was left unweighted. After 36 h, both the control and the weight-loaded axillary branch nodes were sectioned and examined for GUS expression.

Wounding of plant tissue, RNA extraction, and northern hybridization

In order to study the movement of wound-induced signals which induce extensin gene expression, *B. napus* var. Bienvenue plants were used which had been hydroponically grown in Hoagland's solution and which were approximately 10 weeks old-with five to nine fully emerged leaves. In the leaf wounding experiments, leaves were wounded by crushing the leaf surface with a dialysis mediclip and total RNA extracted from the root at 12, 24, 36, 48 and 72 h. To simulate a continuous wounding stimulus, the leaf wounding was repeated at each sample extraction time. In the root wounding experiments, roots were wounded by crushing the root system using mediclips (applying enough pressure to indent the roots, but not to break them) and RNA samples were extracted from leaves at 12, 24 and 36 h. As in the leaf wounding experiment, root wounding was repeated at each time point in order to simulate a continuous wounding stimulus. Total RNA was extracted from both leaves and roots, electrophoresed on agarose gels, blotted and hybridized to radiolabelled *extA* probes, as detailed in

Elliott & Shirsat (1998). Filters were washed to maximum stringency and exposed to photographic films.

Electrophoretic mobility shift assays

Nuclear protein extracts were prepared from *B. napus* leaf and petiole tissues according to Deryckere & Gannon (1994). The amount of nuclear protein in each extract was assayed using the Bradford method (Bradford 1976), aliquoted into single-use fractions and immediately snap frozen in liquid nitrogen. Some 25 bp double-stranded oligonucleotides corresponding to the identified NRR sequence and an unrelated sequence from *Drosophila* (control) were synthesized (MWG-Biotech, Ebersberg, Germany), annealed, and labelled using γ^{32}P-ATP according to standard techniques (Sambrook, Fritsch & Maniatis 1989). Binding reactions were performed in a total volume of 10 μL and contained 1× binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM ethylenediaminetetraacetic acid (pH 8.0), 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCL (pH 7.5) and 0.5 μg poly (dIdC : dIdC)], and 5 μg of nuclear protein extract. Reactants were mixed and incubated on ice for 20 min. 1 μL of radiolabelled oligonucleotide was then added to the reaction and incubation continued for a further 20 min 0.1 pmols of probe DNA were used per reaction (about 1 ng of oligo DNA) and 15 pmols of competitor DNA were used per reaction (where appropriate). 1 μL of 10× loading dye [250 mM Tris-HCL (pH 7.5), 0.2% bromophenol blue, 0.2% xylene cyanol and 40% glycerol] was then added to each reaction, mixed, and electrophoresed on 4% polyacrylamide gels according to standard techniques (Sambrook *et al.* 1989). After electrophoresis was complete, the gel was transferred to 3MM Whatman (Maidstone, Kent, UK) chromatography paper and exposed to photographic film.

RESULTS

Mechanical loading is able to induce GUS expression in transgenic axillary branch nodes of plants containing the –664, –789 and –940 bp extA promoter truncations, but not in transgenics containing the –159 and –433 bp truncations

Truncations of the *extA* promoter and the precise end points of the various promoter deletions used in the transformations are shown in Fig. 1 and have been detailed in previous work (Elliott & Shirsat 1998). These truncations were used to transform *N. tabacum* plants, and GUS expression patterns at the internodes, nodes, unweighted axillary nodes and weighted axillary nodes of the transgenics are shown (Fig. 2). Sections presented are representative of 20 sections per plant, with three plants, each containing single copy insertions, being analysed. In mature stem internodes of plants containing the –664, –789 and –940 bp *extA* promoter truncations, expression was restricted to the internal and external phloem, but the internodes of plants carrying the –159 and –433 bp truncations showed expression in all tissues, including the cortex and pith. This indicates that an element located between –433 to –664 bp is acting as a negative regulator, restricting expression to phloem tissues, and that the non-specific expression seen at the internodes of plants containing the –159 and –433 bp constructs is due to the absence of this controlling sequence. At the axillary nodes this pattern of expression changed radically; all sections, irrespective of promoter length showed expression in all tissue and cell types at the node, with no specificity being exhibited. We have proposed (Elliott & Shirsat 1998), that this expression pattern is due to a negatively regulating region (NRR) situated between –433 and –664 bp and that this region acts to repress expression in tissues (such as the internodes) that do not experience mechanical loading. In tissues that are subject to loading (nodes), this repression is overcome, leading to the observed expression in all tissues. In order to confirm that the non-specific expression seen in all tissues at the nodes was indeed due to mechanical stress experienced at the nodes due to the weight of the axillary branch and was not a developmentally controlled expression pattern, we performed a weight-loading experiment in which all axillary nodes of plants containing the promoter deletions were weight-loaded by hanging a 10 g weight on the nodal axillary branch. In order that the non-specific expression patterns already observed in the high expressers did not mask any additional effects due to the experimental weight-loading, low expressers in which GUS expression at the nodes was barely detectable were chosen for each promoter deletion – the expression patterns at the unweighted axillary nodes of these low expressers are shown. In transgenics containing the –940, –789 and –664 bp truncations, hanging a weight on the axillary branch resulted in the induction of GUS synthesis in a distinct band that extended through the cortical parenchyma and into the pith (weighted axillary nodes), which is exactly where we would have expected the stress to be exerted. The control unweighted axillary nodes from the same plant showed no such pattern. The weight-loaded axillary stalks from plants containing the –433 and –159 (bp) truncations (lacking the NRR) did not show GUS synthesis at the node – the observed expression patterns were identical to the unweighted node sections.

Electrophoretic mobility shift assays show that sequences in the NRR bind nuclear proteins

Examination of the NRR sequence between –433 to –664 bp has revealed the sequence 5′-TAGTGGAT-3′. This sequence contains the core motif *GTGG* that has been shown (in the promoter of the 1.8 kb glycine-rich protein gene from bean) to bind nuclear proteins and behave as a negatively acting regulatory element essential for vascular specific expression (Torres-Schumann *et al.* 1996). As the *extA* promoter also appears to be negatively regulated, we used a synthesized 25 bp sequence 5′-TAAAATATAGTG GATTTTGCATATG-3′ (which contains the TAGTGGAT element) in gel-shift assays to see whether it was capable

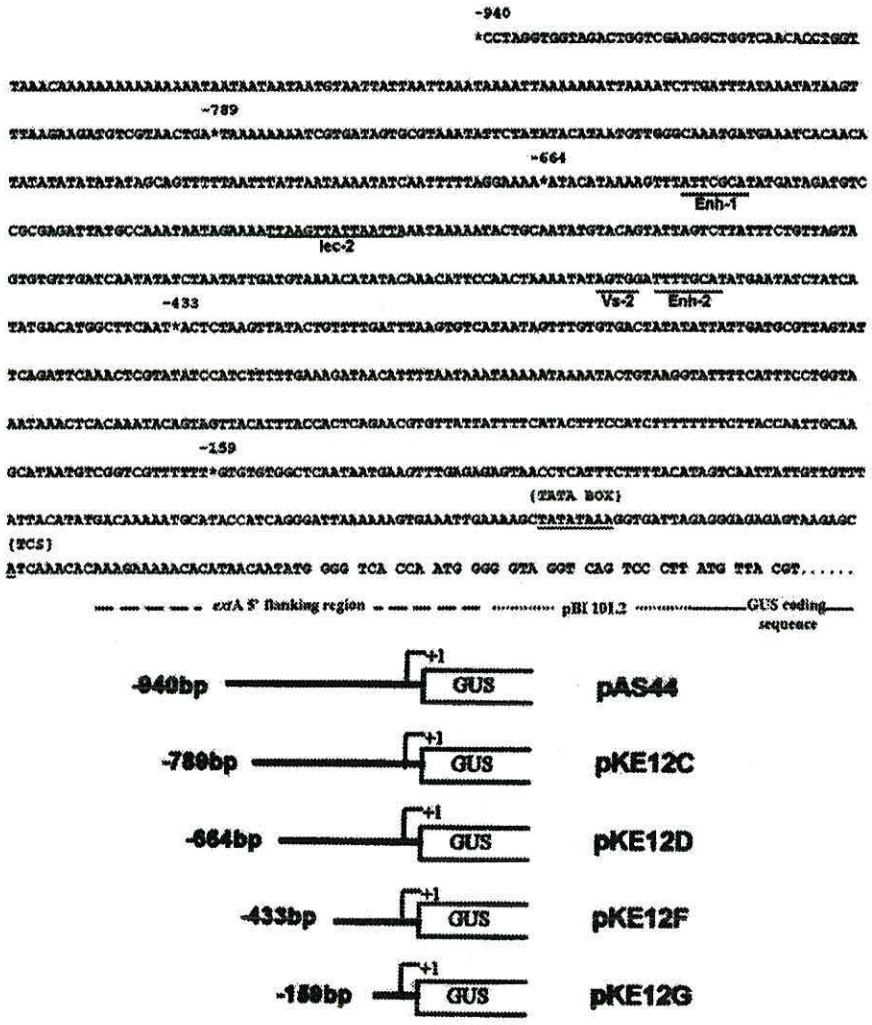

Figure 1. The sequence of the *extA* promoter truncations showing the fusion to the GUS reporter coding sequence in the binary transformation vector pBI 101.2 – the end points of the various truncations are shown by * and a diagrammatic representation of the promoter truncations is shown below the sequence as a line diagram. Underlined transcription factor sites are referred to in the Discussion. The TATA box and the transcription start site (TCS) are underlined.

of binding nuclear proteins (Fig. 3). Initial results showed that a strong shift was seen when rape leaf nuclear protein extract were used in the assay (Fig. 3 track 2) but not when petiole nuclear protein extract was used (Fig. 3 track 3). Adding a 150 M excess of cold homologous probe (compared to the labelled probe) resulted in a small reduction in the intensity of the shifted band (Fig. 3 track 4) – this reduction was not observed when an unrelated 25 bp sequence from *Drosophila* was used as a non-specific competitor (Fig. 3 track 6). As expected, shifts are not seen when petiole nuclear extracts are used in the competition experiments (Fig. 3 tracks 5, 7).

Expression patterns in the developing lateral roots of transgenics containing the various promoter deletions mirror those seen in the nodes and internodes, showing that mechanical forces experienced by the lateral roots also result in extA promoter activation

The cells at the apex of developing lateral roots are under considerable mechanical pressure as they break through the cortex of the parental root. To investigate root expression patterns in the promoter deletion transgenics, roots were taken from 10-week-old-plants grown on half-strength MS agar and used for a GUS histochemical assay (Fig. 4). It is important to note that these plants were the same low expressers as were used in the weight-loading experiments on the aerial tissues – this is why the normal pattern of phloem specific expression in the roots is not seen. Three independently regenerated transgenics from each of the five promoter truncations were used in GUS histochemical assays, and the same pattern of expression (Fig. 4) was seen in all transgenics derived from a particular promoter truncation. Roots from plants carrying the –159 and –433 bp truncations showed expression in all tissues; this is similar to the non-specific expression pattern seen in the internodes of transgenics carrying the –159 and –433 bp truncations (Fig. 2). By contrast, roots from plants carrying the –664, –789 and –940 bp truncations did not show any expression in the cortex and pith of the main root; expression was only seen in the cell types associated with the developing lateral roots. This result confirms the expression patterns seen at the weighted axillary node regions (Fig. 2); at root regions experiencing mechanical stress (presumably

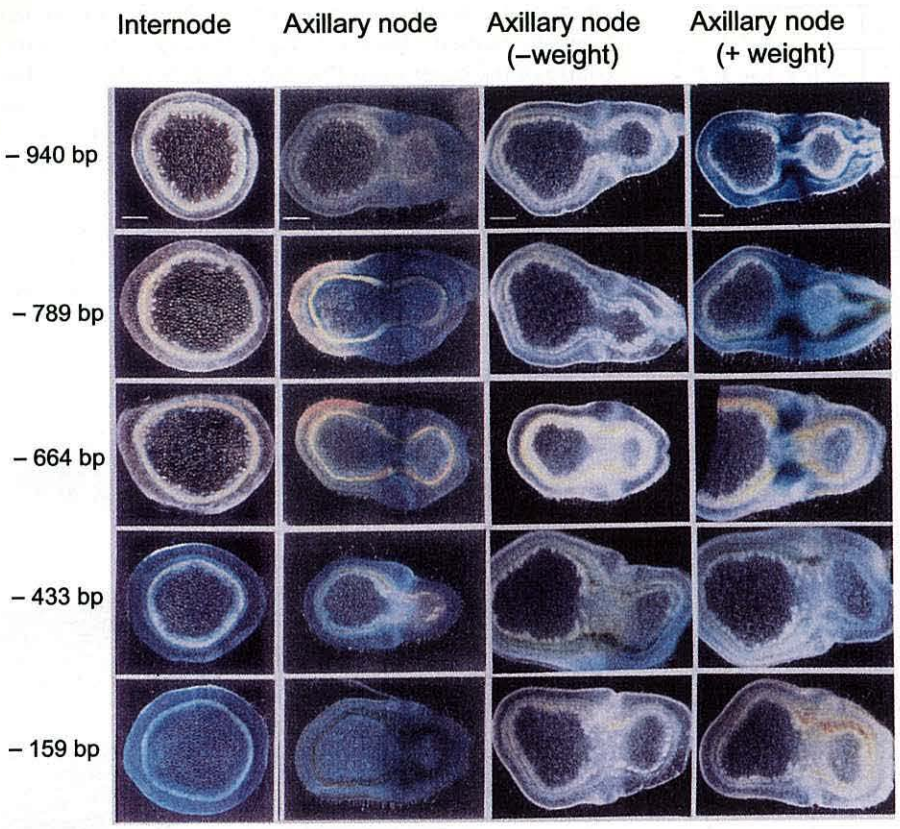

Figure 2. Histochemical analysis of *extA* promoter-driven GUS expression in internodes and axillary branch nodes of transgenic tobacco plants containing the –940, –789, –664, –433 and –159 bp promoter truncations. Internode, transverse sections through the internode of mature plants; Axillary node, transverse sections through the axillary branch node of high expressing plants; Axillary node (– weight), transverse sections through the axillary branch node of unweighted low expressing plants; Axillary node (+ weight), transverse sections through weight-loaded axillary branch nodes from the same low expresser plants used for the unweighted node sections. Bar = 1 mm.

due to the pressure exerted by the lateral root pushing through the cortex of the main root), the normal repression exerted by the NRR is overcome, leading to expression in the stressed organ (lateral root).

High levels of extA expression in the phloem of wild-type roots are not due to the mechanical pressures roots experience as they force their way through the soil

High levels of extensin mRNA are always seen in the roots of pot grown *B. napus* plants – this pattern is also maintained in the roots of *Nicotiana* transgenics carrying the *B. napus extA* promoter – GUS reporter gene fusions. In order to see whether this expression was an induced response due to mechanical stresses imposed on the roots during growth, or whether it was an example of developmentally regulated constitutive expression, one set of wild-type *B. napus* seedlings were grown hydroponically (no restriction on root growth) and the other set on glass beads of 1 mm diameter where the roots were subject to mechanical stresses as they forced their way between the beads. After 25 d of growth on the two media, it was obvious that the roots of seedlings grown on in the glass beads had experienced growth restriction – they were much shorter and thicker in comparison with the roots of hydroponically grown plants. RNA was extracted from both sets of roots, and northern hybridized to the radiolabelled *extA* coding sequence. High levels of 1260 b *extA* mRNA transcripts were seen in both sets of samples (Fig. 5 tracks 1 and 2), both hydroponically grown (no restriction to root growth – track 1) and grown through the glass beads (roots under mechanical stress – track 2) indicating that the high levels of extensin mRNA transcripts which are always seen in the roots are due to developmentally regulated constitutive expression and are not due to the mechanical stresses that the roots experience as they force their way through the soil.

Wound signals regulating extensin gene expression travel bi-directionally through the plant, from root to leaf and vice versa

We have already shown (Elliott & Shirsat 1998) that wounding *B. napus* leaves leads to expression of *extA* mRNA, and that this expression follows a set time-course of transcript accumulation. We also showed in the same work that wounding the root led to a rapid decline in the high levels of *extA* transcripts that are always found in root tissues. Extensin cDNAs hybridize to two RNA species of 1260 and 1450 b from oil seed rape roots (Evans *et al.* 1990), but we have only ever detected a 1260 b transcript in the root and the leaf of oil seed rape using a probe comprising the *extA* coding sequence and the 3′-untranslated region when northern blots have been washed to very high stringency. We know that the 1260 b transcript is the product of the *extA* gene because in previous work, S1 nuclease

Rape leaf NE	-	+	-	+	-	+	-
Rape petiole NE	-	-	+	-	+	-	+
Sp. competitor	-	-	-	+	+	-	-
Non-sp. competitor	-	-	-	-	-	+	+

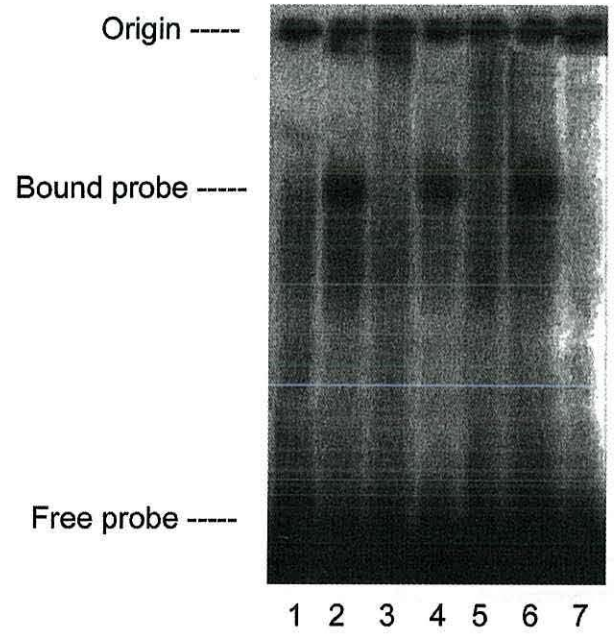

Figure 3. Electrophoretic mobility shift assays using nuclear protein extracts (NE) from rape leaves and petioles and the 5′-TAAAATATAGTGGATTTTGCATATG-3′ oligonucleotide from the NRR as a probe. Components of each track are shown in the table. Sp. (Specific) competitor tracks contain an excess of the NRR oligonucleotide, and Non-sp. (non-specific) competitor tracks contain an excess of a unrelated 25 bp sequence from *Drosophila* which does not bind nuclear extracts.

mapping of the *extA* genomic clone predicted a transcript size of 1260 b for this gene (Evans *et al.* 1990) and further, expression of the *extA* gene in a heterologous transgenic tobacco system (Shirsat *et al.* 1991) resulted in the synthesis of a transcript close to the predicted size of 1260 b.

We investigated whether travelling wound-generated signals within the plant could give the same results (increase in extensin transcripts in the leaf, decrease in the roots) without directly wounding the roots or leaves as was done previously. When the roots of hydroponically grown plants were wounded and expression in the unwounded leaves was analysed (Fig. 6 tracks 3–6), it was found that expression broadly paralleled the results obtained when the leaves were directly wounded. The roots were wounded repetitively throughout the time course, and expression of the 1260 b extensin transcript in the leaves was first seen at 12 h (Fig. 6 track 3) reaching a peak of maximum expression at 72 h (Fig. 6 track 6). When the reverse experiment was performed, in which the leaves were wounded and expression in the roots analysed, there was a small decrease in the amount of extensin root transcripts – the signal declined from the normal high level (Fig. 6 track1) over the time course of the experiment (Fig. 6 tracks 7–10), showing that wounding the leaves resulted in a small decline in extensin expression in the root. This indicates that wound-induced signals travel bi-directionally through the plant. It is interesting that the decline in expression of *extA* RNA in the root as a result of leaf wounding does not follow the same kinetics as experiments in which the roots themselves are wounded (Elliott & Shirsat 1998) – this may indicate

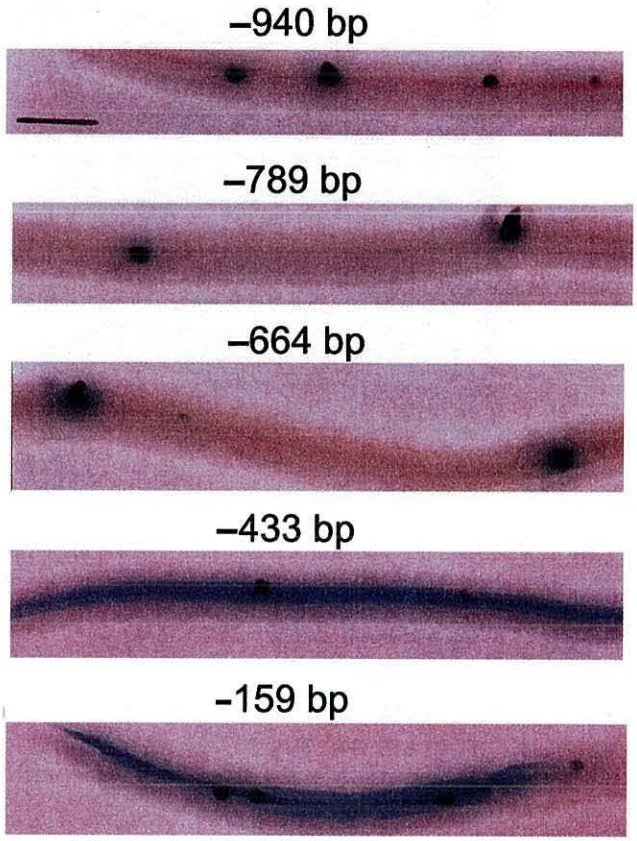

Figure 4. Expression of GUS in transgenic lateral roots from agar-grown transgenic plants containing the different promoter truncations. Bar = 1 mm.

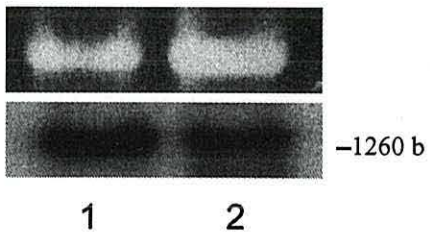

Figure 5. Expression of the *extA* gene in the roots of *B. napus* plants which were either grown hydroponically (1) or through glass beads to simulate mechanical impedance (2). 28S RNA bands are shown as loading controls, and the size of the transcript is indicated.

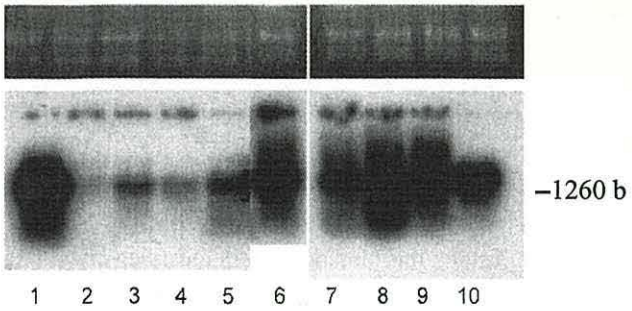

Figure 6. Travelling wound-induced signals affect *extA* expression in leaves and roots. Roots were wounded and total RNA extracted from leaves after 12 h (track 3), 24 h (track 4), 36 h (track 5) and 72 h (track 7). Leaves were wounded and total RNA extracted from roots after 12 h (track 7), 24 h (track 8), 36 h (track 9) and 72 h (track 10). Track 1 shows expression of *extA* transcripts in unwounded root, and track 2 expression of *extA* transcripts in unwounded leaf. 28S RNA bands are shown as loading controls and the size of the transcript in bases is indicated.

differences in the nature of the signals produced in the two experiments.

DISCUSSION

Extensins have been found to be expressed in plant regions that require strengthening, for example, the sclereid cells of the seed coat (Cassab *et al.* 1987) or in the tips of lateral roots that require strengthening to break through the surrounding tissues of the main root (Keller, Schmid & Lamb 1989). Extensin expression has also been shown to occur in cells in response to mechanical pressure. Shirsat *et al.* (1996a) found that extensin transcripts accumulated in the cells of the carpel wall in oilseed rape that are under considerable tensile stress in the later stages of maturation as they begin to desiccate, prior to the explosive shedding of seeds. In *Nicotiana plumbaginifolia*, extensin expression was seen around leaf traces and in cortical cells associated with vascular bundles, and it was suggested that these cells required a strong cell wall to withstand the compression stresses exerted by developing vascular tissues (Tire *et al.* 1994). We have previously shown (Shirsat *et al.* 1996a; Elliott & Shirsat 1998) that the *B. napus extA* extensin gene is expressed in plant regions subject to tensile stresses, and have identified promoter regions responsible for this activation. The NRR, located between –433 and –664 bp was proposed (Elliott & Shirsat 1998) to act as a negative regulator of *extA* expression, acting to restrict expression to phloem tissues in non-stressed regions (e.g. internodes) and that this repression was overcome at stressed regions (e.g. nodes). It could be argued, however, that this observed nodal expression pattern is developmentally controlled and is not at all related to tensile stresses experienced at the nodes. Indeed, all of the published papers on the association of cell wall protein genes with plant regions experiencing mechanical stresses are purely correlative, with the exception of our earlier work (Shirsat *et al.* 1996a). We have now shown by weight-loading axillary nodal branches in transgenics carrying the various *extA* promoter deletions, that the expression patterns seen at the node are indeed produced in response to mechanical forces experienced by the tissue, and moreover, that this expression pattern is controlled by the –433 to –664 bp promoter region.

Further evidence to implicate the NRR in the response to tensile stress comes from studying developing transgenic lateral roots. In transgenic plants containing promoter truncations that include the NRR, the localization of GUS is phloem specific in the main root. This pattern of GUS localization alters in developing lateral roots from the same plants, in which high levels of GUS activity are seen in all cell types within the developing lateral root. As the cells in the lateral root have to withstand considerable stresses to break through the cortex of the main root, this is another instance where mechanical stress is able to override the negative regulation exerted by the NRR, resulting in expression in all cell types of the lateral root. In transgenic plants that do not contain the NRR, as expected, expression is seen in all cell types in both the main root and the developing lateral. It is notable that the same pattern of expression is seen in regions experiencing mechanical stresses in two different organs – the axillary nodal branches and the roots. In both organs, the NRR region appears to function as a negative regulator of *extA* expression. Our data indicate that the application of weight stress results in the activation of the *extA* gene. The fact that deletion of a *cis*-acting regulatory region, the NRR, results in constitutive expression of *extA* suggests that up-regulation is due to alleviation of NRR-dependent repression. If the NRR functioned as a positive *cis*-acting regulator one would expect to maintain the repressed state when the NRR is deleted in the simple reporter system we have employed. However, it is clear that there is a positive regulatory system working to de-repress the *extA* gene expression in response to weight stress, the nature of which remains unknown. Work is in progress to investigate this.

To the authors' knowledge, all extensin genes so far isolated express strongly and constitutively in the root, and in view of the results obtained in the transgenics, we hypothesized that this strong constitutive expression was due to the mechanical impedance the roots experienced as they forced their way through the soil. Seedlings were grown in hydroponics and in mechanically impeded substrates, but the results obtained did not support the hypothesis. Roots of plants grown hydroponically (without mechanical impedance) showed high levels of *extA* mRNA, as did the roots of plants grown through glass beads (high degree of mechanical impedance) indicating that the high levels of *extA* expression seen in the root were not in response to externally applied mechanical stresses. It is interesting that the soybean SbHRGP3 extensin gene has been shown to be expressed primarily in the maturation zone of hydroponically grown developing secondary soybean roots (Ahn *et al.* 1998) rather than the primary root, and in this regard, our results also show that developing lateral secondary

roots express high levels of *extA*-driven GUS. It is possible therefore that expression of extensin genes in the early stages of lateral root development serves to 'harden' the cells of the emerging lateral roots by intra- and intermolecular cross-linking (Carpita & Gibeaut 1993). This may reduce any damaging effects of lateral root penetration through the primary root. Intramolecular cross-linking has been hypothesized to involve the VYK motif and to be catalysed by peroxidase (Kieliszewski & Lamport 1994), and the isodityrosine motif Xaa-Tyr-Xaa-Tyr, has been proposed to be the site whereby intramolecular cross-linking takes place (Epstein & Lamport 1984). Both mechanisms have been proposed to turn soluble extensin monomers into a network conferring strength and rigidity to the wall. This may reduce any damaging effects of lateral root penetration through the primary root.

There are three main models for long-distance signalling in plants – phloem transmission that is bi-directional (Pearce *et al.* 1991), hydraulic dispersal that is postulated to occur via mass flow through the xylem (Malone & Alarcon 1995), and electrical signalling (Wildon *et al.* 1992). As we have previously shown that wounding the root leads to a rapid decline in the amount of root extensin transcripts and that wounding the leaves leads to an accumulation of leaf extensin transcripts (Shirsat, Wieczorek & Kozbial 1996b), we examined the effect of wounding the leaf on the high levels of extensin transcripts in the root, and the effect of wounding the root on extensin transcript accumulation in the leaf, in order to see whether wounding induced the generation of signals that translocated through the plant and regulated extensin mRNA levels at sites distal from the initial wound. The results indicate that wounding the root leads to an accumulation of extensin transcripts in unwounded leaves, and wounding the leaf has a small effect on reducing the high levels of extensin transcripts in the root. These results indicate that bi-directional flow of wound-induced signals through the phloem is probably a factor in our experiments, and that hydraulic dispersal of signals through the xylem via the transpiration stream (as proposed by Malone & Alarcon 1995) may not be the sole route of transmission.

It is apparent that the *extA* NRR contains sequence motifs that are negatively regulating *extA* expression, and we were interested to see if defined sequence elements within this region bound nuclear proteins. We chose a 25 bp sequence which contained the sequence 5′-TAGTGGAT-3′ located between –472 to –479 bp in the NRR because it contains the core GTGG motif which has been shown to bind bZip nuclear proteins (Torres-Schumann *et al.* 1996). In addition, a sequence (5′-ATGTGGAA-3′) in the bean 1.8 glycine-rich protein gene promoter, which also contains the core GTGG motif, has been shown to specifically bind nuclear proteins and to function as a negative regulator that is essential for vascular-specific expression (Schindler, Beckmann & Cashmore 1992; Torres-Schumann *et al.* 1996). The gel shift assays clearly show that rape leaf nuclear protein extracts bind to the NRR G-box-like half site sequence and produce a strong shift, whereas rape petiole nuclear proteins do not bind. This is important because *B. napus* leaves contain the least amount of extensin mRNA, whereas petioles contain higher amounts (Shirsat *et al.* 1996b). If the *extA* gene is indeed under negative regulation, then this is exactly the result one would expect to see – binding specificities to sequence motifs within the NRR should be altered, depending on whether the extensin gene is either activated or repressed. This is exactly what has been observed. Further, this binding protein must be very abundant in the leaf, as a 150 M excess of cold homologous probe cannot compete it out. These results are being investigated further by a combination of gel shift assays linked to an analysis of transgenic plants containing internal truncations of the NRR. It is likely that these further detailed experiments will reveal intricacies of binding that may not be apparent in these initial experiments.

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REFERENCES

Ahn J.I., Choi Y., Kim S.-G., Kwon Y.M., Choi Y.D. & Lee J.S. (1998) Expression of a soybean hydroxyproline-rich glycoprotein gene is correlated with maturation of roots. *Plant Physiology* **116,** 671–679.

Braam J. & Davis R. (1990) Rain, wind and touch induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. *Cell* **60,** 357–364.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgramme quantities of protein utilising the principle of protein dye binding. *Analytical Biochemistry* **72,** 246–254.

Carpita N.C. & Gibeaut D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant Journal* **3,** 1–30.

Cassab G.I. (1998) Plant cell wall proteins. *Annual Reviews of Plant Physiology and Plant Molecular Biology* **49,** 281–309.

Cassab G.I., Nieto-Sotelo J., Cooper J.B., Van Holst G.J. & Varner J.E. (1987) A developmentally regulated hydroxyproline-rich glycoprotein from the cell walls of soybean seed coats. *Plant Physiology* **77,** 532–535.

Deryckere F. & Gannon F. (1994) A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. *Biotechniques* **16,** 405.

Elliott K.A. & Shirsat A.H. (1998) Promoter regions of the *extA* extensin gene from *Brassica napus* control activation in response to wounding and tensile stress. *Plant Molecular Biology* **37,** 675–687.

Ellis J.R., Shirsat A.H., Hepher A., Yarwood J.N., Boulter D. & Croy R.R.D. (1988) Tissue specific expression of a pea legumin gene in seeds of *N. plumbaginifolia*. *Plant Molecular Biology* **10,** 203–214.

Epstein L. & Lamport D.T.A. (1984) An intramolecular linkage involving isodityrosine in extensin. *Phytochemisty* **23,** 1241–1246.

Evans I.M., Gatehouse L.N., Gatehouse J.A., Yarwood J.N., Boulter D. & Croy R.R.D. (1990) The extensin gene family in oilseed rape (*Brassica napus* L.): characterisation of sequences of representative members of the family. *Molecular and General Genetics* **223,** 273–287.

Jaffe M. & Biro R. (1979) Thigmomorphogenesis: the effect of mechanical perturbation on the growth of plants., with special reference to anatomical changes., the role of ethylene. & interaction with other environmental stresses. In *Stress Physiology in Crop Plants* (eds H. Mussell & R. Staples), pp. 25–29. John Wiley, New York, USA.

Jefferson R.A. (1987) Assaying chimaeric genes in plants; the GUS gene fusion system. *Plant Molecular Biology Reporter* **5,** 387–405.

Keller B., Schmid J. & Lamb C.J. (1989) Vascular expression of a bean cell wall glycine rich protein B-glucuronidase gene fusion in transgenic tobacco. *European Molecular Biology Organisation Journal* **6,** 3901–3907.

Kieliszewski M.J. & Lamport D.T.A. (1994) Extensin: repetitive motifs, functional sites, post translational codes and phylogeny. *Plant Journal* **5,** 157–172.

Knight M., Smith S. & Trewavas A. (1992) Wind-induced plant motion immediately increases cytosolic calcium. *Proceedings of the National Academy of Sciences USA* **89,** 4967–4971.

Lamport D.T.A. (2001) Life behind cell walls: paradigm lost, paradigm regained. *Cellular and Molecular Life Sciences* **58,** 1363–1385.

Malone M. & Alarcon J.-J. (1995) Only xylem-borne factors can account for systemic wound signalling in the tomato plant. *Planta* **196,** 740–746.

Merkouropoulos G., Barnett D.C. & Shirsat A.H. (1999) The *Arabidopsis* extensin gene is developmentally regulated, is induced by wounding., methyl jasmonate, abscisic and salicylic acid and codes for a protein with unusual motifs. *Planta* **208,** 212–219.

Mitchell C. & Myers P.N. (1995) Mechanical stress regulation of plant growth and development. *Horticultural Reviews* **17,** 1–42.

Mitchell C., Severson C., Wott J. & Hammer P. (1975) Seismomorphogenic regulation of plant growth. *Journal of the American Society for Horticultural Science* **100,** 605–608.

Murashige T. & Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologica Plantarum* **15,** 473.

Pearce G., Strydon D., Johnson S. & Ryan C.A. (1991) A polypeptide from tomato leaves induces wound-inducible PI proteins. *Science* **253,** 895–989.

Sambrook J., Fritsch E.F. & Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Press, New York, USA.

Schindler U., Beckmann H. & Cashmore A.R. (1992) TGA1 and G-box binding factors. Two distinct classes of *Arabidopsis* leucine zipper proteins compete for the G-box-like element TGACGTGG. *Plant Cell* **4,** 1309–1319.

Shirsat A.H., Bell A., Spence J. & Harris N.J. (1996a) The *Brassica napus extA* extensin gene is expressed in regions of the plant subject to tensile stress. *Planta* **199,** 618–624.

Shirsat A.H., Wieczorek D. & Kozbial P. (1996b) A gene for *Brassica napus* extensin is differentially expressed on wounding. *Plant Molecular Biology* **30,** 1291–1300.

Shirsat A.H., Wilford N., Evans I.M., Gatehouse L.N. & Croy R.R.D. (1991) Expression of a *Brassica napus* extensin gene in the vascular system of transgenic tobacco and rape plants. *Plant Molecular Biology* **17,** 701–709.

Tire C., De Rycke R., De Loose M., Inze D., Van Montagu M. & Engler G. (1994) Extensin gene expression is induced by mechanical stimuli leading to local cell wall strengthening in *Nicotiana plumbaginifolia*. *Planta* **195,** 175–181.

Torres-Schumann S., Ringli C., Heierli D., Amrhein N. & Keller B. (1996) *In-vitro* binding of the tomato bZip transcriptional activator VSF-1 to a regulatory element that controls xylem-specific gene expression. *Plant Journal* **9,** 283–296.

Wildon D.C., Thain J.F., Minchin P.E.H., Gubb I.R., Reilly A.J., Skipper Y.D., Doherty H.M., O'Donnell P.J. & Bowles D.J. (1992) Electrical signalling and systemic proteinase inhibitor induction in the wounded plant. *Nature* **360,** 62–65.

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