Molecular Biological Studies of the Induction of Flowering

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Doctor of Philosophy

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List of Abbreviations

The following list contains abbreviations, other than SI units, not described in the text.

BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
poly(A)$^+$	polyadenylated
cpm	counts per minute
DMSO	dimethyl sulphoxide
dNTP	deoxynucleoside triphosphate
Na_2EDTA	ethylenediaminetetra- acetic acid (disodium salt)
Tris	tris [hydroxymethyl] aminomethane
kD	kilo Daltons
HEPES	N -2- hydroxyethylpiperazine - N' - 2 - ethanesulphonic acid
TEMED	N, N, N', N' - tetramethylethylenediamine
PPO	2, 5 - diphenyl - oxazole
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

rpm	revolutions per minute
bp	base pairs
v/v	volume to volume
w/v	weight to volume
nm	nanometer
A_{260}	absorbance at wavelength 260 nm
DEPC	diethyl pyrocarbonate

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Summary

Potential changes in gene expression which occur in the leaves during photoperiodic floral induction were investigated in a variety of plant species including *Nicotiana plumbaginifolia* (day neutral plant), *Amaranthus caudatus* (short day plant), *Silene coeli-rosa* (long day plant), and *Anagallis arvensis* (long day plant) which exhibit a range of photoperiodic responses. The approach taken was to employ techniques which would demonstrate differential gene expression at the level of mRNA or protein including *in vitro* translation of leaf mRNA and analysis of products using one- or two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), analysis of leaf total protein using one- or two-dimensional SDS-PAGE, differential immunoadsorbtion techniques for the identification of 'induced' specific proteins, and differential screening of an 'induced' cDNA library.

Isolation and analysis of mRNA from *Nicotiana plumbaginifolia* over a time course beginning 3 weeks after seeds were sown and 3 weeks prior to shooting of the stem (associated with flowering) initially showed that an mRNA which directed synthesis of an *in vitro* translation product with a molecular mass of 36.5 kD was differentially expressed prior to shooting. This mRNA was present in all of the individual leaves of a plant harvested one week prior to shooting but it was absent in individual leaves of plants harvested before or after one week prior to shooting. Later experiments showed that differential expression of this mRNA was apparent at other times during the time course of sampling of *Nicotiana plumbaginifolia* leaves and therefore could not be associated with a specific stage of development.

Studies investigating changes in the presence of specific mRNAs or proteins in *Amaranthus caudatus* leaves after transfer to floral inducing short day conditions showed that no differential expression could be detected which was contrary to a previous report. When mRNA isolated after each cycle of short days was translated *in vitro* and the products were analysed using one-dimensional SDS-PAGE, there was no difference when comparing the products synthesized from mRNA isolated after 1, 2, or 4 short day cycles.

Two-dimensional SDS-PAGE was used to compare *in vitro* translation products synthesized from mRNA isolated from leaves of *Silene coeli-rosa* kept in short day conditions with *in vitro* translation products synthesized from mRNA isolated from leaves exposed to 3 long days. The results of one experiment suggested that there were two translation products synthesized from mRNA isolated from leaves exposed to 3 long days that were not present in the translation products synthesized from mRNA isolated from leaves exposed ot short days only; however, due to problems in obtaining consistent resolution on the gels of the translation products, no conclusions could be made.

One-dimensional and two-dimensional SDS-PAGE was used to analyse and compare *in vitro* translation products synthesized by mRNA isolated from *Anagallis arvensis* leaves exposed to short days only or to one long day. One-dimensional analysis showed no differences, but in one experiment, two-dimensional analysis showed one product with a molecular mass of 14-15 kD that was present in products synthesized from mRNA extracted from leaves exposed to one long day that was not

among the products synthesized from mRNA extracted from leaves exposed to short days only. Again, however, problems were encountered in obtaining consistent resolution of the translation products.

One-dimensional and two-dimensional SDS-PAGE was also used to analyse and compare total proteins extracted from *Anagallis arvensis* leaves exposed to short days only or to one long day. One-dimensional analysis showed no difference when proteins from the two sources were compared. Comparison of two-dimensional SDS-PAGE gels showed that one protein with a molecular mass of 57.5 kD was present in short day leaf extracts and absent in long day leaf extracts. Also, two proteins with molecular masses of 33 and 21.5 kD, respectively, were present in long day leaf extracts but not in short day leaf extracts.

An antiserum was made against total protein from long day *Anagallis arvensis* leaf extracts so that the technique of differential immunoadsorption could be used to produce an antiserum that was specific to the 'induced' proteins (33 and 21.5 kD). Incubation of the antiserum with protein extracted from short day leaves removed much of the activity against protein common to both short day and long day leaves, but the technique was not effective enough to be useful.

Differential screening of a cDNA library produced from mRNA isolated from *Anagallis arvensis* leaves exposed to one long day allowed for the initial selection of 13 'induced' specific cDNA clones. Further screening, however, showed that these positives were likely to be false.

An examination of these results and discussion of potential future experimentation is provided.

Chapter 1 - Introduction

1.1 Introduction

Flowering is an exteremely important stage in the life cycle of all Angiosperms. There are several stages in the flowering process which can be defined as follows. The initial event is the detection of the appropriate developmental or environmental signals and the consequent production of a floral stimulus, both of which processes, as discussed below, are believed to take place in the leaves. These events are defined as floral induction. The floral stimulus is transmitted from the leaves to the shoot apex whereupon the events of floral evocation are initiated. Evocation leads to the morphogenesis of the floral organs, which can be regarded as the final stage of the flowering process. The following discussion is concerned specifically with floral induction.

The regulation of the switch from vegetative to reproductive growth in plants appears to be a particularly complex process involving the interaction of genetic factors with several environmental variables. The induction of flowering has been the subject of extensive research and a wealth of data has been obtained. For instance, in 1985, it was reported that 26,000 papers concerning flowering had been published since 1971 including 8000 specifically regarding the role of daylength (Evans, 1987). Also, it was pointed out that although thousands of species have been studied, only 2% of all flowering plants are represented of which 80 - 85% show some photoperiodic behaviour (Rees, 1987). Thus despite an enormous effort on the part of plant physiologists over many years, a thorough understanding of the mechanism of floral induction has not been

obtained.

1.2 Photoperiodism

1.2.1 Daylength requirement

The effect of daylength on flowering was noted as early as 1852 by Henfrey who reasoned "that the natural distribution of plants was at least partially due to latitudinal variations in summer daylength" (Vince-Prue, 1975). The importance of daylength was first definitively demonstrated by Tournois in 1912 when he showed that precocious flowering in *Humulus* was due to shortening of the daylength as oppposed to other environmental factors including light quantity (Vince-Prue, 1975). The work of Tournois was complemented by experiments on *Sempervivum funkii*, carried out by Klebs, which showed that continous illumination induced flowering in plants which otherwise remained vegetative in short days (Vince-Prue, 1975). Further experiments in the early twenties by Garner and Allard provided information on the photoperiodic behavior of a wide range of species (Vince-Prue,1975).

Eventually it became clear that photoperiod influenced floral induction in a species specific manner. In general, plants are mainly categorized as either long day plants (LDPs), short day plants (SDPs) or day neutral plants (DNPs): flowering in LDPs is accelerated or will only occur in daylengths longer than a specified duration ; flowering in short day plants is accelerated or only occurs in daylengths shorter than a specified duration; flowering in day neutral plants is unaffected by daylength. If the daylength requirement for flowering is absolute, a plant will be referred to as 'qualitatively

photoperiodic', and if daylength merely enhances the flowering response of a given plant it is referred to as 'quantitatively photoperiodic.' Table 1.1 lists some of the more commonly studied plants that fall into one of the main categories.

The behaviour of some plants does not fall into the main categories. These cases include species which must be exposed to certain photoperiods in succession in order to achieve flowering or where the photoperiodic response includes treatment at a specific temperature. For example, in *Bryophyllum crenatum* or *Cestrum diurnum* there is a requirement for a certain number of long day exposures prior to transfer into short days for induction, and consequently, this species is referred to as a long-short-day plant (LSDP). The reverse is true for such species as *Echeveria harmsii and Scbiosa succisa* and these plants are referred to as short-long-day plants (SLDP). There are also plants requiring intermediate daylength treatments for induction such as *Coleus hybrida* cv Autumn and others that are inhibited by intermediate daylengths. Many plants require vernalization (treatment at low temperature) in combination with transfer to the specific daylength, as with *Beta vulgaris* or biennial strains of *Arabidopsis thaliana*.. (The process of vernalization is a large topic unto itself and will not be discussed in detail here.) Lastly, photoperiodic requirements of some plants can be modified or replaced by a specific temperature exposure, such as in *Pharbitis nil* (an SDP) which is day-neutral at low temperatures or *Silene armeria* (an LDP) where growth at 32°C in short days substitutes for the long day effect (Vince-Prue, 1975).

Every species has specific requirements for floral induction and it is therefore very difficult to group plants into general classifications. Species specific variables typically include the number of cycles of a certain photoperiod which will facilitate floral

Short day plants	Type	Other Influences
Bryophyllum pinnatum	qualitative	
Kalanchoë blossfeldiana	qualitative	
Cannabis sativa cv Kentucky	quantitative	
Chrysanthumum indicum	qualitative	
Glycine max cv Biloxi	qualitative	
Glycine max cv Mandell	quantitative	
Parbitis nil (Chois) cv Violet	qualitative	day neutral at low temperature
Perilla crispa	qualitative	quantitative at low temperature
Xanthium strumarium (L.)	qualitative	
Long day plants		
Anagallis arvensis (L.)	qualitative	
Brassica campestris	quantitative	
Hyoscyamus niger , annual strain	qualitative	
Lemna gibba G3	qualitative	
Lolium temulentum cvs 6139, 6137	qualitative	requires vernalization
Nicotiana sylvestris	qualitative	
Day neutral plants		
Cestrum elegans		
Nicotiana tabacum		
Phaseolus vulgaris		
Pisum sativum		

Table 1.1 The major photoperiodic categories with examples. (Data compiled and adapted from Vince-Prue, 1975.)

induction, the effects of temperature, light intensity and quality, nutrition as well as other environmental factors. With regard to the assignment of quantitative or qualitative requirements, the lack of complete (or conflicting) information on many species causes further complications since these distinctions are assigned based on the length of time in which any given plant was allowed to grow under non-inducing conditions. For example, it is the opinion of some researchers that virtually all plants will eventually flower under non-inducing conditions (Bernier, 1988) even though some plants have demonstrated vegetative growth for several years such has been the case with *Anagallis arvensis* (Ballard, 1969; Brulfert, *et al.*, 1985). However, the known details of many plants that have been studied is contained in Halevy's Handbook of Flowering, vols I - VI (Halevy, 1985; Halevy, 1986; Halevy, 1987).

1.2.2 Perception of Photoperiod

In 1934, experiments carried out on the LDP spinach, by Knott, demonstrated that flowering occurred when only the leaves of the plant were exposed to inducing conditions (Vince-Prue, 1975). Similar work where leaves and apex were exposed to inducing conditions separately, published shortly thereafter, provided similar results with other species including *Xanthium strumarium* and *Chrysanthemum morifolium*. Grafting experiments conducted since then have implied that exposure of a variety of plants to inducing conditions causes the leaves to produce a transmissible substance. Such experiments show that leaves of such an induced plant (donor) can be grafted onto a non-induced plant (vegetative receptor) so that it will flower in non-inducing conditions. Grafting experiments of this type have been conducted successfully between plants of different family, genus, species, and photoperiodic category (for

comprehensive review see Zeevart, 1976). Examples which illustrate this point are given in Table 1.2.

The induction of the leaves appears to be an autonomous process. For example, when leaves of *Perilla* are detached from the parent plant prior to induction, they can still act as donors and stimulate flowering in receptor plants (Thomas and Vince-Prue,1984). The induction process is thus independent from the ultimate response at the apex and therefore these events can be considered separately.

Tremendous variation can occur among the leaves of a given species with regard to ability to respond to photoperiod and promote flowering. These qualities can vary based on leaf age and position relative to the apex. For example, studies of *Perilla* leaves showed that when 30 cm^2 of leaf taken from the fifth leaf pair of a photoperiodically induced older plant was grafted onto a vegetative receptor, its ability to promote flowering was twice that observed from the same amount of leaf tissue taken from the second leaf pair of a photoperiodically induced younger plant (Vince-Prue, 1975). Therefore, in this species, ability to respond to photoperiod and promote flowering was shown to increase with higher ontogeny as opposed to physiological age of the leaf during induction. Conversely, by defoliating the subject plant to a single leaf, it was shown that the ability to promote flowering following photoperiodic induction in leaves of *Xanthium strumarium* peaked when they reached half their final length, i.e. the peak of their growth rate (Vince-Prue, 1975).

Defoliation experiments are used to determine the efficiency of a leaf or group of leaves by removing all of the leaves except those to be tested, and this allows for the

Donor				Receptor			
Species	Family	Response type	Conditions	Species	Family	Response type	Conditions
Xanthium strunarium	Compositae	SDP	short day	*Silene armeria*	Caryophyllaceae	LDP	short day
Helianthus annuus	Compositae	DNP	long day	*Xanthium strumarium*	Compositae	SDP	long day
Calendula officinalis	Compositae	DNP	long day	*Xanthium strumarium*	Compositae	SDP	long day
Bryophyllum daigremontianum	Crassulaceae	LSDP	long day - short day - long day	*Kalanchöe blossfeldiana*	Crassulacaceae	SDP	long day
Kalanchöe blossfeldiana	Crassulacaea	SDP	short day - long day	*Bryophyllum daigremontianum*	Crassulaceae	LSDP	long day
Perilla crispa	Labiatae	SDP	short day	*Silene armeria*	Caryophyllaceae	LDP	short day
Chenopodium polyspermum, C. rubrum	Chenopodiaceae	SDP	short day	*Blitum capitum B. virgatum*	Chenopodiaceae	LDP	short day
Blitum capitatum B. virgatum	Chenopodiaceae	LDP	long day	*Chenopodium rubrum*	Chenopodiaceae	SDP	long day

Table 1.2 Examples of successful grafts between different species. Data compiled and adapted from Zeevart (1976).

identification of the leaves of a given plant which are most essential for flowering at a given time under specified conditions. Such experiments have also provided further proof for the primary role of the leaves in perception of photoperiod in that complete defoliation does not yield flowering while tiny portions of sensitive leaves from *Xanthium*, *Perilla*, and *Lolium* are sufficient for a response in the appropriate photoperiod (Vince-Prue, 1975).

There is some evidence for the perception of daylength by other tissues other than leaves. For example, defoliated *Chenopodium amaranticolor* can be induced to flower and it is assumed that either the stem or the very young leaves present in the apex must be capable of daylength perception and mediation of the response (Vince-Prue, 1975). Another example is the parasitic and virtually leafless plant *Cuscuta reflex* where stem tips have been shown to be inducible in culture (Vince-Prue, 1975). Examples where the apex is capable of solely mediating floral induction are limited and it therefore seems that with the exception of special cases, leaves play the dominant role in the perception of an inducing photoperiodic treatment.

1.2.3 Role of Phytochrome

Prior to the discovery and purification of the photoreceptor phytochrome, much work had been done to establish and quantify the action of defined wavelength bands on the promotion or inhibition of flowering when plants were subjected to brief exposures during the dark period (night breaks). The action spectra produced showed that red light between 600 and 660 nm was most effective for inhibition of flowering in SDPs and for promotion of flowering in LDPs, and these results were comparable to action spectra

determined for other light-dependent responses such as seed germination and de-etiolation (Vince-Prue, 1975). It was clear that there was a common primary photoreceptor for a variety of responses and it was distinguished by the characteristic "red/far red" photoreversibility, i.e. the response obtained by red light could be reversed or suppressed by subsequent illumination with far red light. This characteristic was initially demonstrated in the germination of lettuce seeds in the dark where stimulation by a brief exposure to red light could be suppressed when followed by exposure to far red light. If a series of alternating exposures was given, the last exposure determined the ultimate response (Borthwick *et al.*, 1952).

More recent data show that the photoreceptor involved in such "red/far red" responses was phytochrome which is now known to exist as a chromoprotein consisting of an apoprotein and a linear tetrapyrrole chromophore attatched covalently to its N-terminal domain (Pill Soon Song, 1984). Phytochrome is present in two photo-interconvertible, spectrally distinct forms designated as P_r and P_{fr}. P_r is converted to the P_{fr} form by the absoption of red light and P_{fr} is subsequently changed backed to the P_r form by the absorption of far-red light. There are also two immunologically distinct types of phytochrome which are referred to as type I or "light labile" phytochrome, which is abundant in etiolated tissue and becomes depleted upon illumination, and type II phytochrome which is stable in light (Kay *et al.*, 1989).

With the data collected to date, the way in which the components of the phytochrome system function in photoperiodic induction of flowering, or any other light dependent process, is still unknown. The effect of light on photoperiodic induction of flowering is much more complicated than implied by the early experiments. Photoperiodic control is associated with rhythmic changes in the response to light as

demonstrated by experiments where plants were given night breaks at various times during the dark period (Thomas and Vince-Prue, 1984). The relationship of light with rhythm was seen in both SDP and LDP and was further demonstrated by experiments which showed that the flowering response changed rhythmically in response to changes in the length of the dark period (Thomas and Vince-Prue, 1984). Therefore, phytochrome is involved in both photoperception and time measurement.

The regulation of gene expression by phytochrome is well documented (for review see Nagy *et al.*, 1988; Simpson and Herrera-Estrella, 1990), and it is also well established that there are specific *cis* -acting elements and *trans* -acting factors which are essential for the mediation of light regulated transcription (for review see Nagy *et al.*, 1988). A molecular approach to research is now being directed towards characterization of the mode of action of phytochrome and the signal transduction pathways that lead to the control of gene expression, determination of the functions of the type I and type II species, determination of the effects of altering cellular levels of the protein in plants, and identification of the structural domains of the protein required for biological action.

A number of results during the past year have shown that cDNA corresponding to the phytochrome mRNA from one species can be introduced into a distantly related species and expressed such that the apoprotein can be a substrate for chromophore addition and act as a functional photoreceptor (Kay *et al.*, 1989; Keller *et al.P*, 1989; Boylan and Quail, 1989). The introduction of heterologous phytochrome has produced mutant phenotypes in some of the cases reported. For example, when oat cDNA representing type I phytochrome was introduced into the dicot tobacco, phenotypic changes including semi-dwarfism, darker green leaves, increased tillering, and reduced

apical dominance were produced. Therefore, in this case, type I phytochrome was shown to function in light grown plants and its overexpression caused an exaggerated light grown phenotype. This demonstrates that even though type I phytochrome normally only accumulates in dark-grown plants it is, nevertheless, able to function in light-grown plants (Keller *et al.*, 1989; Boylan and Quail, 1989). The mutant phenotype is similar to the changes observed when plants were treated with gibberellin antagonists (Keller, 1989), or when plants were transformed with a cytokinin biosynthetic gene (Keller, 1989) and therefore provides further evidence for the participation of phytochrome in the regulation of the balance of phytohormones. (Phytohormone levels may play a role in floral induction and this will be discussed in section 1.4.) Similar results were reported for tomato plants which were also transformed with type I phytochrome cDNA from oat (Boylan and Quail, 1989). When rice type I phytochrome cDNA was introduced into tobacco, altered expression of the *Cab* (chlorophyll *a/b* binding protein) gene, which is normally controlled by the interaction of phytochrome with the circadian clock in light grown tissues, was seen (Kay *et al.*, 1989). Normal oscillation of *Cab* gene expression (alternation between high and low levels every 12 hours) becomes rapidly dampened to undetectable levels after 48 hours when plants are placed in continuous darkness. When the transgenic plants were placed in continuous darkness, the *Cab* mRNA level continued to cycle each day, but the levels decreased much less rapidly and appeared highly abundant 48 hours later. Therefore, the overproduction of the rice phytochrome can effect the abundance but not the timing of *Cab* gene expression.

All of the work so far described has been performed to establish the feasibility of transgenic approaches to the study of phytochrome and is clearly only preliminary.

Caution should therefore be exercised in attempting to draw any firm conclusions until further detailed analysis has been done. Future work of this kind will, hopefully, provide a greater understanding of the role of phytochrome in the photoperiodic control of flowering.

1.3 Investigations into possible floral inducing substances

Grafting and defoliation experiments, as described in section 1.2.2, have provided circumstantial evidence for the presence of transmissible floral inducing substances. Based on these results, the dominant theory for over 50 years has presented a simplistic model of the flowering process as being controlled by the absence or presence of a hypothetical plant hormone referred to as "florigen".

Later experiments provided similar evidence for a transmissible floral inhibitor (Thomas and Vince-Prue, 1984). Initially, it was shown that some plants, for example the LDP *Hyoscyamus niger* and the SDP *Fragaria* X *ananassa*, will flower in non-inducing conditions when all of the leaves are removed. Further evidence was provided by experiments where the effects of inductive cycles could be antagonized by intercalation of non-inductive cycles as was demonstrated in the SDP *Kalanchoë blossfeldiana*. Demonstration of the transport of an inhibitory substance came from one experiment where parent *Fragaria* X *ananassa* grown in long days inhibited flowering in daughters attached by runners and maintained in short days (Thomas and Vince-Prue, 1984).

Much effort has been directed towards the identification of a floral stimulus or

inhibitor, but all attempts to date have failed. Attempts to purify the transmissible substance involved the preparation of extracts from induced leaves which when applied to non-induced leaves would stimulate flowering (Hammer and Bonner, 1938; Bonner and Bonner, 1948; Roberts, 1954; Lincoln, 1961; Paré *et al.*, 1989). Although extracts such as these could be made, any attempt to further purify the activity has has led to the loss of the activity. One claim of purification of a floral inhibitor has been reported (Jaffe, 1987), but the identified substance, *Bis* (2-ethylhexyl)hexane dioate, was later found to be a contaminant in plastics that were used in plant culture (Bernier, 1988). Early work attempted to link the translocation of the floral stimulus with assimilate flow from the leaves to the apex but velocity of the stimulus flow appears to vary dependent upon the species under study. For example, velocity of stimulus and assimilate flow are comparable in *Pharbitis nil* , but in *Lolium*, movement of floral stimulus is much slower than assimilate flow (Vince-Prue, 1975).

These studies on assimilate flow are not ignored as it is believed by some that "nutrient diversion", or a modification of the source/sink relationships within the plant play a major role in floral induction. Evidence in support of this theory is mainly based on the fact that many treatments which can control floral induction and initiation also affect photosynthesis and/or assimilate availability (Bernier, 1988). For example, in some species increased irradiance or CO_2 levels in the air as well as sugar application have been noted to stimulate or have an effect on flowering and in some cases replace photoperiodic requirements (Bernier, 1988).

Efforts to define the pathway of stimulus flow indicate that it is predominantly via the phloem, as demonstrated by grafting experiments where flowering was delayed or

inhibited in receptor plants by treatments which would interefere with the normal flow of material through this tissue (Vince-Prue,1975). As with most aspects of flowering, there is always the exception, such as a report of a successful graft of a donor leaf from *Xanthium strumarium* to a receptor, *Silene armeria*, which stimulated flowering without development of phloem tissue (Vince-Prue, 1975).

1.4 Role of known plant growth regulators in floral induction

A vast number of experiments have demonstrated the effect of the application of plant growth regulators, including gibberellins, auxins, abscisic acid, ethylene, and cytokinins, on flowering in non-inducing and inducing conditions (for review see Bernier, 1988). The results are grossly varied. Some substances, when applied exogenously, cause floral promotion in certain plants and inhibition in others or no effect at all. The response can be affected by the concentration of the substance applied, age of plant, genetics, and various environmental conditions.

Of the known plant growth regulators, the gibberellins have been the most thoroughly studied and elicit responses spanning many species (a comprehensive list of subject plants and their responses to gibberellin application is given in Vince-Prue, 1975; Halevy 1985, 1986, and 1987 will provide information on specific plants if it is available). The more consistent ability of gibberellins to replace the photoperiodic requirement of many long day plants as compared to other agents and to overcome the effects of floral suppressive agents has led to the proposal of a primary role for these hormones in floral induction (Krishnamoorthy, 1975).

Changes in the presence of various endogenous gibberellins have been cited in some plants including *Hyoscyamus* (Bernier, 1988), *Lolium* (Pharis *et al.*, 1987), and *Bryophyllum* (Pharis and King, 1985). The role of endogenous gibberellins in various physiological processes has been investigated genetically by the isolation of mutants where gibberellin synthesis is blocked or altered (for review see Graebe, 1987). Interestingly, in the well studied *Pisum sativum* , it has been shown that the flowering genes *Sn, Dne,* and *Hr* (described in detail in section 1.5) which appear to control the quantitative photoperiodic response, are equally expressed in gibberellin deficient mutants as in wild type plants.

The difficulty in demonstrating the same effects in all species, particularly SDPs where there are only a few examples of responsive plants, suggests that gibberellins are not the universal stimulus suggested by the grafting experiments between distantly related species of different photoperiodic classes as described in section 1.2.2. However, it has been considered that a negative result might be due to the use of an inappropriate type of gibberellin [there at least 62 individual gibberellins known (Jones and MacMillan, 1985)], inadequate timing of the application, or administration of the wrong concentration of the hormone used (Krishnamoorthy, 1975). Therefore, further work on the role of gibberellins and flowering is necessary and valuable.

Work on other plant growth regulators is tremendously fragmented but is not ignored (Bernier, 1988). This is particularly true with regard to cytokinins especially with work concerning evidence for the influence of roots on flowering. Changes in cytokinin levels in the roots as well as root exudates have been noted in a number of plants during photoperiodic induction including *Xanthium* and *Sinapis* and it has been

postulated that a signal produced in the leaves travels to the roots where it affects the course of cytokinin production and/or release (Bernier,1988). Also, floral induction in many plants can be stimulated by treatments that restrict root growth (Bernier,1988), and alternatively, rerooting of some plants encourages vegetative growth (Meeks-Wagner *et al.*, 1989; Brulfert *et al.*, 1985). Further evidence which indicates the importance of cytokinins as well as auxins in *in vitro* flowering systems is well documented (Tran Thanh Van, 1981)

Results concerning plant growth regulators have led to the development of a multifactorial model for the control of flowering such that several factors, promoters and inhibitors, play a role in the control of the flowering process (Bernier *et al.*, 1981). In this way, substances (i.e. assimilates and plant growth regulators) common to all plants might be present in species specific amounts as determined by genetic variation and growing conditions such that the limiting factor(s) will be different in each case. Flowering, presumably, proceeds when the necessary factors become available to the apex at the appropriate time and at the correct levels (Bernier *et al.*, 1981). This model was presented in order to account for the complexity in the observations made to date.

1.5 Genetics of floral induction

Agicultural breeding practices have historically demonstrated genetic control over a number of aspects of flowering. These include the sensitivity and control of the response to daylength and the length of the period of early growth when some plants are unresponsive to inducing stimuli (juvenility period), the number of cycles of daylength exposure required for induction, conditions necessary for inflorescence development,

speed of inflorescence development, sensitivity to gibberellin treatment, and sensitivity to other environmental conditions. Natural variation within a species has obviously evolved to adapt reproductive strategies to a particular environment and to allow for the avoidance of unfavorable climatic extremes (Evans, 1987). For example, genotypes of the perenial grass*Themeda australis* from northern Australia are SDPs while those from the south are LDPs. The photoperiodic requirements of long day plants weaken in the drier inland regions but are enhanced by vernalization in the coldest regions. Therefore, species which have become widely adapted have gene pools allowing for an array of environmental responses for the control of flowering (Evans, 1987).

Use of selective breeding has made the cultivation of many plants more flexible. For example, rice varieties have changed during this century such that while flowering was previously limited to the end of the wet season, which allowed for drying and harvesting in sunny weather, the introduction of irrigation techniques means that up to four crops a year can be grown and this has necessitated selection for day length insensitivity.

Attempts to characterize specific genes that are involved with flowering are best typified by work done with *pisum sativum* where six major loci, *Lf*, *E*, *Sn*, *Dne*, *Hr*, and *Veg*, have ben identified (Murfet, 1985). The characteristics of each gene are listed in Table 1.3.

The model put forth by Murfet (1985) is that the *Sn*, *Dne* system produces a floral inhibitor (or a vegetative promoter) with pleiotropic effects such that the plant favours juvenile characteristics. This system may work by directing metabolic flow towards vegetative growth and away from reproduction as exemplified by the lower flower to leaf

Lf - The four alleles lf^a, *lf*, *Lf*, and Lf^d, determine the minimum flowering nodes as 5, 8, 11, and 15, respectively, and appear to function by determining the threshold level of the flowering signal required to initiate flowering at the apex.

Sn, Dne - The interaction of these two genes produce a photoperiodically regulated inhibitor which delays the initiation of flower primordia, development of primordia into mature flowers and apical senescence (apical arrest), and it promotes basal branching (**29**). It appears to act as a juvenility factor.

Hr - This gene acts to prevent the decline of *Sn, Dne* function with age.

E - This gene acts in the cotyledons to inhibit the *Sn, Dne* function and promotes early floral initiation in certain combinations.

Veg - This mutant blocks the response to the flowering signal received at the apex in plants that are otherwise normal.

Table 1.3 Characteristics of the flowering genes identified in *Pisum sativum*. (Data compiled and adapted from Murfet, 1985)

ratio in plants where the *Sn, Dne* system is active. It is suggested that the metabolic changes which occur within the plant with the passage of time are such that vegetative growth is favoured early in life and then metabolism is later directed to reproduction and reproductive development. The level of the metabolic state is referred to as 'vitality' which is high at the beginning of life and then declines. In the photoperiodically long day types, the *Sn, Dne* system might increase vitality and delay flowering until the system is switched off. Vitality will then fall below the critical threshold, determined by an *lf* allele, and flowering is then stimulated by meristem perception of a reproductive state. In day neutral types, the same would happen autonomously with the passage of time. Obviously, an examination of the role of the *Sn* gene on the partitioning anddistribution of assimilates is necessary for determining the possible validity of this model.

Other species which have been well studied with regard to the genetics of flowering include the following species : wheat, where multiple alleles at two loci, designated *Pdp1* and *Pdp2* , have been descibed for daylength insensitivity (Law, 1987); *Cestrum nocturnum*, where a single gene is considered to control the long-short day photoperiodic response (Rees, 1987); *Silene armeria*, where one major dominant gene and several modifying genes for earliness (which reflects differences in the rate of induction) have been described (Wellensiek, 1985); and *Sorghum bicolor*, where four loci have been described which influence the photoperiodic requirement in this quantitative SDP (Morgan and Quinby, 1987). However, the site of action of the genes does not yet appear to be as thoroughly investigated as the flowering genes cited for pea.

1.6 The molecular biology of floral induction

1.6.1 Early work

The demonstration of a genetic role in the control of flowering has created great interest in possible molecular biological mechanisms involved. With leaves being the site of perception of photoperiod, some work has been done to attempt to characterise molecular changes which might occur in this organ during induction.

Early work with short day induced *Pharbitis nil* seedlings showed that a clear change occurred in the base composition of mRNA from cotyledons exposed to one inductive photoperiod (Oota and Unemura, 1970). A brief light break during the night cancelled the change seen in mRNA base composition and flower induction did not proceed. Cycloheximide (an inhibitor of protein synthesis) treatment of *Xanthium* leaves prevented floral induction and therefore showed the possible importance of *de novo* protein synthesis for floral induction (Ross, 1970). Similar results were also seen with application of the amino acid analogues ethionine and *p* - fluorophenylalanine (Ross, 1970). It should be noted that the mode of action of these treatments was never determined, i.e. the substances mentioned above may have other physiological effects other than inhibition of protein synthesis or protein function which might affect flowering.

1.6.2 Analysis of gene expression associated with floral induction using current techniques

1.6.2.1 Analysis of protein and *in vitro* translation products using polyacrylamide gel electrophoresis

More advanced techniques have been used recently with flowering in *Impatiens balsamina* (Sawney *et al.*, 1976), various species of *Amaranthus* (Kohli *et al.*, 1980), *Hyoscyamus niger* (Warm, 1984) and *Pharbitis nil* (Lay-Yee *et al.*, 1987a; Lay- Yee *et al.*, 1987b; Felsheim and Das, 1988). Work on *Impatiens balsamina* and *Amaranthus* involved the preparation of water soluble protein extracts during the course of photoperiodic induction which were subsequently analysed by disc gel electrophoresis (Ornstein, 1964; Davis, 1964). In both plant types, new electrophoretically separable proteins became apparent in extracts taken from leaves during the inductive treatment. Although interesting, this work must be considered with caution since no obvious controls were included to distinguish changes arising from a non-specific light effect (i.e. changes arising from the difference in light quality or quantity) from those related to floral induction *per se*.

The strategy taken in more recent work has been to extract mRNA from leaves at specific stages of photoperiodic induction and translate it *in vitro* . The translation products were then subjected to two-dimensional SDS-PAGE and visualized by autoradiography so that products of mRNA from induced leaves could be compared to products of mRNA from uninduced leaves. In this way, differences in mRNA species present can be observed and may represent differences in gene expression.

Warm (1984) demonstrated that the LDP *Hyoscyamus niger* showed changes in *in vitro* translatable leaf mRNA as a result of photoperiodic floral induction. Test plants were exposed to continuous light for eighty two hours (three cycles) which was followed by fourteen hours of darkness while control plants were kept in short daylengths. Leaves of test (induced) and control (non-induced) plants were harvested

after the exposure to the dark phase so that non-specific light effects would be presumably minimized. Two-dimensional electrophoresis of *in vitro* translation products showed ten obvious changes in mRNA seen as increases or decreases in particular translation products when comparing test plants to controls. Three of the translation products, which were noted as increases, were undetectable in the control samples, and one of the translation products, which was noted as a decrease, was undetectable in the test sample. The significance of these "induced-specific" mRNAs is unknown, and although this work was published in 1984, cloning of the corresponding genes, which would possibly yeild information on the nature of the proteins they encode, has not yet been been reported.

Two groups have presented data regarding changes in gene expression in *Pharbitis nil* . In Reid's laboratory (Lay-Yee *et al.*, 1987a; Lay-Yee *et al.*, 1987b), two dimensional electrophoresis was performed on *in vitro* translation products of mRNA obtained from leaves harvested after exposure to an inductive long night, a long night with a ten minute night break after the first eight hours, or continuous light. Only one mRNA, seen as a translation product with a molecular mass of 28 kilodaltons, expressed in *Pharbitis nil* during the inductive photoperiod, was of particular interest because it appeared in significantly lower levels in plants where flowering was specifically inhibited by the night break and by ethylene treatment (Lay-Yee *et al.*, 1987a; Lay-Yee *et al.*, 1987b). Based on these results, it was suggested that this mRNA may be involved in the induction process. However, it is well established that ethylene treatment alone can have an effect on gene expression and there was no control for this (Grierson and Covey, 1988).

Unfortunately, similar work (by a different group) with *Pharbitis nil* has shown different results from those decribed above (Felsheim and Das, 1988). mRNA extracted from the induced plants showed three qualitative differences in translation products with molecular masses of 28, 33, and 46 kilodaltons which were undetectable in control plants given a ten minute night break eight hours into the dark period. One translation product of 16 kilodaltons present in control samples was absent in test samples. These results clearly demonstrate the tremendous frustration in producing comparable results when dealing with this difficult developmental problem.

1.6.2.2. The thin cell-layer system for the study of floral induction

The *in vitro* thin cell-layer system has been used successfully to investigate various aspects of floral induction. Sections taken (up to four cell layers thick) from tobacco stems are floated on liquid culture media and induced to form flowers, vegetative buds, roots, or callus by adding specific exogenous factors to the media and altering the pH (Tran Thanh Van *et al.*, 1985)

Meeks - Wagner *et al.* (1989) have used this system to obtain cDNA clones for genes that are expressed in explants initiating floral meristems but not explants initiating vegetative shoot meristems. Morphogenesis was regulated in this case by adding to the medium either zeatin, which resulted in vegetative shoots, or kinetin, which resulted in the direct formation of flowers or floral branches without any leaf production. mRNA was harvested on the seventh day of culture which is the stage of active cell division that precedes meristem organization and organ differentiation for both zeatin and kinetin

treated cultures.

Differential screening of a cDNA library (as described in section 2.15), prepared from the mRNA from floral explants, with mRNA from the vegetative explants led to the identification of six gene families. The expression of three of these gene families, referred to as FB7-1, FB7-2, and FB7-5 (FB refers to floral bud and 7 to the day the tisssue was harvested), was analyzed and shown to have interesting temporal and spatial patterns when tissue was examined from explants and, more inportantly, from plants grown from seed. In most tissues examined, expression was highest preceeding the transition of the apex to flowering. A notable amount of expression occurred in the leaves and roots with strikingly high levels in the roots. The authors suggested that although a direct relation of FB7 expression to either floral induction (production of the floral stimulus) or evocation (response at the apex) cannot be made until it is possible to block the activities of the gene products in a tissue specific manner, the results provide strong support for the multifactorial model which brings together evidence for the involvement of roots as well as leaves in the floral induction process.

Two of the clones, FB7-2 and FB7-5, show interesting homologies with other known genes (personal communication from D. Ry Meeks-Wagner, University of Oregon). FB7-2 has been sequenced and shows limited homology to PR-S (pathogenisis related protein, type S) and FB7-5 has been shown by Southern hybridization to be homologous to a ß - glucanase. PR-S has been described but has not been characterized so its enzymatic function is yet unknown (Rigden and Coutts, 1988), although it has been shown to be serologically related to other pathogenesis response proteins that have (1,3) - ß - glucanase function. ß - glucanase is an enzyme which can

digest plant cell walls generating fragments which act as powerful signalling agents that activate plant defence genes.

The important role of oligosaccharide signalling in plants has recently been reviewed (Ryan, 1987) and it is clear that cell wall fragments can influence development as well as mediate the pathogenesis response. For example, auxin stimulated elongation of pea stem segments is inhibited by a xyloglucan isolated from sycamore cell walls (Ryan, 1987), and fragments of sycamore and of *Lemna* cell walls enhanced fond growth of *Lemna gibba* G3 and inhibited its flowering at certain concentrations (Ryan, 1987). Particularly interesting is work performed by Tran Thanh Van *et al.* (1985) where oligosaccharide fragments (referred to as oligosaccharins in cases where they are capable of regulating physiological processes), produced by treatment of sycamore cell walls with endo - α - (1,4) - polygalacturonase, were shown to be capable of controlling morphogenesis in "thin cell - layer" explants of tobacco.

Further recent work has shown that morphogenesis can be controlled using cell wall fragments from both sycamore and tobacco cell walls (Eberhard et al., 1989) and although the culture conditions were modified from those used by Tran Thanh Van *et al.* (1985), the conclusion that the pectic fragments may play a role in morphogenesis is the same. Future work must be done to purify the active component (s) of the fragment mixture, elucidate the mechanism by which the fragments affect "thin cell-layer" explant morphogenesis, and determine whether the fragments are *in situ* regulators of morphogenesis.

PR proteins were initially described as proteins induced by pathogen infection and

not found in healthy untreated plants. However, PR proteins were subsequently found to accumulate in untreated tobacco plants (highest amounts in the lower, older leaves) at the onset of flowering (Fraser, 1981). It is extremely tempting to suggest a model based on the results obtained with the thin cell-layer" system that would include the differential regulation of an enzyme such as ß - glucanase that would produce floral inducing cell wall fragments in the leaves which could be transported to and act at the apex. (1,3)-ß-glucanase is developmentally and hormonally regulated in tobacco (Felix and Meins, 1986) where a gradient of enzyme activity exists with the highest amount found in the lower part of the plant. This gradient is presumably caused by the lower concentration in older leaves of auxin and cytokinin which together act to inhibit the enzyme (Felix and Meins, 1985). It would be useful to see if fragments produced by a ß-glucanase have morphogenesis regulating properties with regard to the "thin cell-layer" system.

1.6.2.3 Studies on post translational modifications associated with floral induction

It is clear that much of the molecular studies on floral induction has focussed on differential gene expression as measured by direct protein analysis, *in vitro* translations, or differential screening of libraries. Obviously, gene activity can be regulated at other levels such as post - translational modification. Recent work by Kato and Fujii (1988) has shown that changes occur in protein kinase activity during floral induction of *Lemna puacicostata.*, where three soluble protein kinases, designated as PI (cAMP inhibited), PII (cAMP activated), and PIII (cAMP independent), and one microsomal protein kinase (cAMP independent) have been identified and studied. Protein kinase activity was

measured as the amount of enzyme which transfers 1 picomole of phosphate from (γ -^{32}P) - ATP to type II calf thymus histone substrate protein per minute at 30 °C, and of the different protein kinases, PII and PIII showed distinctly elevated levels of activity under inducing conditions. This activity could also be seen when soluble extracts were incubated with (γ -^{32}P) - ATP and subjected to SDS-PAGE. Four proteins with molecular masses of 59, 51, 16, and 14 kilodaltons, were seen to be more highly phosphorylated in extracts taken from plants grown under floral inducing conditions. Three of these proteins were previously noted to become more highly phosphorylated in the presence of cAMP (Kato *et al.*, 1983). These observations are interesting because inhibition of flowering in *Lemna paucicostata* 6746 and *Lemna gibba* G3 (Khurana *et al.*, 1988) by sucrose is partially overcome by cAMP, and cAMP is able to stimulate flowering in *Lemna paucicostata* 6746 strongly (Khurana *et al.*, 1988). Since, cAMP is converted to a cytokinin in plant cells (Trewavas, 1976), these results obtained from studies of *Lemna* should be interpreted with caution.

1.7 Purpose of this Work

The information presented in the previous sections provides convincing evidence for a role of specific gene products in the floral induction process in the leaves, although no genes with a confirmed function have yet been molecularly isolated. The focus of this study has been to select plant species which would be useful in attempts to identify genes associated with flowering. The difficulty in attempting to identify these genes is that there is no way of predicting the behaviour of their expression which would be helpful in cloning. For example, gene products involved in the production of inhibitor(s) might be down-regulated upon induction, or those involved with the

production of a promoter(s) might be up-regulated. It is also possible that gene products are produced at any time prior to expression and remain inactive until they are post-translationally modified as a result of some environmental or physiological stimulus. Whenever and however the genes are expressed, it is not easy to anticipate where or when the expression might be visible, i.e. at the RNA level, protein level, or both. Given the numerous ways that gene expression can be regulated, any number of scenarios could be suggested.

Despite these uncertainties, no advancement in furthering the understanding of the floral induction mechanism will be made until experiments are done to describe what can be observed with regard to gene expresssion using current techniques and in preparation for techniques that will soon become available. A description of the possible techniques that might be used is presented in section 1.8.

1.8 Potential technology used to identify and clone genes of interest

1.8.1 Molecular techniques

1.8.1.2 *in vitro* translation analysis

In the molecular biological studies on flowering reported to date, the assumption has been made that genes regulating the floral induction process are differentially expressed, as is the case with many other developmental processes such as seed development and fruit ripening (Grierson and Covey, 1988), and techniques have been

used which allow for distinguishing differences in the mRNA populations which may exist when comparing induced and non-induced tissue. A common approach used to identify differential gene expression is to extract mRNA from tissues to be compared and translate it *in vitro*. Comparisons of autoradiographs can be made when the translation products, synthesized in the presence of [^{35}S] - L - methionine, are subjected to electrophoresis through a one-dimensional or two-dimensional SDS polyacrylamide gel. Qualitative or quantitative differences in protein bands (in the case of one-dimensional electrophoresis) or spots (in the case of two-dimensional electrophoresis) are presumed to represent an mRNA that is differentially expressed in the compared samples which should otherwise have identical mRNA populations.

1.8.1.2 Differential screening of cDNA libraries and subtractive hybridisation techniques

Once it is established that differential gene expression occurs, the gene or genes of interest can be cloned by differential screening of complementary DNA (cDNA) libraries (Sargent, 1988). Differential screening can be used in any system when attempting to characterize genes whose expression is spatially or temporally regulated, occurs at different stages of development, or is altered after a specific treatment.

The technique involves the preparation of a cDNA library by first extracting and isolating polyadenylated mRNA from the 'induced tissue', i.e. the tissue containing the genes which are differentially expressed. This is used in a synthesis reaction to make cDNA which is subsequently made double stranded by further reactions. The double stranded cDNA can then be cloned into plasmid vectors or into one of the more popularly

used λgt bacteriophage vectors. Libraries are plated out and replica blots are taken from the surface of the dishes using nylon or nitrocellulose filters. One blot is incubated in a hybridization mixture containing radioactive cDNA prepared from mRNA taken from the induced tissue, and the other blot is hybridized with radioactive cDNA prepared from mRNA taken from the uninduced tissue. The radioactive cDNA prepared from the induced tissue will hybridize to all of the colonies (when plasmid vectors are used) or plaques (when bacteriophage vectors are used) since the library was made from the same mRNA, and the radioactive cDNA prepared from the uninduced control will hybridize to most of the colonies or plaques except for the select ones which contain copies of genes expressed only, or more abundantly, in the induced tissue. These plaques are then harvested, rescreened, and purified to allow for subcloning and further analysis of the insert.

The differential screening technique is routinely used for cloning genes which represent 0.1% and in some cases 0.01% of the total mRNA population depending on the care taken with experimental conditions. When designing such experiments, the abundance of the mRNAs of interest must be considered as this allows for the determination of the number of clones from the library which need to be screened for successful results. The following equation is used for calculating the number of clones which must be screened when the relative abundance of the mRNA of interest can be estimated :

$$N = \log P / \log(1-A)$$

where N is the number of clones, P is the probability that the clone will be missed, and A is the relative abundance of the mRNA of interest (Sargent, 1988). Therefore, when

the screening conditions are optimal and the clone of interest represents 0.01% of the total mRNA, it will be necessary to screen approximately 46,000 clones in order to have a 99% chance of obtaining the clone of interest. If the mRNA is expressed in low abundance, for example 0.001%, the number of clones to be screened increases ten-fold and the differential screening procedure becomes very tedious and impractical.

Attempts have been made to improve upon the original differential screening procedure to provide greater ease in the identification of positive clones (Olszewski *et al.*, 1989), but the most common approach is to employ subtractive cloning techniques when the clone(s) of interest are of low abundance (Rhyner *et al.*, 1986; Duguid *et al.*, 1988). The premise of these techniques is that cDNA prepared from total mRNA from the 'induced' tissue can be hybridised to completion with an excess of mRNA from the 'uninduced' tissue such that induced specific cDNAs will remain single stranded and can be purified by hydroxylapatite chromatography. The single stranded induced specific cDNA can be subsequently used to construct an enriched library which can be screened as described above or using the subtracted cDNA as a probe. Using these techniques, cloning of certain mRNA species with an abundance of 0.0001% has been reported (Rhyner *et al.*, 1986). The disadvantages of this technique are (1) the need for a large quantity of mRNA to drive the subtractive hybridisation to completion, and (2) messages whose difference in relative abundance is not great enough will be lost in the subtraction, i.e. the typical ratio of RNA to cDNA in the hybridisation is 30:1, and therefore, the abundance of the message present in the cDNA must be thirty times the amount in the driver RNA.

The subtraction approach has been improved upon and kits are now commercially

available (Invitrogen, California) that are based on the development of a procedure which allows for the subtraction of the library itself (Duguid *et al.*, 1988). In this case, two libraries are prepared representing total mRNA populations from induced and non-induced tissue using special plasmid vectors. These vectors contain the M13 origin of replication so that they can be converted to single stranded cDNA libraries by employing a helper phage. The induced library is then hybridised with biotinylated sonicated DNA from the non-induced library, and this allows for the removal of the hybrid DNA by the addition of avidin and simple phenol extraction. The avidin-biotin complex is more soluble in phenol, so only the induced-specific cDNA remains in the aqueous phase. The advantage of this technique over the traditional subractive method is that only the amount of mRNA needed to construct the library (1μg) is necessary for the subtraction procedure as well as the synthesis of probes for screening, which can be produced from restriction digests of the library in its double stranded form (Duguid *et al.*, 1988). Also, the synthesis of two libraries in this fashion allows for easier identification of cDNAs that are more highly expressed in the induced tissue as well as cDNA expressed more highly in non-induced tissue.

Differential gene expression can also be visualized by comparison of one- or two-dimensional electrophoretic patterns of total proteins extracted from induced and non-induced tissues. If enough protein is available, it can be purified by direct elution from the polyacrylamide gel using commercially available systems (Pharmacia/LKB multiphor system) and then sequenced using standard protein sequencing techniques. In some cases, the protein can be transferred to nitrocellulose and sequenced directly (Bauw *et al.*, 1989). Using either approach, protein sequence data can be used to produce a degenerate oligonucleotide probe which can be used to screen a cDNA library.

1.8.1.3 cDNA cloning using immunoadsorbed antisera

An alternative method for cloning genes coding for proteins of interest employs the differential immunoadsorption technique which has been used successfully in the isolation of cDNA clones representing genes that are developmentally regulated during carrot embryogenesis (Choi *et al.*, 1987). The procedure followed in this approach is to prepare an antiserum against total protein extracted from the induced tissue and subsequently incubate it with protein extracted from non-induced tissue which should bind to all antibodies that are against proteins which the induced and non-induced tissues have in common. The immunoadsorbed antiserum can then be used to screen cDNA libraries which have been prepared using a vector such as λgt11 which expresses the cDNA insert such that the protein produced can be detected with antibodies. This technique has been reported to yield cDNA clones for messages at an abundance of 0.01% of the total mRNA population, but the basic assumptions which must be considered are that the mRNA coding for any specific protein of interest is present at a high enough level to facilitate cloning, and the expression vectors used to construct the library must produce proteins that are detectable by the immunoadsorbed antibodies.

1.8.1.4 Molecular approaches to studying the function of identified genes

Once clones for genes of interest are identified, there are three strategies which are available, at least theoretically, to analyze the biological function of the gene product. Intially, the cDNA clone can be sequenced which may provide insight into its function if,

by searching computer sequence libraries, homology with other known proteins is apparent. A second approach is to block gene activity in the wild type plant by using methods which employ antisense RNA (Inouye, 1988), ribozymes (Haseloff and Gerlach, 1988), or antibodies (Hiatt, 1990) . The antisense method, which has been used successfully to study the effects of blocked expression of the polygalacturonase gene in tomato (Smith *et al.*, 1988), is based on the idea that if the antisense version of the gene of interest is expressed in a transgenic plant, the mRNA that is synthesized will bind to the sense mRNA and prevent its translation by an as yet undefined mechanism. The success of this method depends on the ability to introduce the antisense gene into the system of interest and block activity so that an observable phenotype is produced. It is possible that if the activity of the gene is not blocked completely, the effect may not be visible and this must be considered in the interpretation of results. The use of ribozymes, which also act by specifically inactivating the mRNA of interest, in gene analysis is still being developed and no examples are available for its implementation in plants. If monoclonal antibodies were expressed in a transgenic plant, they could bind and inactivate the protein product of the gene of interest. Although successful expression of an antibody in tobacco has been reported recently (Hiatt, 1990), future practical applications for this approach are only speculative.

The other strategy that might be considered is the dominant negative mutation approach (Herskowitz, 1987)) which involves the introduction of a mutagenised form of the gene of interest into the wild type system in the hope that production of a mutant protein will inhibit the function of the wild type protein presumably by competition. There are no examples yet of the successful use of this technique.

1.8.2 Genetic techniques

1.8.2.1 Gene tagging

Genetic techniques have been widely used in the isolation of genes in many eukaryotic systems. The most promising technique for the cloning of plant genes using genetics is the implementation of transposon tagging which has been thoroughly successful in the identification of developmentally important genes in *Drosophila* . The possibilities and problems regarding gene tagging using currently identified plant transposons as well as T-DNA from the Ti plasmid of *Agrobacterium tumefaciens*, have recently been reviewed (Hanson, 1989). The theory for transposon tagging is that when introduced into the plant, the transposon will insert into a gene, disrupt its function, and hopefully result in a visible phenotype. The gene can then be cloned on the basis of its hybridisation to a probe containing the transposon sequence.

Using this technique, genes have only been cloned in plants with the use of endogenous transposons (for example, see Federhoff *et al.*, 1984), but no gene has yet been cloned using a foreign transposon in a transgenic plant. The cloning of the *Glabrous 1* gene, which is involved with trichome development in *Arabidopsis thaliana*, was accomplished by T-DNA tagging (Marks and Feldman, 1989; Herman and Marks, 1989), so it is possible that the use of T-DNA over transposons may become preferred for the identification and isolation of genes controlling developmental processes in plants. A major problem with both approaches (transposon/T-DNA) will continue to be the need to screen large numbers of mutagenized individuals (as many as 100,000 in a typical plant species), and therefore, the techniques will be most valuable in identifying

genes controlling major visible phenotypes.

1.8.2.2 RFLP analysis

The application of restriction fragment length polymorphisms (RFLPs) has been very useful in the cloning of genes in mammalian systems as in the recent cloning of the cystic fibrosis gene (Rommens *et al.*, 1989). RFLPs are used to produce genetic linkage maps by isolating single copy clones of DNA from the species of interest and using them to follow the homologous areas of the genome in individuals from segregating populations such as an F2 or backcross. Also, RFLP analysis can be applied to plants as most species of current interest are sexually reproductive and techniques are available for selectively cloning the single copy DNA fraction from plant genomes (Tanksley *et al.*, 1988), and RFLP maps are now available for several plant species (for review see Tanksley, 1989) and are currently being used in plant breeding programmes.

Once linkage to a RFLP marker is established, genes can be cloned by chromosome walking or hopping (for review see Tanksley *et al.*, 1989), i.e. moving away from the RFLP region towards the gene. The two most common problems with this approach is that it is difficult to identify the clone containing the gene of interest and in some cases, very tight genetic linkage can still mean the RFLP marker is a great distance away, e.g. one centimorgan of tomato DNA is approximately equivilent to 550 kilobase pairs of DNA. The most promising species for which RFLP map based cloning will be implemented is *Arabidobsis thaliana* which is particularly suited to molecular genetic studies due to its very small genome consisting of five chromosomes equalling approximately 70,000 kilobase pairs (in the haploid genome) (Meyerowitz, 1989)

1.9 Plants Systems Used in This Work

To design an experimental approach which would enable the identification and characterization of potentially important genes employing one or a combination of the techniques described in section 1.8, several considerations should be made to maximize chances for success. Initially, plant subjects should be selected which allow for (1) ease in nucleic acid and protein extraction, (2) mutant isolation and genetic studies, (3) introduction of exogenous DNA by using *Agrobacterium* vectors or other available techniques, (4) defined induction protocol with well studied flowering physiology, and (5) short growth time prior to readiness for experiments.

There is no ideal plant that satisfies all of the above requirements, but for preliminary studies of this sort, it was thought that it would be best to choose a selection of species encompassing an array of flowering behaviours. The following plants were investigated and the reasons for their choice are given.

1.9.1 *Nicotianna plumbaginifolia*

Nicotianna plumbaginifolia was obtained for use based on the recommendation of Dr. P.J. King, of the Freidrich Meischer Institute in Basel, who has extensive experience in utilizing this plant for developmental genetic studies. This plant is a true diploid that demonstrates great success in transfection with *Agrobacterium*, direct DNA transfer to protoplasts, regeneration from culture, protoplast fusion to facilitate complementation analysis of mutants, graft treatment, and production of mutants from seed and haploid protoplasts. In addition, it has been used to clone many genes of

biochemical or developmental interest including those coding for glutamine synthetase (Tingey and Coruzzi, 1987), the beta subunit of the mitochondrial ATPase (Boutry and Chua, 1985), the gene for *rbcS* -8B (Poulsen and Chua, 1988), and the gene for *cab* (Castresana *et al.*, 1988). *N. plumbaginifolia* grows in a rosette form and then upon induction produces a flower bearing shoot from the apex. According to Dr. King, flowering occurred when plants grown in short days of ten hours at 25 °C were transferred to long days of sixteen hours at 25 °C.

Duplicate conditions for growth and induction used in this work showed that *N. plumbaginifolia* will flower regardless of daylength and appeared to be only enhanced by exposure to long days. Daylength neutrality was not considered a problem and merely provided the study with a subject plant displaying a flowering behaviour which was not initially included in the original choices (i.e. the choices were preferentially limited to photoperiodic species). The benefit of using a non-photoperiodic plant is that difficulties which normally arise in controlling for non-specific influences of gene expression from the change in light conditions used for induction will be avoided.

1.9.2 *Amaranthus caudatus*

Amaranthus caudatus was selected primarily because qualitative changes in water soluble protein extracts, when analysed by one-dimensional electrophoresis, had been observed during floral induction as previously described by Kohli (1984), and no further work had been attempted to characterize the genes that might be involved. Several contradictory descriptions for the photoperiodic classification of this plant exist (Fuller, 1949; Zabka 1961; Kigel and Rubin, 1985) in that the short day requirement has been reported as either qualitative or quantitative. This is possibly due to differences in

experimental conditions, the length of time the control plants were observed, or possibly the genotypes of the plants used in each case which was not clearly defined. In any case, the number of short day cycles is minimal (2 to 3) to fully induce flowering which can become evident only 4 to 5 days after the first inducing cycle. Flowering can be inhibited by brief exposure to red light during the inductive dark period and this aspect provides an attractive control for non-specific light effects on gene expression which was not considered in the work described by Kohli *et al.* (1980).

1.9.3 *Silene coeli-rosa*

The flowering physiology of *Silene coeli-rosa* is well studied and is a strictly photoperiodic qualitative long day plant (Lyndon, 1985). This plant is induced maximally by exposure to seven cycles of long photoperiods composed of eight hours of high intensity light extended to twenty four hours by low intensity light. Also, much information has been reported concerning events occurring at the apex at various points during the induction process including very detailed analyses of changes in cell cycle and total RNA levels (Lyndon, 1985). Although 100% induction in all plants during any given experiment requires seven long day exposures, changes in cell cycle at the apex begin to occur after the first long day exposure and may therefore indicate the arrival of stimuli produced in the leaves. When plants are subjected to twenty minutes of darkness at the end of the eight hour photoperiod and prior to the low intensity light extension used for induction, flowering is inhibited and this should provide the control for non-specific light effects. Light perception is possibly not limited to specific leaves because with the related species *Silene armeria* the number of leaves which keeps the plants alive is all that is necessary. Experiments have shown that plants grown from

seed are available for experimentation 28 days after germination.

1.9.4 *Anagallis arvensis*

Anagallis arvensis was selected because of the enormous amount of physiological data available regarding its flowering behaviour (Ballard, 1969; Brulfert *et al.*, 1985). This plant is a very strict qualitative LDP species that can be induced by only one cycle of the appropriate light treatment. Defoliation experiments have established that the perception of daylength appears to be localised predominantly in the young leaves which are not fully expanded and the youngest two pairs of fully expanded leaves. Exposure to inducing conditions of plants where only older leaves remain results in virually no flowering. Thus, the older photoperiodically insensitive leaves provide a control for changes in gene expression which may result from light conditions and not from photoperiod *per se* . Defoliation experiments have also shown that the floral stimulus is produced and transported out of the leaves within 48 hours from the beginning of the inducing treatment, so therefore, only a short period of rigorous sampling would be required to determine if any changes in gene expression have taken place.

There are many inducing regimes that can be employed and this would allow for comparative analysis. For example, night break experiments, where three to five hour exposures of red light given at various points during the dark period, shows that plants are most sensitive to light during the second part of the period. Thus, plants exposed during the first half of the period and plant exposed to the second half can be compared. Plants from the first half will, again, control for changes due only to the change in the amount of light.

Cuttings of the clone which originated from a single plant collected in the surroundings of Paris are available and allow for easy propagation of plant material from cuttings which are ready for experimentation after 20 days of growth. Use of clonal tissue should minimize natural variation in responses and has the benefit of being identical to the tissue used in most of the physiological studies reported.

Chapter 2 - Materials and Methods

2.1 Materials

Most chemicals were obtained from Formachem® or Analar® (distributed by BDH). Fine chemicals were obtained from Sigma unless otherwise indicated. Radiochemicals were obtained from Amersham International, plc unless otherwise indicated. Sources of enzymes are indicated appropriately throughout the text.

2.2 General experimental procedures

2.2.1 Sterilisation of equipment and solutions

Equipment and solutions used in all experiments were sterilized in a Lab Thermal Equipment 225E autoclave for 20 minutes at a pressure of 15 pounds per square inch or in a Prestige Hi-Dome pressure cooker. Solutions were always prepared with Analar® water unless otherwise indicated.

2.2.2 Preparation of equipment and solutions for RNA work

When working with RNA, all solutions, glassware, or any equipment which would come in contact with the RNA were treated with diethyl pyrocarbonate (Sigma). Solutions were treated by adding diethyl pyrocarbonate to a concentration of 0.1 % (v/v), leaving overnight at room temperature, and autoclaving for 20 minutes at a pressure of 15 pounds per square inch. Glassware and other equipment were treated by soaking overnight at room temperature in a 0.1 % solution of diethyl pyrocarbonate followed by autoclaving for 20 minutes at a pressure of 15 pounds per square inch.

2.2.3 Preparation of equipment and solutions for DNA and protein work

Equipment and solutions required for the manipulation, handling, and storage of DNA or protein was sterilised prior to use by autoclaving.

2.2.4 pH measurement

A Corning pH meter 220 and combination electrode was used to determine the pH of solutions.

2.2.5 Spectrophotometric determination of nucleic acid concentration

For quantitating the amount of nucleic acid, concentrated samples were diluted 1 : 50 or 1 : 100 and placed into a 1 cm path length quartz cuvette. The cuvette was placed in a LKB Ultrospec II spectrophotometer and absorbance was measured at wavelengths of 260 and 280 nm. For RNA, an optical density of 1 corresponded to a concentration of 40 µg/ml (Maniatis, *et al.*, 1982). The ratio of the readings at 260 nm and 280 nm was calculated to estimate the purity of the preparation.

2.3 Growth of plants and experimental conditions and procedures used in floral induction studies

2.3.1 Growth, maintenance and sampling of *Nicotiana*

plumbaginifolia

Seeds (obtained from Dr. P. J. King, The Freidrich Meischer Institute, Basel) were sown on fresh compost and left under glass to germinate in an environment controlled growth room at 25 °C with fluorescent (OSRAM warm white, 85 Watts) light illumination at 150 μmol • m^{-2} • sec^{-1} (400 - 700 nm) as measured with a Li-Cor LI - 185B quantum sensor. Day length was maintained at eight hours, and after approximately one to two weeks, the seedings were transferred to four inch plant pots containing fresh compost. Samples were taken by cutting the rosette of leaves from the roots and then individually removing each leaf from the stem, making a note of its leaf number (with number one being the first leaf produced after the cotyledons), and wrapping it in aluminium foil prior to immersion in liquid nitrogen and storage at -80 °C.

2.3.2 Growth and induction of *Amaranthus caudatus*

Seeds (obtained from the Royal Botanic Garden at Kew) were sown on fresh compost and left under glass to germinate in an environment controlled growth room at 25 ° C under continous illumination by fluorescent light (OSRAM warm white, 85 Watts) at an intensity of 150 μmoles • m^{-2} • sec^{-1}. After approximately one to two weeks, seedlings of uniform size were transferred to four inch plant pots containing fresh compost and allowed to grow for thirty days. Plants were grown for a minimum of 28 days and induced to flower by placing them in a short day length growth room illuminated with 8 hours of fluorescent light (intensity at 150 μmoles • m^{-2} • sec^{-1}). Samples were taken by removing all healthy leaves and wrapping them together in aluminium foil and immersing in liquid nitrogen. Samples were stored at -80 °C.

2.3.3 Growth and induction of *Silene coeli-rosa*

Seeds, obtained as a gift from Dr. Robert Lyndon, Department of Botany, University of Edinburgh, were sown on fresh compost and left under glass to germinate in an environment controlled growth room at 25 °C with a combination of fluorescent light (OSRAM warm white, 85 Watts) at an intensity of 150 μmoles • m^{-2} • sec^{-1} and tungsten light at an intensity of 120 - 130 μmoles • m^{-2} • sec^{-1} and a day length of eight hours. Seedlings were transferred to four inch plant pots and grown for a minimum of 28 days. Plants were induced to flower by extending the daylength to 24 hours with tungsten light at a low intensity of 10 - 15 μmoles • m^{-2} • sec^{-1}. Samples were taken by removing all healthy leaves, wrapping them in aluminium foil and immersing in liquid nitrogen. Samples were stored at -80 °C.

2.3.4 Growth and induction of *Anagallis arvensis*

Cuttings of the Paris clone (Ballard, 1969; Brulfert, 1985) obtained as a gift from Jeanne Brulfert (Centre Nationale de la Rechearch Scientifique, Gif - sur - Yvette, France) were placed in water for one week in an environment controlled growth room at 25 °C and light intensity and short day length as described in section 2.3.3. Rooted cuttings were transferred to four inch plant pots (four to six cuttings each) containing fresh compost and allowed to grow for a minumum of two weeks. Plants were induced to flower by extending the day length to 24 hours as described in section 2.3.3. Samples were taken by first removing the stem and dipping it into liquid nitrogen using a pair of forceps to hold on the end. After snap freezing, the stem was withdrawn from

the liquid nitrogen and the first two pairs of expanded leaves (those closest to the apex) and the remaining unexpanded leaves were quickly broken off and allowed to fall directly into a plastic weigh boat filled with liquid nitrogen. After sampling was complete, the leaves collected in the weigh boat were poured into a plastic universal punched with small holes and stored in liquid nitrogen.

2.3.5 Harvesting and microscopy of apical tissue

Leaves of the plant were carefully removed to expose the apex. A razor blade was used to cut the apex from the stem beneath it. The apex was fixed for a minimum of 24 hours in 3% (v/v) glutaraldehyde (Sigma) prior to dehydration and embedding. The embedding, sectioning and staining of the apical tissue was carried out by Mr. Ian Robertson in the Department of Chemistry, University of Glasgow.

2.3.6 Measurement of Leaf Area

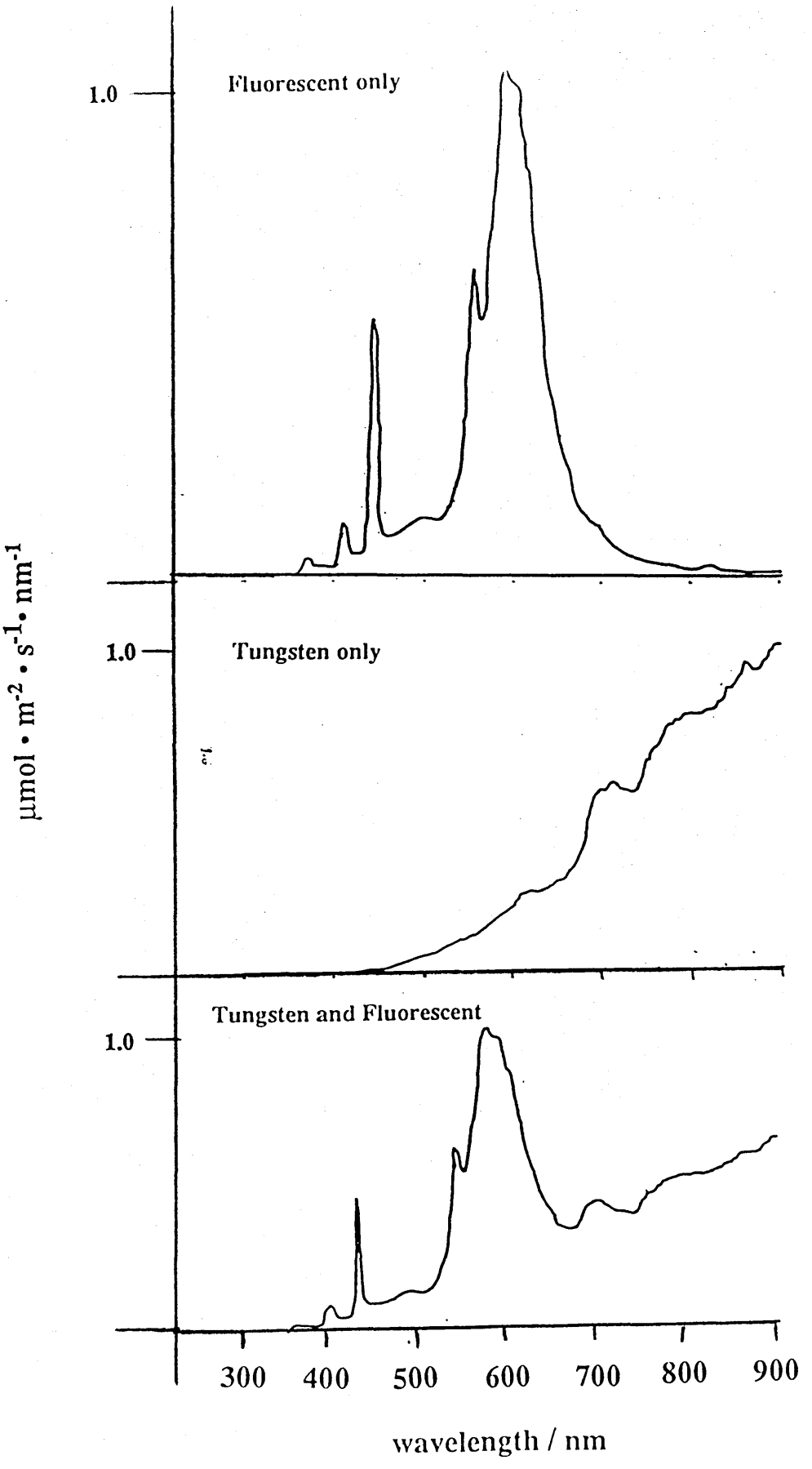
Leaf area was measured in cm^2 using a Delta T area meter (Delta Devices, Cambridge, England).

2.3.7 Measurement of spectral quality of the light sources used in the growth and induction of the plants

Spectral photon distributions of the light sources used in the various experiments were determined using a Macam spectroradiometer and are shown in figure 2.1

Figure 2.1 Spectral photon distributions of light sources used in growth, maintenance, and induction of plants

Spectral photo distributions were measured at wavelengths from 300 to 900 nm using a spectroradiometer as described in section 2..3.7 for light sources including (A) flourescent light only, (B) incandescent light only, (C) combination of fluorescent and incandescent light.


Fluorescent only
1.0
Tungsten only
1.0
Tungsten and Fluorescent
1.0
µmol • m-2 • s-1• nm-1
300
400
500
600
700
800
900
wavelength / nm

2.4 Isolation of total RNA

2.4.1 Preparation of total RNA from *Nicotiana plumbaginifolia*

Leaves were ground under liquid nitrogen using a mortar and pestle and then transferred to a DEPC treated flask containing a solution made up of one part 5X extraction buffer [0.25 M sodium chloride, 0.025 M sodium acetate, 0.0025 M Na_2 EDTA, and 5% (w/v) sodium dodecyl sulphate], four parts water and five parts of a one to one mixture of phenol (prepared by redistillation followed by the addition of 8-hydroxyquinoline to a concentration of 0.1 % (w/v) and saturation with water or 10 mM Tris-HCl, pH 7.6, and chloroform. The flask was then placed in a water bath at 60 - 65 °C and gently shaken for 3 minutes. The solution was then transferred to a Corex® tube and centrifuged at 5000 rpm in a MSE Mistral 2L centrifuge for 20 minutes at 10 °C. The aqueous phase was then placed into a clean Corex® tube and extracted two more times with a one to one mixture of phenol and chloroform. The RNA was then precipitated by adding one tenth the volume of 3M sodium acetate pH 5.5 and two and one half the combined volume of absolute ethanol and stored overnight at -20 °C . The RNA was pelleted by centrifugation at 12,000 rpm in a MSE High Speed 18 centrifuge using a 8 x 50 ml rotor for 15 minutes at 4°C. The supernatant was discarded and the pellet was then washed with 80% (v/v) ethanol and centrifuged again at 12,000 rpm at 4 °C. After the supernatant was discarded, the pellet was dried under vacuum and resuspended in water and stored at -80 °C. Prior to use in experiments, spectrophotometric determination of the concentration of the RNA preparation was carried out as described in section 2.2.5. If an optical density ratio (260 nm/280 nm) was less than 1.8, indicating protein or phenol contamination (Maniatis *et al.*, 1982),

phenol extraction and ethanol precipitation was repeated.

2.4.2 Preparation of RNA from other species

RNA from all species other than *N. plumbaginifolia* was prepared according to Lichtenstein, and Draper, (1985) with some modifications. Tissue was ground under liquid nitrogen using a mortar and pestle. Homogenization buffer (0.2 M Tris - HCl, pH 8.5, 0.2 M sucrose, 30 mM magnesium acetate, 60 mM KCl) was then added and the ground tissue was ground further until completely thawed. The mixture was then transferred to a Corex® tube and centrifuged at 7 °C for 15 minutes at 10,000 rpm in a MSE High Speed 18 centrifuge using a 8 x 50 ml rotor. The supernatant was placed into a clean Corex® tube and sodium dodecyl sulphate was added to a concentration of 0.5% (w/v). An equal volume of a one to one mixture of phenol and chloroform was added and the tube was vortexed for 30 seconds. The extraction was centrifuged at 5,000 rpm in a MSE Mistral 2L centrifuge for 10 minutes at 7 °C. The aqueous phase was transferred to another clean Corex® tube, and the phenol and chloroform extraction was repeated until the denatured protein which collects at the interface of the organic and aqueous phases was no longer visible. The RNA was precipitated by adding one tenth the volume of 3M sodium acetate pH 6.0 and two times the combined volume of absolute ethanol and then storing at -20 °C overnight. The RNA pellet was collected by centrifugation at 10,000 rpm for 20 minutes at 4 °C in a Beckman J2 - 21 centrifuge using a JS - 131 rotor. The supernatant was discarded and the pellet was washed in 80% (v/v) ethanol and then centrifuged again at 10,000 rpm for 20 minutes at 4 °C. The pellet was dried under vacuum and resuspended in water. 50 Units of human placental ribonuclease inhibitor (Amersham International plc) were added and then the RNA was stored at -80

°C.

2.4.3 Preparation of poly(A)$^+$ RNA

The method used to purify messenger RNA from total RNA was that of Clemens (1985) with some modification. 0.15g of the oligo-dT cellulose type 7 (Pharmacia) was suspended in sterile loading buffer consisting of 0.5 M sodium chloride, 1 mM Na_2 EDTA, 0.1% (w/v) sodium lauryl sarcosine, and 10 mM Tris-HCl, pH 7.5. The suspension was then poured into a 2ml syringe containing a glass wool plug and washed successively with one column volume of water, a solution of 0.1 M sodium hydroxide and 5 mM Na_2EDTA, and again, water. The final water wash was continued until the pH of the effluent reached pH 7.0. The column was then re-equilibrated with loading buffer. The total RNA was heated in a water bath at 65°C for 5 minutes and then quickly chilled on ice. An equal volume of two-fold concentrated loading buffer was added to the RNA, and, after mixing, the RNA was loaded onto the column. The unbound RNA which eluted was collected, heated again at 65°C, cooled on ice, and then reapplied to the column. The column was then washed with loading buffer until the A_{260} of the effluent was very close to zero. The poly(A)$^+$ RNA was collected by washing the column with elution buffer, containing 1mM Na_2EDTA, 0.05% (w/v) sodium lauryl sarcosine, and 10 mM Tris-HCl, pH 7.5, at 65 °C. The poly(A)$^+$ RNA was then precipitated as described aboved in the precipitation of total RNA. After resuspension in water, 50 Units of human ribonuclease inhibitor (Amersham International plc) was added to the poly(A)$^+$ RNA which was then stored at -80 °C.

2.5 Procedures concerning the preparation and use of cell free wheat germ extracts in the *in vitro* translation analysis of mRNA

2.5.1 Preparation of dialysis tubing

Dialysis tubing was first boiled two times in 1% (w/v) Na_2EDTA, pH 7.0, for twenty minutes and two times in 1% (w/v) sodium bicarbonate for twenty minutes. The tubing was then washed successively in water and 25% (v/v) ethanol follwed by storage in 25% (v/v) ethanol. The tubing was washed in water prior to use.

2.5.2 Preparation of wheat germ extract

The preparation of the wheat germ extract was done as previously described (Davies, 1977) Wheat germ obtained from General Mills (Vallejo, California) was added and stirred into a mixture of 75ml cyclohexane and 250 ml carbon tetrachloride. The wheat germ which floated on the top was collected with a tea strainer and spread out to dry on filter paper. 0.5 g of the floated wheat germ was ground in a chilled mortar containing a small amount of sand for 15 seconds. 1 ml of grinding buffer (120 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, 5 mM HEPES, ph 6.7) was added to the mortar and grinding was resumed for one minute. A further 1 ml of grinding buffer was added and grinding was continued for another minute. Finally, 1 ml more was added and the slurry formed was transferred to an ice-cold Corex® tube using a further 2ml of grinding buffer to wash out the mortar. The tube was then centrifuged at 12,000 rpm for 10 minutes at 4 °C in a MSE High Speed 18 using a 8 x

50 rotor. The supernatant was transferred to another ice cold Corex® tube containg 50 µl of 0.5 M HEPES, pH 7.6, and centrifuged for a further 15 minutes. A pasteur pipette was used to remove the supernatant avoiding the upper 0.5 ml and the 0.2 ml at the bottom. The extract was then dialysed overnight against 1 l of ice cold dialysis buffer (20 mM tris-acetate, pH 7.6, 120 mM potassium acetate, 5 mM magnesium acetate, and 1mM dithiothreitol) in an ice bucket at 4 °C. The buffer was changed and dialysed a further 3 hours. The extract was then transferred to a ice cold Corex® tube and centrifuged at 5,000 rpm for 5 miutes at 4 °C in a MSE High Speed centrifuge using a 8 x 50 rotor. The supernatant was then divided into 0.5 ml aliquots in tubes suitable for storage in liquid nitrogen.

2.5.3 *In vitro* translation of total or poly(A)$^+$ RNA using prepared wheat germ extract

Each reaction contained 12.5 µl of extract, 2.5 µl of HKMS (200 mM HEPES pH 7.6, 400 mM potassium acetate, 4mM magnesium acetate, 4 mM spermidine), 2.5 µl of Mix 6 (3.75mM GTP, 0.375 mM amino acids mixture minus methionine (Amersham International, plc), 2.5 mM ATP, 90mM creatine phosphate, 0.1 mg/ml creatine phosphate kinase), 25 µCi of [^{35}S] - L - methionine at 1000 Ci/mMol (NEN-Dupont), 5µg of total RNA or 0.5 µg of poly(A)$^+$ RNA, and water to a total volume of 25 µl. The reaction was incubated for 90 minutes at 30 °C. Translation products were visualized by either one-dimensional or two-dimensional SDS-PAGE in which 2 x 10^5 or 1.5 x 10^6 cpm were loaded respectively. In preparation for one dimensional electrophoresis, an equal volume of loading buffer was added to the sample which was then boiled for two minutes prior to loading. In preparation for two-dimensional

electrophoresis, samples were incubated with ribonuclease A at 5 µg/ml for 15 minutes at 42 °C. Electrophoresis was usually followed by fluorography employing one of the methods described in sections 2.8.1 or 2.8.2.

2.5.4 *In vitro* translation of total or poly(A)$^+$ RNA using wheat germ extract obtained from Amersham International plc

5 µg of total RNA or 1 µg of poly(A)+ RNA was added in a volume of 7.5 µl to a mixture containing 2 µl of 1mM amino acid mixture minus methionine, 2.5 µl of [^{35}S] - L - methionine totalling 25 µCi, 15 µl of the wheat germ extract, and 3 µl of 1M potassium acetate. The reaction mixture was incubated at 25 °C for 90 minutes. Translation products were visualized by either one- or two-dimensional SDS-PAGE by loading 2x 10^5 or 1.5 x 10^6 cpm, respectively. Electrophoresis was usually followed by fluorography employing one of the methods described in sections 2.8.1 or 2.8.2.

2.5.5 *In vitro* translation of total RNA using wheat germ extract obtained from Promega (Madison, WI, USA)

5 µg of total RNA was added in a total volume of 13 µl to a reaction mixture containing 25 µl of wheat germ extract, 4 µl of 1 mM amino acid mixture minus methionine, 5.0 µl of [^{35}S] - L - methionine totalling 50 µCi, and 3 µl of 1 M potassium acetate. The reaction was incubated at 25 °C for 90 minutes. Translation products were visualized by either one-dimensional or two-dimensional SDS-PAGE by loading 2 x 10^5 or 2 x 10^6 cpm, respectively. Electrophoresis was usually followed by fixation and

fluorography employing one of the methods described in section 2.8.1 or 2.8.2.

2.5.6 Estimation of incorporated activity of *in vitro* translation products using TCA precipitations

A 100% stock of TCA was prepared by dissolving 500 g in 227 ml of water. Using an Eppendorf® micropipettor, 2 µl of the *in vitro* translation reaction was removed and placed into 1 ml of water in a six inch test tube. 500 µl of 1.0 M sodium hydroxide containing 0.5 M hydrogen peroxide and 1 mg/ml of unlabeled L - methionine was added and the mixture was incubated at 37 °C for 10 minutes. 1 ml of 25% TCA was added with 10 µl of 2% (w/v) BSA and the tube was stored on ice for 15 minutes. The precipitate was then collected by filtration on a Whatman® GF/A glass fiber filter disc and washed twice with 5 ml of ice cold 8% TCA and once with 5 ml of absolute ethanol. The filter was then dried in an oven and the radioactivity was measured by counting in a scintillation counter using Ecoscint A© biodegradable scintillation solution for aqueous and non-aqueous samples (National Diagnostics, Manville, NJ, USA).

2.6 Preparation and quantitation of total protein extracts from plant leaves

2.6.1 Preparation of total protein from plant leaves for one-dimensional SDS-PAGE

A small amount (~0.4 g) of leaf tissue were ground under liquid nitrogen in a mortar and to the resulting powder was added 1 ml of the sample loading buffer

(described in section 2.7.1). After thawing, the powder in the sample loading buffer was ground further for about 15 seconds and then transferred to a 1.5 ml microcentrifuge tube. The tube was centrifuged for 5 minutes in an Eppendorf® microcentrifuge at high speed and then the supernatant was transferred to a fresh tube which was stored in aliquots at -80 °C until needed. 20µg of total protein was loaded onto one-dimensional SDS-PAGE gels for analysis.

2.6.2 Preparation of total protein from plant leaves for two-dimensional SDS-PAGE

Total protein was prepared according to Colas des Francs (1985). Leaf tissue was ground to a powder under liquid nitrogen using a mortar containg a small amount of sand. The powder was transferred to a Corex® tube containing 3ml of extraction buffer [50 mM Tris-HCl pH 8.5, 4% SDS, 5% ß-mercaptoethanol, 1 mM PMSF, 25µg/ml leupeptin, 40 mg/ml polyvinylpyrrolidone (average molecular weight 40,000)] per 0.5 of tissue. The contents of the tube was vortexed for 20 seconds and then heated at 100 °C for 3 minutes prior to centrifugation at 10,000 rpm for 20 minutes in an MSE High Speed 18 centrifuge using a 8 x 50 rotor at 4°C. 100 µl of the supernatant was transferred to a 1.5 ml polyethylene microcentrifuge tube to be used for the determination of protein concentration in a Bio-Rad protein microassay. The volume of the remainder of the supernatant was accurately measured and then transferred to a clean Corex® tube and the protein was precipitated by the addition of 10 volumes of cold acetone followed by incubation overnight at -20°C. The protein was recovered by centrifugation at 4 °C for 20 minutes at 10,000 rpm in an MSE High Speed 18 using a 8

x 50 ml rotor. Total protein was visualized by applying 100 µg to a two-dimensional gel followed by staining with silver or Coomassie blue.

2.6.3 Protein concentration estimation using the Bio-Rad Protein Microassay

A stock solution of BSA at 1µg/µl was prepared by reconstitution in the same solution in which the sample proteins had been suspended. Duplicate sets of protein standards were prepared by adding 5, 10, 15, and 20 µl of the BSA stock solution into separate 1.5 ml microcentrifuge tubes and bringing the total volume to 20 µl in each tube using sample solution. Duplicate sets of unknown protein samples were prepared by adding 5, 10, and 15 µl into separate tubes and bringing the volume up to 20 µl with sample solution. 780 µl of water and 200 µl of Bio-Rad dye reagent concentrate were added to each tube which were then mixed gently by inversion. After incubation at room temperature for between 5 to 30 minutes, the BSA standards and the unknown protein samples were transferred to 1 ml polystyrene cuvettes. The OD_{595} of all samples was measured using a spectrophotometer that had been prepared for use by placing a sample blank (20 µl of sample solution, 780 µl of water, and 200 µl of dye reagent concentrate) in a 1 ml polystyrene cuvette into the machine and setting the absorbance to zero at OD_{595}. A plot of the average OD_{595} versus the concentration of each duplicate standard provided a standard curve which was used to determine the concentration of the unknown protein samples.

2.7 Electrophoretic techniques used in the analysis of proteins

2.7.1 One-dimensional SDS-PAGE

Electrophoresis was carried out according to previously described protocols (Laemmli, 1970). A 12% resolving gel was first prepared by combining 16.4 ml of water, 12 ml of buffer I [1.5 M Tris-HCl, 0.4% (w/v) SDS, pH 8.8], 16 ml of a solution of 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide, 3.2 ml of 50% (v/v) glycerol, 16 µl of TEMED, and 180 µl of 10% (w/v) ammonium persulphate. After mixing, the solution was poured into a set of glass plates with 1.5 mm spacers leaving enough room at the top for the stacking gel and sample well former. Immediately after pouring the gel, 1 ml of distilled water was applied to the top to ensure the formation of a level surface. When the gel had polymerised (1 -2 hours), the stacking gel was prepared by combining 9.75 ml of water, 3.75 ml of buffer II [0.5M Tris-HCl, 0.4% (w/v) SDS, pH 6.8], 1.5 ml of a solution of 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide, 8 µl of TEMED, and 150 µl of 10% (w/v) ammonium persulphate. After mixing, the stacking gel solution was poured on top of the resolving gel and the sample well former was set in place. Samples were boiled for 2 minutes and applied in loading buffer [50mM Tris-HCl, 2% (w/v) SDS, 10% (w/v) glycerol, 2% (w/v) ß - mercaptoethanol , 0.1 % (w/v) bromophenol blue, pH 8.0]. One lane typically contained prestained protein molecular weight markers (BRL) or ^{14}C labelled protein molecular weight markers (Amersham International plc). Running buffer [0.025M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS] was placed in the upper and lower reservoirs and electrophoresis was conducted at 65 mA for ~2.5 hours (until the dye front reached the bottom of the gel). Gels were removed from the plates and fixed in 7% (v/v) acetic acid for at least one hour.

2.7.2 Two-dimensional SDS-PAGE

Two-dimensional SDS-PAGE was carried out according to O'Farrel,(1975) with slight modification. Tube gels were prepared for the first dimension (isoelectric focussing) by combining 5.5 g of urea, 1.33 ml of a solution containing 28.3% (w/v) acrylamide and 1.62% (w/v) bis-acrylamide, 2 ml of 10 % (v/v) NP-40, 1.95 ml water, 0.4 ml Ampholine carrier ampholytes pH 5 - 7 (LKB), 0.1 ml Ampholine carrier ampholytes pH 3.5 - 10 (LKB), 10 µl of 10% (w/v) ammonium persulphate, and 5 µl of TEMED. After polymerisation, the tubes containing the gels were placed into the electrophoresis apparatus (LKB) and to the top of each one was applied 25 µl of sample buffer [9.5 M urea, 5% (v/v) ß - mercaptoethanol, 2% NP-40, 1.6% Ampholines (pH 5-7), 0.4% Ampholines (pH 3.5 -10)]. 25 µl of overlay buffer (sample buffer diluted with water two fold) was next loaded followed by the addition of 20 mM NaOH which was loaded until it completely filled the tube. 10 mM H_3PO_4 was placed in the lower reservoir (anode) and 20 mM NaOH was placed in the upper reservoir (cathode) and the gels were pre-run for one hour at 400 Volts. After the pre-run, the tubes were removed from the apparatus and the liquid layered on the top of the gels was carefully removed by aspiration. The tubes were then put back into the apparatus and the samples suspended in sample buffer were loaded. 25 µl of overlay buffer was then applied to the top of each sample and the remaining space in the tubes was completely filled with 20 mM NaOH. Electrophoresis was conducted for 18 hours at 500 Volts. After electrophoresis was complete the gels were removed from the tubes, by applying gentle pressure on one end, placed into 5 ml of equilibration buffer [2.3% (w/v) SDS, 5% (v/v) ß - mercaptoethanol, 10% (v/v) glycerol, 62.5 mM Tris-HCl pH 6.8, 0.1% (w/v) bromophenol blue], and left at room temperature for 1 hour. The tubes were then taken

from the equilibration buffer, wrapped in foil and stored at -80°C until ready for the second dimension.

The second dimension proceeded as described in section 2.7.1 for one-dimensional SDS-PAGE. Instead of forming sample wells, however, 1 ml of water was applied to the top of the freshly poured stacking gel to provide a flat surface on which to lay the tube gel prior to electrophoresis.

2.8 Fluorographic procedures

2.8.1 Fluorography of SDS-PAGE gels containing *in vitro* translation products using PPO

Fluoragraphic methods for detection of labelled proteins were used as described by Laskey and Mills,(1975). After fixing, gels were washed three times in DMSO for 30 minutes at room temperature with gentle agitation. The gels were then incubated with 22% (w/v) PPO dissolved in DMSO for two hours at room temperature with gentle agitation. The PPO was removed and the gels were rinsed for two hours under a water tap. Gel were dried using a gel dryer (Bio-Rad) under vacuum and autoradiographed.

2.8.2 Fluorography of SDS-PAGE gels containing *in vitro* translation products using EN^{3}HANCE™ (NEN-Dupont)

After fixing, gels were incubated with EN^{3}HANCE for 1 hour at room

temperature with gentle agitation. The EN^{3}HANCE solution was removed and the gels were rinsed under a water tap for thirty minutes.

2.9 Autoradiography of radioactive SDS-PAGE or agarose gels and radioactive hybridisation filters

Gels were dried under vacuum and then placed in a film cassette with Fuji X-Ray film type RX. Intensifying screens were used for gels containing samples labelled with ^{32}P. Exposure times depended on activity, but generally, forty eight hours were required for one-dimensional SDS-PAGE gels containing *in vitro* translation products, one week for two-dimensional SDS-PAGE gels containing *in vitro* translation products, twelve hours for agarose gels containing cDNA synthesis products, and twenty four to forty eight hours for differential screening filters.

2.10 Procedures for staining proteins in SDS-PAGE gels

2.10.1 Staining proteins in SDS-PAGE gels with Coomassie Brilliant Blue R25

After fixation, gels were immersed in Coomassie brilliant blue R250 staining solution [50 % ethanol, 45% water, 5% acetic acid, by volume, and 0.2% (w/v) Coomassie brilliant blue R250] for 1 hour. The stain was discarded and gels were submerged in destaining solution (20% ethanol, 75% water, and 5% acetic acid, by volume) for several hours (until background staining disappeared).

2.10.2 Staining of protein in SDS-PAGE gels with silver

Silver staining was done according to Morrissey (1981). The gels were first fixed in plastic food storage boxes for thirty minutes in a solution of 50% methanol, 40% water, and 10% acetic acid, by volume, which was then changed to a solution of 88% water, 5% methanol and 7% acetic acid, by volume, for a further thirty minutes. The gels were next soaked in 10% (v/v) glutaraldehyde for thirty minutes, after which, they were washed in distilled water for two hours. This was done by filling the boxes with water which was changed at least four times during the incubation. After the final water wash was discarded, the gels were soaked in a solution of dithiothreitol at a concentration of 5 mg per liter for thirty minutes. The dithiothreitol solution was then poured off, and without rinsing, the gels were immersed in a solution of 0.1% (w/v) silver nitrate for thirty minutes. The gels were quickly rinsed in distilled water before development in a solution containing 3% (w/v) sodium carbonate and 0.02% (v/v) formaldehyde. When the proteins were clearly visible and the background staining was minimal, staining was stopped by pouring off the sodium carbonate solution and adding 0.1% (v/v) acetic acid. Gels were placed in distilled water for long term storage.

When necessary, gels were destained as follows. Two solutions were made for the destaining and referred to as solutions A and B. Solution A was made by first adding together 14.8 g of NaCl, 14.8 g of Cu (II) SO_4, and 150 mls of water, and mixing until the constituents dissolved. Concentrated NH_4 OH solution was added until a precipitate that forms upon its addition redissolves and the solution takes on a deep blue color. The volume was adjusted to 200 ml with water. Solution B was prepared by

adding 87.7 g of sodium thiosulphate to 200 ml of water and stirring until all was dissolved. Prior to beginning the destaining procedure, solutions A and B were mixed in equal volumes. The gel was submerged in the mixed solution and agitated for 5-10 minutes until stain was removed. The solution was removed and the gel was soaked overnight in 1 l of water.

2.11 Preparation of polyclonal antisera against 'induced' proteins

2.11.1 Preparation of total protein for injection into rabbits

Two separate protein extracts from the leaves of plants exposed to one and three long days were prepared as described in section 2.6.2 for use in two-dimensional electrophoresis, with the exception that, after precipitation, the protein pellet was resuspended in a solution consisting of 6M urea, 1 M ß - mercaptoethanol, and 0.1% (w/v) SDS. 25 µl of the resuspended protein was reserved for protein concentration estimation, and after careful measurement of the volume, the remainder was applied to a Sephadex - G25 (medium) column (Pharmacia) that had been equilibrated with 20 ml of 0.1 M sodium phosphate buffer (prepared by combining equal volumes of 0.1 M disodium hydrogen phosphate and 0.1 M sodium dihydrogen phosphate by adding one to the other until the pH reaches 7.0). Ten 1 ml fractions were taken of protein eluted in sodium phosphate buffer. Fractions four and five, which contained the eluted protein, were pooled and and placed in Amicon centriprep concentrators (Amicon Div., W.R. Grace and Co., Danvers, MA, USA) so that the total volume could be reduced to 0.5 ml.

Two lots of antisera were made: one (referred to as MB-1) against proteins from plants exposed to one long day and the other (referred to as MB-3) against proteins from plants exposed to three long days. 600 µg of total protein, prepared as described in section 2.11.1, contained in 300 µl was mixed with an equal volume of Freund's complete adjuvant (Sigma) and injected into New Zealand White rabbits. The rabbits were given a booster injection of 400 µg of total protein mixed with an equal volume of Freund's incomplete adjuvant (Sigma) after four weeks. After a total of six weeks, 20 ml of blood was drawn from the rabbits, coagulated with heparin (Sigma), and centrifuged in a Beckman Model TJ-6 benchtop centrifuge. The supernatant was retained and stored at -20 °C in 250 µl aliquots.

2.12 Immunoadsorption procedures

2.12.1 Immunoadsorption of antisera (according to Choi *et al.*, 1987) prepared against MB-1

Protein extracts from short day control leaves were prepared by grinding the leaves in a solution of 50 mM Tris-HCl, pH 6.8, 1 mM dithiothreitol, 0.1% (w/v) SDS and used to saturate 2 cm x 6 cm nitrocellulose filter strips (Schleicher and Schuell). 1 ml of MB-1 antiserum was added to 50 ml of blocking buffer [1% fat free powdered milk (Cadbury's Marvel®), 1X PBS (Dulbecco's 10X PBS without calcium or magnesium was made by first combining 2g of KCl, 2g of KH_2PO_4, 80 g of NaCl, and 14.24 g of $Na_2HPO_4 \cdot 2H_2O$, and then bringing the volume to one liter) , 0.2% (v/v)

NP-40] which was then incubated 3 times for four hours with four of the control protein saturated nitrocellulose filter strips at 4 °C. The immunoadsorbed antiserum was ready for direct use in the western blotting procedure described in section 2.13.

2.12.2 Modified immunoadsorption of antisera prepared against 'Induced' proteins

0.4 g of leaf tissue from plants grown in short days was used in a total protein preparation as described above except for that the acetone precipitate was resuspended in 250 µl of a solution consisting of 5 mM, Tris-HCl, pH 8.0, 0.1% (w/v) SDS and 10 mM NaCl and transferred to a 1.5 ml microcentrifuge tube. 250µl of a solution consisting of 1% (v/v) Triton X - 100 and 1% (w/v) deoxycholic acid was added to the resuspended protein. 500 µl of antiserum was added to the mixure which was then incubated at 4 °C for 6 hours. 100 µl of protein A - Sepharose [2.5 mg/ml suspended in a solution consisting of 0.5 mM Tris - HCl, pH 8.0, 0.01% (w/v) SDS, 1 mM NaCl, 0.1 % (v/v) Triton X - 100, and 0.1 % (w/v) deoxycholic acid] was added to the mixture which was then incubated overnight at 4 °C. The tube was centrifuged for 1 minute in an Eppendorf® microcentrifuge at high speed and the supernatant was stored at 4 °C until ready for use in a western blot.

2.13 Western blotting procedures

2.13.1 Transfer of protein from SDS-PAGE gels to nitrocellulose membranes

Proteins separated on polyacrylamide gels were transferred to nitrocellulose membranes (Schleicher and Schuell) by using a commercial electroblotting unit (LKB). Gels that were to be blotted were fixed for one hour in protein transfer buffer [0.025 M Tris-HCl, pH 8.3, 0.15 M glycine, 20 % (v/v) methanol] and then positioned in the electroblotting apparatus. The current was set to 1.5 A and transfer was complete after two hours.

2.13.2 Detection of antiserum reactivity against protein bound to nitrocellulose membranes

The nitrocellulose membranes, prepared as described in section 2.13.1, were placed into a plastic food storage box containing 100 ml of 5% (w/v) fat free powdered milk (Cadburry's Marvel®) in 1X PBS (Dulbecco's 10X PBS without calcium or magnesium was made by first combining 2g of KCl, 2g of KH_2PO_4, 80 g of NaCl, and 14.24 g of $Na_2HPO_4 \cdot 2H_2O$, and then bringing the volume to one liter) for 2 hours at room temperature with gentle agitation. The liquid was drained and replaced with a solution consisting of 1% (w/v) fat free powdered milk, 1X PBS, 0.2% (v/v) NP-40 and a 1 to 200 dilution of the polyclonal antiserum. The filter was incubated with the antiserum overnight at room temperature. The antiserum was removed and the filter was washed three times for ten minutes in a solution consisting of 1X PBS and 0.2% (v/v) NP-40. After the washes, the filter was next incubated for two hours at room temperature with peroxidase linked donkey anti - rabbit antiserum (provided by the Scottish Antibody Production Unit) which was diluted one to four hundred in 100 ml of

a solution consisting of 1% (w/v) fat free milk, 1X PBS and 0.2% (v/v) NP-40. The antiserum was discarded and the filter was washed twice, as described above, and once for 10 minutes in 1X PBS. The filter was developed by incubation in a solution containing 40 ml of PBS, 10 mg of orthodianisidine (Sigma), and 20 µl of hydrogen peroxide, until the proteins were clearly visible and the background remained minimal. The filters were rinsed well in distilled water and dried on filter paper.

2.14 cDNA synthesis and cloning procedures

2.14.1 Preparation of siliconized 1.5 ml microcentrifuge tubes

Siliconized microcentrifuge tubes were used throughout cDNA synthesis and purification steps. Tubes were prepared under a fume hood by briefly immersing them in dichlorodimethylsilane and then allowing them to drain upside down on absorbant tissues. After the tubes were dry, they were rinsed in several changes of distilled water and then autoclaved.

2.14.2 cDNA Synthesis and product analysis

2.14.2.1 1st and 2nd strand synthesis reactions

cDNA synthesis proceeded according to protocols included with the cDNA synthesis system obtained from Amersham International plc. The first strand reaction contained the following solutions (provided with the kit, excluding radioactive nucleotide and mRNA) added together (in order) in a siliconized 1.5 ml microcentrifuge tube : 4.0 µl

5X first strand buffer (information regarding constituents was not provided) , 1.0 µl sodium pyrophosphate (information on concentration was not provided), 1.0 µl (20 units) human placenta ribonuclease inhibitor, 2.0 µl of nucleotide pool (10 mM dATP, 10 mM dGTP, 10 mM TTP, 5 mM dCTP), 1.0 µl (1.6 µg) oligo - dT synthesis primer, 5.0 µl [α -^{32}P] - dCTP (1µCi/µl), 5.0 µl poly(A)$^+$ mRNA (1µg, prepared according to the protocol above) and 1.0 µl (20 units) RAV-reverse transcriptase. The reaction mixture was placed in a water bath at 42 °C for 90 minutes. 1 µl of the reaction was removed for product analysis and to the remainder was added 37.5 µl of second strand buffer (information regarding constituents was not provided), 5.0 µl of [α -^{32}P] - dCTP (10 µCi/µl), 1.0 µl (0.8 units) of ribonuclease H, 6.5 µl (22.75 units) of *E. coli* DNA polymerase I, and 31.0 µl of water. The second strand reaction was incubated at 12 °C for 1 hour and at 22 °C for 1 hour followed by inactivation at 70 °C for 10 minutes. The tube was centrifuged for 5 seconds in an Eppendorf® microcentrifuge at high speed and then placed on ice. 2 units of T4 DNA polymerase was added to the cDNA which was then incubated at 37 °C for 10 minutes. The reaction was stopped by the addition of 4 µl of 0.25 M Na_2 EDTA pH 8. 1µl of the reaction was removed for analysis.

2.14.2.2 Purification of cDNA products

2.14.2.2.1 Phenol/Chloroform extraction of cDNA products

An equal volume of phenol/chloroform solution [*Ultra* PURE™ phenol (redistilled nucleic acid grade phenol from BRL) was saturated with TE buffer (10 mM Tris-HCl, 1 mM Na_2 EDTA, pH 8.0) until the phenol and aqueous phases were equivalent in

volume. 8-hydroxyquinoline was added to a concentration of 0.1 % and then a volume of chloroform was added that was equivalent to the phenol phase. The solution was mixed thoroughly and the phases were left to separate] was added to a microcentrifuge tube containing the cDNA products and mixed by vortexing and centrifuged for one minute. The aqueous phase was removed and placed into a fresh microcentrifuge tube. An equal volume of phenol/chloroform solution was added to the tube which was again mixed and centrifuged. The aqueous phase was removed and placed into a fresh microcentrifuge tube into which was added an equal volume of chloroform. The tube was mixed and centrifuged and the resulting aqueous phase was removed and placed in a fresh tube for ethanol precipitation as described below.

2.14.2.2.2 Ethanol precipitation of double stranded or single stranded cDNA with removal of unincorporated nucleotides

The double stranded cDNA was precipitated by the addition of 20 µg of glycogen (Boehringer/Mannheim), an equal volume of 4 M ammonium acetate and twice the combined volume of ethanol and storage on dry ice for 20 minutes. The tube was removed from dry ice and allowed to warm to room temperature before centrifugation for 20 minutes in an Eppendorf® microcentrifuge at high speed. The pellet was resuspended in 100 µl of 2 M ammonium acetate followed by the addition of 200 µl of cold ethanol (-20 °C) and storage on dry ice for 20 minutes. The tube was again allowed to warm to room temperature with gentle shaking before centrifugation as before for 20 minutes. The pellet was washed with 80% (v/v) ethanol, centrifuged again for 20 minutes and dried under vacuum.

2.14.2.3 Calculation of synthesis efficiency

2.14.2.3.1 TCA precipitation of cDNA

A 100% stock of TCA was prepared by dissolving 500 g in 227 ml of water. 1 µl was taken from each first and second strand reaction using an Eppendorf® Comforpipette 4700 and diluted to 20 µl with water. 2 µl of each diluted sample was added to 100 µl of salmon sperm DNA (Sigma) at 500 µg/ml in a six inch test tube and the mixture was precipitated by adding 5 ml of ice cold 10% TCA. The test tube was chilled on wet ice for 15 minutes and then the precipitate was collected by filtration through a Whatman® GF/C glass fiber filter disc. The filter, representing the total cpm incorporated, was washed six times with 5 ml of cold 10% TCA, washed once with 95% ethanol, and then counted in a scintillation counter (LKB) using Ecoscint A© scintillation solution (National Diagnostics, Manville, NJ, USA). Total cpm in the reaction were also measured for 2 ul of diluted sample spotted directly onto the filter.

2.14.2.3.2 Calculation of percentage incorporation

Percentage incorporation in the first strand reaction was calculated using the following equation

percentage incorporation = cpm incorporated/total cpm x 100%

For calculation of percentage incorporation for the second strand, the amount of radioactivity incorporated in the first strand was subtracted from the cpm values obtained

for the incorporated and total radioactivity in the second strand reaction. An equation used for this calculation was as follows

$$\text{percentage in corporation (second strand)} = (A - C/5.5) / (B - C/5.5) \times 100\%$$

where A equals the total cpm incorporated from the second strand reaction, B equals the total cpm in the second strand reaction, C equals the total cpm incorporated from the second strand. In this equation, the counts incorporated for the first strand reaction is divided by 5.5 because the volume of the first strand reaction (19 µl minus the 1 µl removed for analysis) is 1/5.5 of the volume of the 'stopped' second strand reaction (124 µl).

2.14.2.3.3 Calculation of the yields of cDNA synthesized in the 1st and 2nd strand reactions

The amount of cDNA synthesized in both 1st and 2nd strand reactions was calculated by multiplying the percentage incorporation by 140 ng. The total amount of double stranded cDNA produced is twice the amount of cDNA synthesized in the second strand reaction. The yield in terms of the percentage of mRNA transcribed in the 1st strand reaction was calculated with the following equation

$$\text{percentage of mRNA transcribed} = \frac{\text{(amount first strand cDNA synthesized)}}{\text{(amount of input mRNA)}} \times 100\%.$$

The yield in terms of the percentage second strand cDNA transcribed from the first strand was calculated using the following equation

percentage of second strand cDNA transcribed from the first strand cDNA = (amount of second strand cDNA) / (amount of first strand cDNA) x 100%.

The yields for percentage mRNA transcribed into first strand cDNA was typically 15 - 20%. The yields of first strand cDNA transcribed into second strand cDNA was typically greater than 90%.

2.14.2.4 Physical analysis of cDNA sysnthesis products

2.14.2.4.1 Preparation of Hind III molecular weight markers from phage λ DNA

1 µg of λ DNA was added to a 1.5 ml microcentrifuge tube containing 2 µl of 10X restriction buffer (React® 2 from BRL), 20 units of Hind III (BRL), and water to a total volume of 20 µl. The tube was placed in a water bath at 37 °C for one hour.

2.14.2.4.2 End-labelling λ Hind III molecular weight markers with [α ^{32}P] - dATP

2 µCi of [α - ^{32}P] - dATP and 1 unit of *E. coli* DNA polymerase I, Klenow fragment, was added to the 20 µl of restricted DNA prepared by the proceedure described above. The tube was incubated for ten minutes at room temperature. Unincorporated nucteotides were removed by ethanol precipitation in 2 M ammonium

acetate as described in section 2.14.2.2.2.

2.14.2.4.3 Preparation of labelled cDNA products for alkaline agarose gel electrophoresis

A sample of cDNA contained in a microcentrifuge tube and representing between 10,000 to 30,000 cpm was subjected to alkaline hydrolysis by first adding 20 µl of salmon sperm DNA (Sigma) at 100 µg/ml and one third of the combined volume of 1 M NaOH. The mixture was incubated at 46 °C for 30 minutes . After heating, the tube was briefly centrifuged and then a volume equivalent to the volume added of 1 M NaOH was sequentially added of 1 M HCl and 1 M Tris-HCl (pH 8.0). The mixture was then phenol extracted, ethanol precipitated, and resuspended in alkaline agarose gel loading buffer.

2.14.2.4.4 Alkaline agarose gel electrophoresis of cDNA synthesis products

A 1.5 % alkaline agarose gel was prepared by combining in a 250 ml flask 1.5 g of agarose, 10 ml of gel preparation buffer (500 mM NaCl, 10 mM Na_2 EDTA), and 88.5 ml of water followed by heating to boiling. The flask was allowed to cool to 55 °C and the contents were poured into a minigel former which was left at 4 °C until the agarose hardened. Alkaline gel electrophoresis buffer (30 mM NaOH, 1 mM Na_2 EDTA) was added until it covered the gel to a depth of 5 mm and the gel was left to equilibrate for 30 minutes. Labelled cDNA samples and molecular weight markers were prepared for

electrophoresis by resuspension in alkaline loading buffer (50 mM NaOH, 1 mM Na_2 EDTA, 2.5% (w/v) Ficoll 400, 0.025% bromophenol blue) after ethanol precipitation. Samples were loaded onto the gel which was then run at 100 mA for four to five hours or until the dye front had migrated to one third the length of the gel. After electrophoresis was complete, the gel was dried on a gel dryer (Bio-Rad) under vacuum and autoradiographed.

2.14.3 Cloning of double stranded cDNA into λgt10

A cDNA cloning system, including all necessary solutions, enzymes, columns, λgt10 vector arms, *E. coli* strains, and experimental protocols was purchased from Amersham International plc for use in cloning double stranded cDNA. The cDNA was first subjected to a methylation reaction using EcoRI methylase. This was done by adding 1μg of purified (phenol extracted and ethanol precipitated) double stranded cDNA (in a volume of 13 μl) to a 1.5 ml microcentrifuge tube containing 4 μl of EcoRI methylase buffer (information on buffer constituents was not provided), 2 μl of 1X s-adenosyl methionine solution (information on concentration was not provided), and 20 units (1μl) of EcoRI methylase . The tube was mixed gently and incubated at 37 °C for one hour followed by inactivation at 70 °C for 10 minutes. Linkers were ligated to the cDNA by adding the following (in order) : 3 μl of ligase buffer (information on constituents was not provided), 2 μl EcoRI linkers (information on concentration was not provided), 3μl of water, and 2 μl (5 units) of T4 DNA ligase. The tube was mixed gently and placed in a water bath at 15 °C overnight. The reaction was stopped by incubation at 70 °C for 10 minutes. The linkered DNA was then subjected to EcoRI

digestion by adding 10 µl of EcoRI restriction buffer (information on the constituents was not provided), 58.3 µl of water, and 1.7 µl (100 units) of EcoRI and then incubating at 37 °C for a minimum of five hours. The reaction was inactivated by heating at 70 °C for 10 minutes.

The linkered cDNA was then separated from the excess linkers by column chromatography. The columns provided in the kit were prepared for use by first equilibrating with 2 x 3 ml of STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM Na_2 EDTA). All of the 100 µl of linkered cDNA prepared above was loaded on to the column bed. After the 100 µl had entered the column, 100 µl of STE buffer was added to the top of the column, and the eluate was collected in a 1.5 ml microcentrifuge tube labelled as fraction one. A further 200 µl of STE was loaded to the column and again the eluate was collected into a fresh microcentruge tube which was labeled as fraction two. This was repeated until a total of ten fractions was collected. Fractions three and four which contained the cDNA peak (identified by radioactive monitoring) were pooled and ethanol precipitated by the addition of one tenth the volume of 3 M sodium actetate and 2.5 volumes of absolute ethanol followed by storage at -20 °C for two hours. The precipitate was collected by centrifugation in a microcentrifuge for 30 minutes, dried under vacuum, and resuspended in water. The purified linkered cDNA was then ligated to λgt10 vector arms by combining 50 - 150 ng of cDNA with 2 µl of arms (1 µg), 1 µl of ligation buffer (information on buffer constituents was not provided), a volume of water for a total volume of 10 µl, and 1 µl of T4 DNA ligase (2.5 units), and incubating at 15 °C overnight. The ligated cDNA and vector arms were preciptated by the addition of one tenth the volume of 3M sodium acetate and 2.5 volumes of absolute ethanol, stored on dry ice for 15 minutes, and centrifuged for 15

minutes. The pellet was dried under vacuum and resuspended in 2.5 µl of TE (10 mM Tris -HCl, pH 7.5, 1 mM Na_2 EDTA). Ligated vector arms and cDNA were packaged using packaging extracts supplied in the Amersham kit. The volume of the packaged phage was brought to 500 µl with SM buffer (prepared by combining 5.8 g of NaCl, 2 g of $MgSO_4 \cdot 7H_2O$, 5 ml of 1 M Tris-HCl, pH 7.5, and 5 ml of 2% Gelatin and adjusting the volume to one liter with distilled water) and stored at 4 °C.

2.14.4 Procedures used in the analysis of the cDNA library

2.14.4.1 Preparation of phage λgt10 plating cells

A loopful of glycerol stock of the *E. coli* strain required [either L87 (wild type) or NM514 (hfl^+)] was streaked onto a L-agar plate (prepared by adding together 10 g of Bacto-tryptone™, 5 g of Bacto-yeast extract™, 10 g of sodium chloride, and 15 g Bacto-agar™ and adjusting the volume to one liter with water) and incubated overnight at 37 °C. A single colony was picked and used to inoculate 50 ml of sterile L-broth (prepared by combining 10 g Bacto-tryptone™, 5 g Bacto-yeast extract™, and 10 g sodium chloride and adjusting the volume to one liter with water) + 0.4 % (w/v) maltose in a flask which was placed in a shaking incubator overnight at 37 °C. 1 ml of the overnight culture was used to inoculate 50ml of sterile L - broth + 0.4% (w/v) maltose in a flask which was then placed on a shaking incubator at 37 °C until the cells reached an OD_{600} of 0.5. The culture was poured into a sterile 50 ml conical centrifuge tube and centrifuged at 3000 rpm in a Beckman model TJ-6 centrifuge for 10 minutes at 4 °C using a swing out bucket rotor. The cell pellet was resuspended in 15 ml of ice cold 10

mM magnesium sulphate and stored at 4 °C until ready for use.

2.14.4.2 Infection of plating cells and growth of plaques

100 µl of diluted phage in SM buffer [prepared by combining 5.8 g NaCl, 2 g of $MgSO_4 \cdot 7H_2O$, 5 ml of 1 M Tris-HCl pH7.5, and 5 ml of 2% (w/v) gelatin and adjusting the volume to one liter with distilled water] were added to 100 µl of plating cells in a six inch glass test tube which was then placed in a water bath at 37 °C for 15 minutes. 4 ml of liquid top agar (prepared by combining 1 g of Bacto-tryptone™, 0.5 g of Bacto-yeast extract™, 0.5 g NaCl, 0.25g $MgSO_4 \cdot 7H_2O$, and 1 g of Bacto-agar™, and adjusting the volume to 100 ml) at 45 °C were added to the tube and the total contents were poured onto a L-agar plate (90 mm in diameter). The plate was turned upside down and incubated at 37°C until plaques formed (approximately seven hours). Volumes were adjusted for larger plates such that 150 µl of cells and 6 ml of top agar were necessary when using 10 cm^2 plates.

2.14.4.3 Titration of λgt10 recombinants

Dilutions of the final packaged phage mixture were made to be used for infection of plating cells. 30 µl of final packaged phage mixture was added to 270 µl of SM buffer and mixed. This first dilution was referred to as a 10^2 dilution which indicated that 1/100 of 1 ml of the initial phage stock would be plated out when 100 µl of the dilution was used to infect plating cells. 30 µl of the 10^2 dilution was removed and added to 270 µl of SM and this was repeated until a dilution of 10^6 was achieved. 100 µl of the 10^4,

10^5 , and 10^6 dilutions were used to infect plating cells of *E. coli* strain L87 and NM514.

Plaques on plates containing between 20 and 500 plaques were counted and the phage titre per ml of packaged phage stock was calculated by multiplying the number of plaques by the dilution number. Mean of the titration results is described in section 5.2.10.2.

2.14.4.4 Small scale liquid culture of phage particles and purification of phage DNA

A single plaque was cored out from an L-agarose plate (same as an L-agar plate except for the substitution of agarose for agar) using a Pasteur pipette and placed into 0.5 ml of NM514 (hfl+ strain provided with the Amersham kit) plating cells in a 50 ml conical centrifuge tube. The cells were left for 15 minutes at room temperature and then 5 ml of L - broth containing 5 mM $CaCl_2$ was added. The cells were incubated for 4 hours at 37 °C in a shaking incubator after which a few drops of chloroform were added and shaking was allowed to continue for a further 5 minutes. The cells were pelleted by centrifugation in a Beckman model TJ-6 bench top centrifuge for 10 minutes at 3000 rpm at 4° Cand the supernatant was removed and placed into a fresh tube. 4 ml of 20% polyethylene glycol in 2 M NaCl was added to the supernatant and the mixture was placed on ice for one hour. The tube was centrifuged again at 3000 rpm for 15 minutes at 4° C. The supernatant was carefully removed by aspiration and the pellet was

resuspended in 500 µl of TE. 10 µl of 10% (w/v) SDS and 10 µl of 0.5 M Na_2 EDTA was added to the supernatant which was subsequently heated to 70 °C for 15 minutes. The mixture was extracted (in succession) once with an equal volume of phenol, once with an equal volume of a one to one mixture of phenol and chloroform, and once with an equal volume of chloroform only. The phage DNA was precipitated by adding an equal volume of isopropanol and incubating at room temperature for 15 minutes. The DNA was pelleted by centrifugation for 15 minutes in a microcentrifuge, washed in 70 % (v/v) ethanol, centrifuged again for 15 minutes, and then dried under vacuum. The pellet was resuspended in 30 µl of sterile water.

2.14.4.5 Double digestion of phage DNA with Hind III and Bgl II restriction enzymes

10 µl of the small scale phage purification described above was added to a microcentrifuge tube containing 1.5 µl of 10 X restriction buffer (React® 2 from BRL), 2.5 µl of water, and 1.0 µl (10 units) Eco RI restriction enzyme (BRL). The tube was place in a water bath at 37 °C for two hours and then the reaction was inactivated by heating to 70 °C for ten minutes. 1 µl (10 units) of Bgl II restriction enzyme (BRL) was added to the tube and it was incubated again at 37 °C for two hours. The reaction was again inactivated at 70 °C for ten minutes.

2.14.4.6 Agarose gel electrophoresis using the Tris-borate buffering system

Gels were prepared by combining, in a 250 ml flask, 1 g of agarose, 10 ml of 10X

Tris-borate buffer (0.89 M Tris-borate, 0.89 boric acid, 0.02 M Na_2EDTA, pH 8.0), and 89 ml of water, and heating to boiling. The contents of the flask were allowed to cool to 55 °C and then ethidium bromide was added to a concentration of 0.5 µg/ml. The gel was poured into a minigel former and allowed to set at 4 °C. The gel was immersed in electrophoresis buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M Na_2EDTA) and DNA samples mixed with one tenth the volume of loading buffer [125mM Na_2EDTA, 0.1% (w/v) SDS, 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue] (find this out) were applied to gel slots. Electrophoresis was conducted at 50 mA until the dye front had migrated to two thirds the length of the gel. DNA was visualized by placement on top of a UV transilluminator (UVP, Inc., San Gabriel, GA, USA). Photographs were taken using a Polaroid CU-5 Land camera.

2.15 Procedures for the differential screening of cDNA libraries

2.15.1 Preparation of nylon membranes for differential hybridization

Hybond™ N (Amersham International plc) membranes were cut into 9 cm^2 squares for taking plaque lifts. A plaque lift was done by placing a nylon filter on to the top agarose of a 10 cm^2 L-agarose plate. The first filter was left in contact with the plaques for 45 seconds and 30 seconds was added with each subsequent filter. The filters were placed (plaque side up) onto Whatman 3MM filter paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 minutes. The filters were then placed on Whatman 3MM filter paper soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 0.001 M Na_2 EDTA) for 3 minutes and then transferred to fresh filter paper soaked in

the same solution for another 3 minutes. The filters were then washed in 2X SSC (1X SSC is 0.15 M NaCl / 0.015 M sodium citrate pH 7), left to air dry, and baked in an oven at 80 °C for two hours. Filters were immersed in 1 % (v/v) Triton X - 100 and then soaked in 2X SSC for 15 minutes immediately prior to prehybridization.

2.15.2 Preparation of cDNA probes for differential screening

cDNA probes were synthesised using the first strand synthesis protocol from the Amersham cDNA synthesis kit with the exception that the reaction contained 100 - 150 μCi of [α -^{32}P] - dCTP and excluded any cold dCTP (the nucleotide pool contained 10 mM dATP, 10 mM dGTP, and 10 mM TTP, only). After incubation for 60 minutes, 1 μl of cold 10 mM dCTP was added and the reaction was incubated for a further 30 minutes. An equal volume of a solution containing 0.6 M NaOH and 20 mM Na_2 EDTA was added to the reaction which was then incubated at 60 °C for 30 minutes. The cDNA products were purified by ethanol precipitation and phenol/chloroform extraction as described in section 2.14.2.2.

2.15.3 Hybridization procedure for differential screening

All solutions used in the differential screening were of the highest purity obtainable. Prehybridization and hybridization solutions were filtered through a Millipore™ 0.45μm filter prior to use.

Membranes were prehybridized in plastic food storage boxes containing 50 ml of

hybridization solution {6X SSC, 10X Denhardt's solution [0.2% (w/v) BSA, 0.2% (w/v) Ficoll™-400 (Pharmacia) and 0.2% polyvinylpyrollidone (average molecular weight 40,000)], 100 µg/ml sonicated denatured salmon sperm DNA (Sigma), 15 µg/ml polyadenylic acid (Pharmacia), 1% (w/v) SDS (electrophoresis grade from BioRad) , and 0.005% (w/v) sodium pyrophosphate} overnight at 68 °C. Prehybridization fluid was discarded and replaced with 50 ml of hybridization solution containing 0.5 - 1 x 10^8 cpm of cDNA probe (prepared as described in section 2.15.2) and the membranes were hybridized for 36 - 48 hours at 68 °C. Membranes were washed for 30 minutes in 2X SSC at room temperature; for 30 minutes in 2X SSC, 1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate at 68 °C; for 30 minutes in 0.1X SSC, 1% (w/v) SDS, 0.1 % (w/v) sodium pyrophosphate at 68 °C. Membranes were then placed in plastic bags and autoradiographed overnight.

Chapter 3 - Studies on the Floral Induction and Development of *Nicotiana plumbaginifolia*

3.1 Introduction

Nicotiana plumbaginifolia was the first species studied because of its extreme suitability for combining molecular and genetic approaches as discussed in section 1.9.1. Consideration required for this initial selection took into account other possiblities such as *Nicotiana tobacum*, *Nicotiana sylvestris*, *Petunia hybrida*, *Hyoscyamus muticus*, and *Arabidopsis thaliana* . These species were deemed undesirable because of a variety of problems they would present when employing either molecular or genetic techniques. For example, regeneration of fertile plants from tissue culture, which would be necessary in some genetic techniques, is difficult with *Nicotiana sylvestris*, *Petunia hybrida*, and *Hyoscyamus muticus* . Regeneration is not a problem when working with *Nicotiana tobacum* or *Arabidopsis thaliana*, but the former is an allotetraploid, which would make genetic approaches confusing, and the latter is such a small size that total RNA extractions would be tremendously tedious. Also, efficient transformation of *Arabidopsis* with *Agrobacterium tumefaciens* had not yet been reported at the time when this project was initiated.

Nicotiana plumbaginifolia appeared to be the best choice overall with the only drawback being the lack of absolute photoperiodic behaviour which did not represent an important concern at the beginning of the study. If a change in gene expression is associated with floral induction in this species, then initially the approach would be to attempt to monitor gene expression in the leaves over a time course prior to flowering so

that any changes noted might be correlated with a particular stage of development. It is impossible to predict at what time point changes in gene expression specifically involved with floral induction may occur, and it is possible that there might be genes expressed in the leaves during the course of development which control separate aspects of the flowering process. For example, some genes may be required for synthesis of a transmissible floral inducing stimulus which promotes the initiation of the transition at the apex from the vegetative state to the reproductive state, and other genes could be involved with the elongation of the stem (shooting) which accompanies the production of flowers in rosette plants.

Section 3.2.2 presents results acquired in the attempt to monitor gene expression during development of *Nicotiana plumbaginifolia* at the mRNA level using the *in vitro* translation technique described in section 1.8.1.1. Since it was hoped that any changes in gene expression might be associated with a particular time point during the growth of the plants, measurements of plant sizes and individual leaf sizes as well as microscopic examination of apical tissue during the course of development until shooting were recorded, and this information is presented in section 3.2.1.

3.2 Results

3.2.1 Characterization of plant development

For each experiment, when eighty plants were grown in a controlled environment which included a 8 hour photoperiod as described in section 2.3.1, development in the population appeared to be quite uniform. Plants grew in a rosette form producing more leaves with time until shooting began approximately 45 days after the seeds were sown. The appearance of the plants at various time points (beginning at 27 days after sowing up until shooting) during one typical experiment is depicted photographically in figure 3.1. Figure 3.2 shows, by graphical representation, the size of the plants during the same experiment at specific time points after sowing by giving the total areas of the individual leaves present. The growth of the individual leaves is also depicted graphically in figure 3.3. It can be seen that growth rates for the various leaves can be quite different and appear to increase with increasing ontogeny. For example, the growth rate of leaf #6 can be seen as much slower than the growth rate of leaf #11. Since the data presented in figures 3.2 and 3.3 were obtained from measuring the area of all the leaves from single plants randomly selected from the population at the times indicated during one experiment, caution should be exercised in interpreting the observations considering the likely variaton to be found between the plants themselves and between experiments .

The beginning of shooting could be detected by visual examination of the apex as

Figure 3.1 Photographic representation of whole plant growth at time points prior to shooting in *Nicotiana plumbaginifolia*

Photographs were taken of plants randomly selected from a population of *Nicotiana plumbaginifolia* plants grown as described in section 2.3.1 at time points in days after sowing including: (A)21, (B) 25, (C) 27, (D) 29, (E) 32, (F) 34, (G) 37, (H) 42, (I) 46, (J) 51. The day of shooting during this particular experiment was 45 days after sowing.

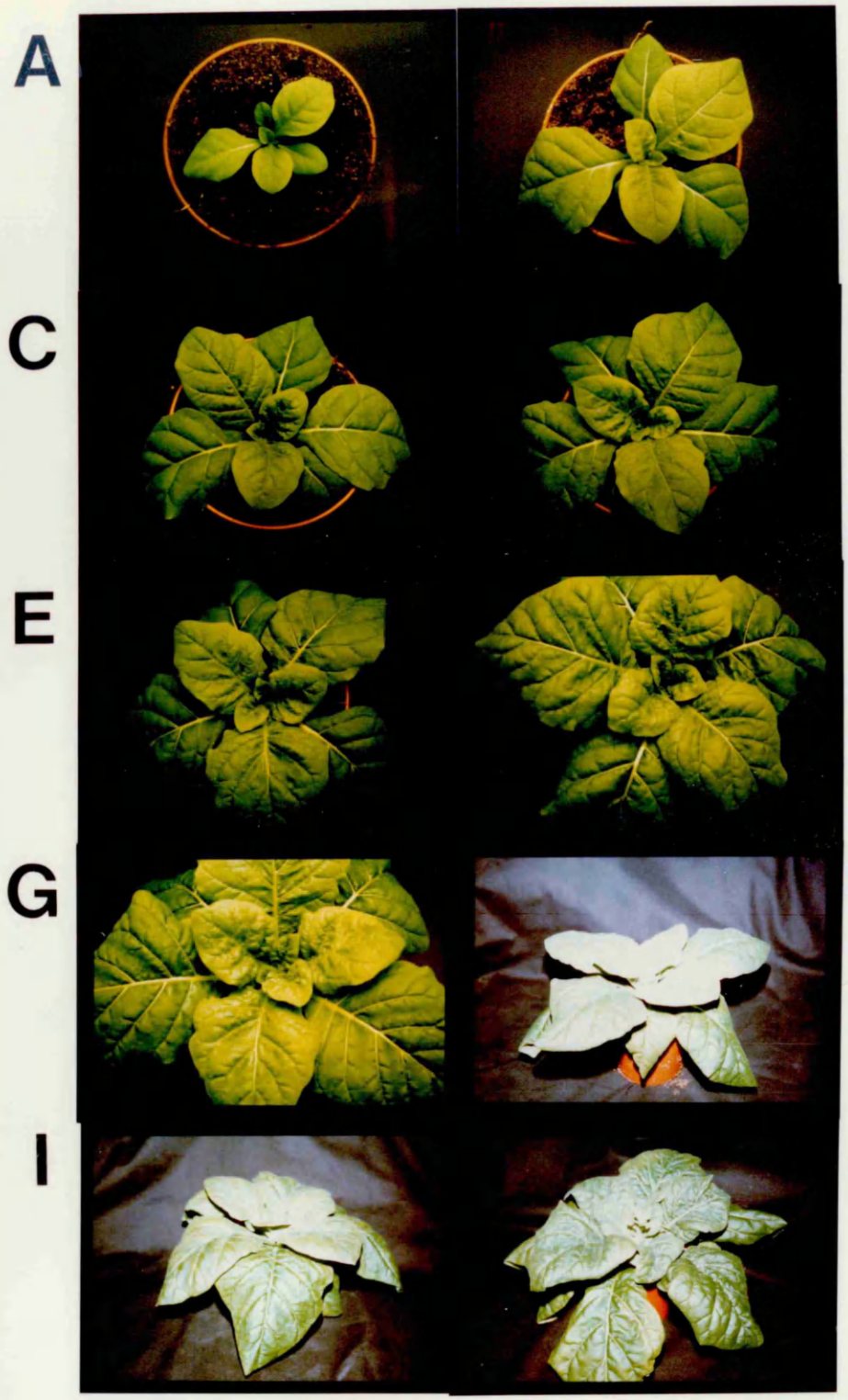
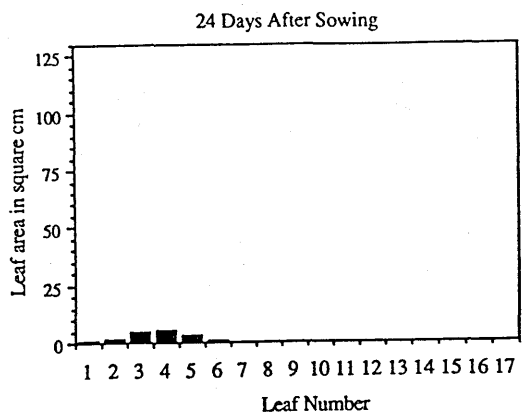
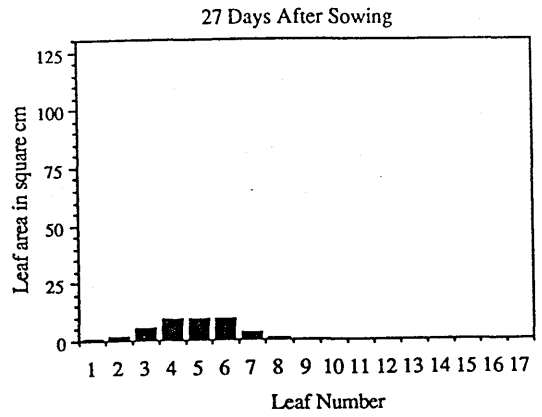
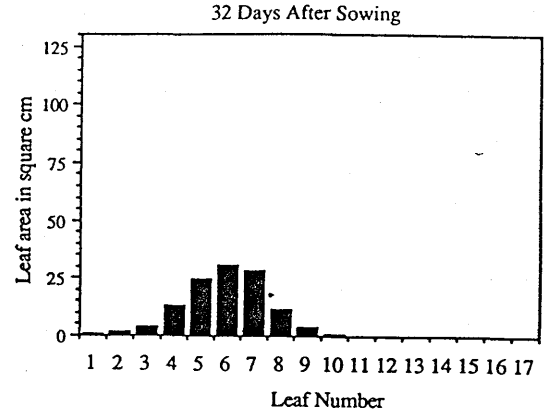

A
B
C
D
E
F
G
H
I
J

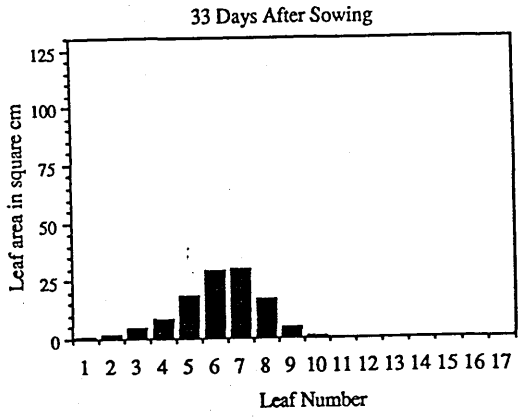
Figure 3.2 Developmental characterization of whole plant growth prior to shooting in *Nicotiana plumbaginifolia*

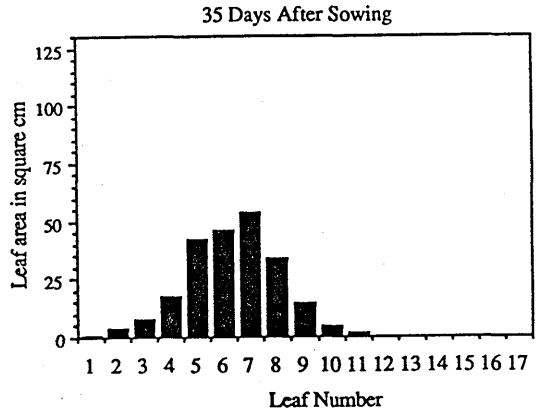
Leaves were individually harvested from randomly selected *Nicotiana plumbaginfolia* plants at time points from 24 days after sowing to 45 days after sowing at which time the plants were shooting (section 2.3.1). The area in square cm of each leaf harvested was measured as described in section 2.3.6. The figure shows a bar graph representation for plant size at each time point by including areas in square cm for all of the leaves present on the plant at the given time.

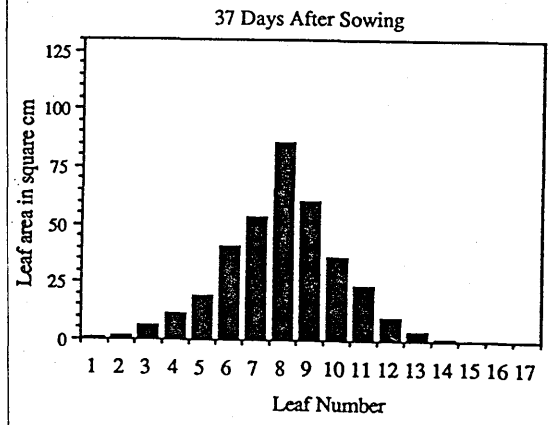

24 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number

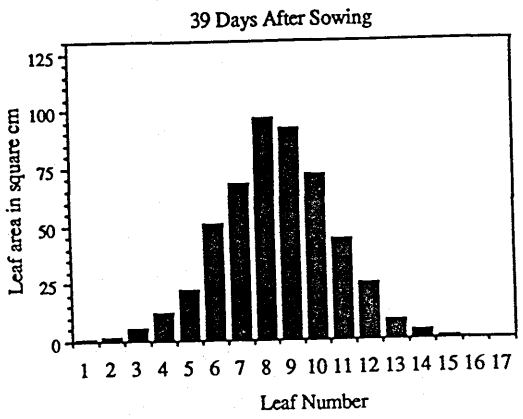

27 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number

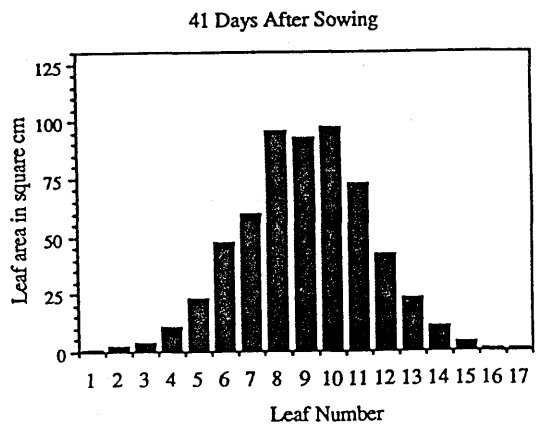

32 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number

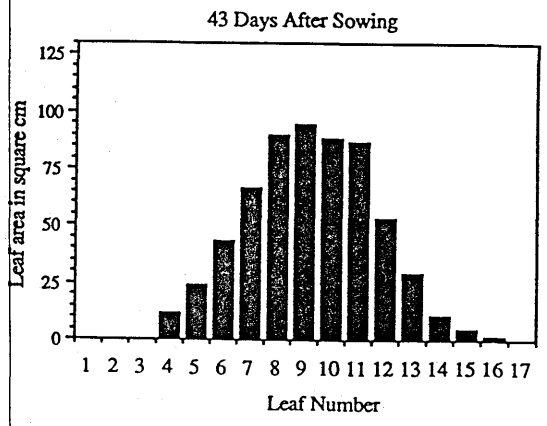

33 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number

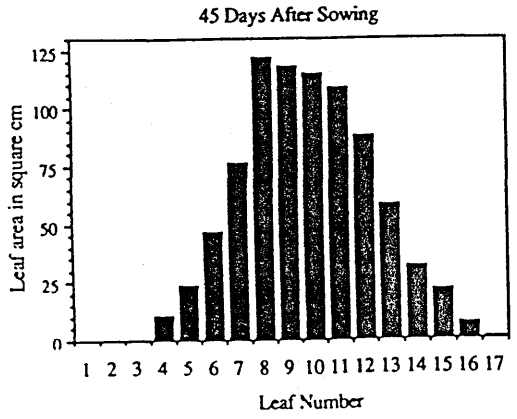

35 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number


37 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number


39 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number


41 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number


43 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number


45 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number

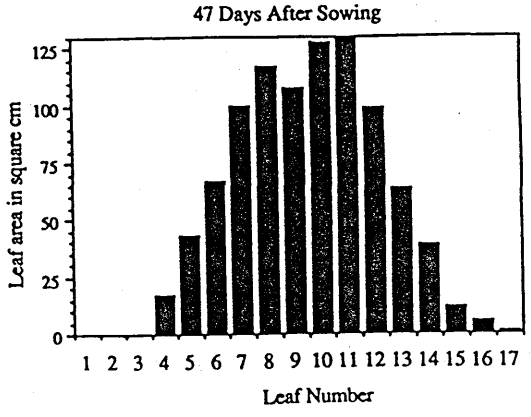
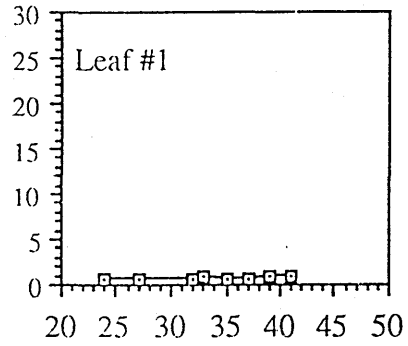
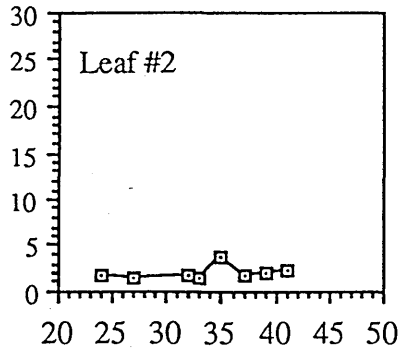

47 Days After Sowing
Leaf area in square cm
0 25 50 75 100 125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leaf Number

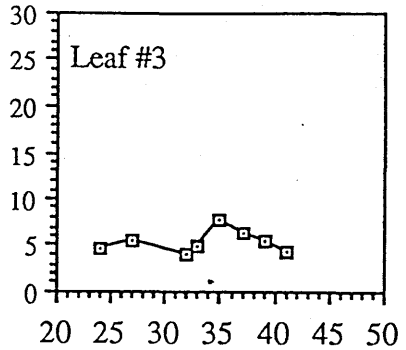
Figure 3.3 Developmental characterization of leaf growth prior to shooting in *Nicotiana plumbaginifolia*

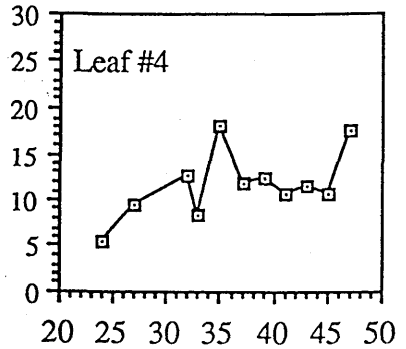
Leaves were individually harvested from randomly selected *Nicotiana plumbaginfolia* plants at time points from 24 days after sowing to 45 days after sowing at which time the plants were shooting (section 2.3.1). The area in square cm of each leaf harvested was measured as described in section 2.3.6. The figure depicts graphical analyses of growth over time prior to shooting of individual *Nicotiana plumbaginifolia* leaves numbered 1 (oldest) to 16 (youngest). Plots indicate the area of the leaf expressed in square cm on the **y-axis** and days after sowing the seeds on the **x-axis**. Leaf areas for older leaves at later time points are not noted because of senescence and leaf areas for younger leaves are noted from the time point when they appear.

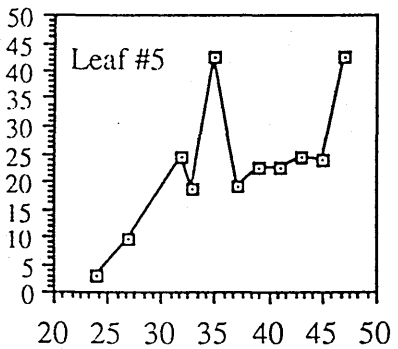
Leaf area in square cm

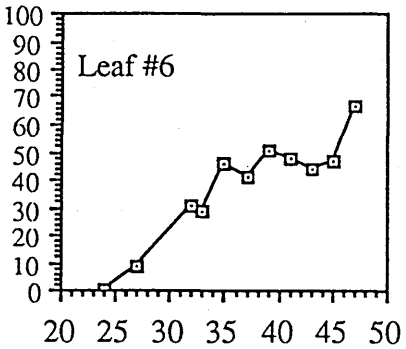


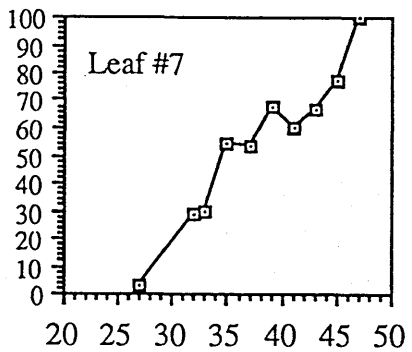


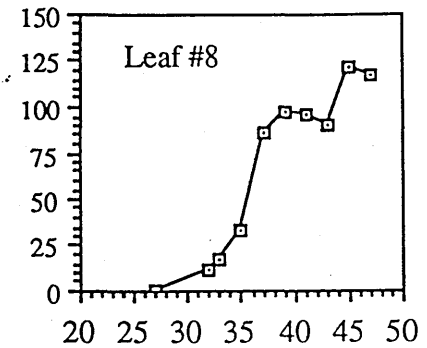


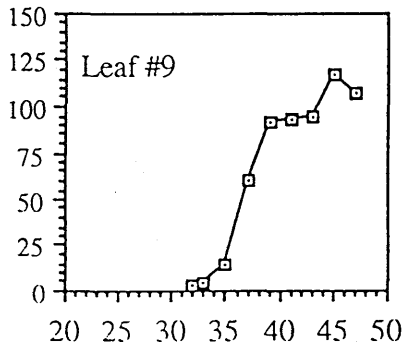


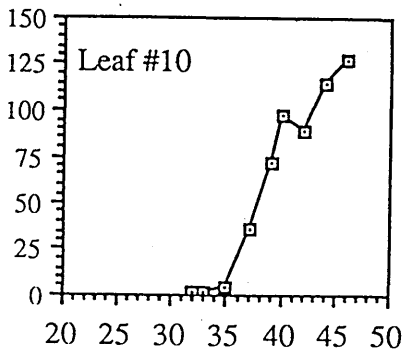


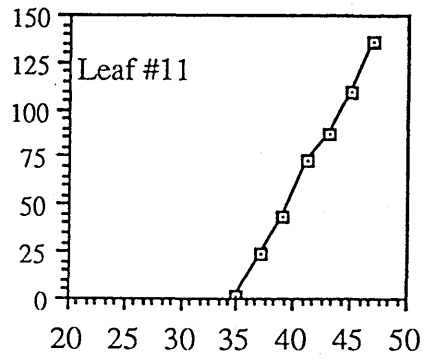


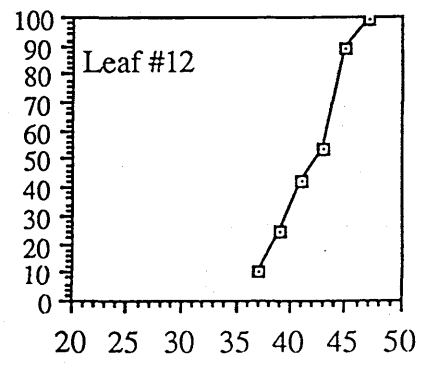


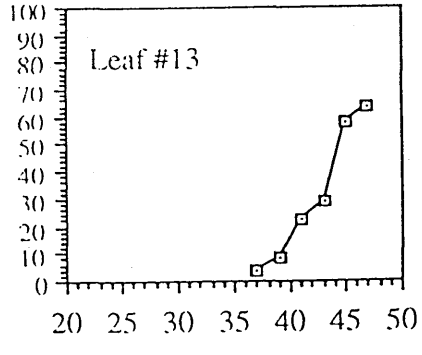


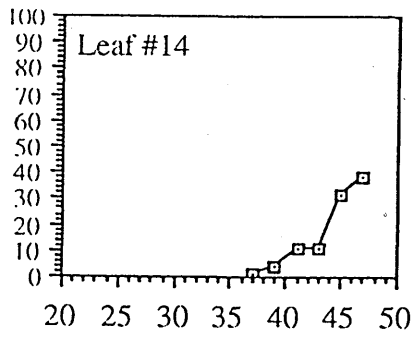


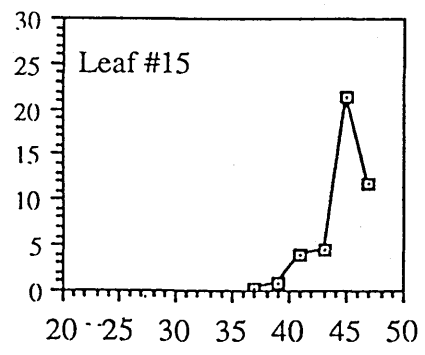


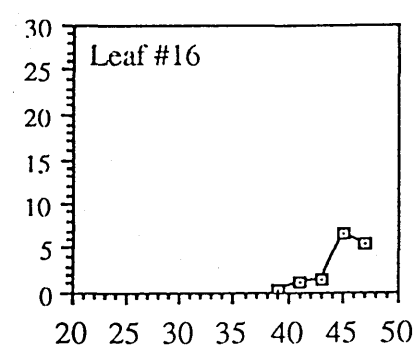












DAYS AFTER SOWING

well as by touching. Prior to shooting, the apex felt flat, but when shooting began, the apex could be felt to be protruding from its base and would press into a finger when gently touched. Once the apex achieved this structure, elongation of the stem followed rapidly with an increase in length of several centimeters each day thereafter. Figure 3.4 shows that the appearance of the apex remained similar prior to the day of shooting (figures 3.4 A to 3.4 D) until 41 days after sowing (figure 3.4 E) where it looks rounded and slightly elongated. Although shooting was recorded to be 45 days after sowing, it could be argued that the beginning of shooting was actually at 41 days post sowing since this seems to be at the time point where the apex changes.

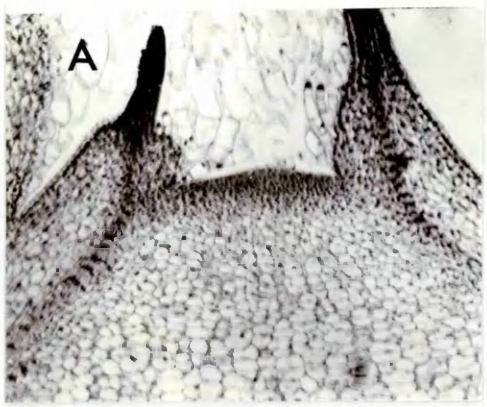
The shoot of each plant, bearing developing flowers, would gradually elongate to approximately 60 cm and then after flower development was complete, senescence of the leaves would become greatly apparent.

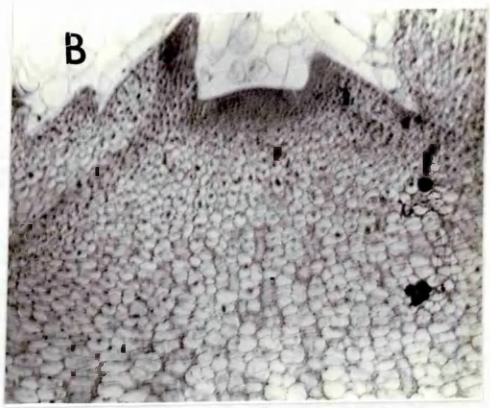
3.2.2 *In vitro* translation analysis

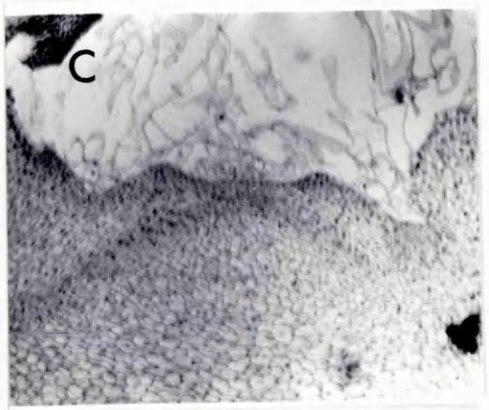
Total RNA for translation was isolated from the leaves of plants selected at random from the population over a time course prior to shooting. Two plants were harvested every other day beginning approximately three weeks after *Nicotiana plumbaginifolia* seeds had been sown. Sampling continued until shooting was clearly apparent and shoots had extended to several centimeters. Plant samples were taken by individually removing each leaf and noting its leaf number with number 1 being the first leaf produced.

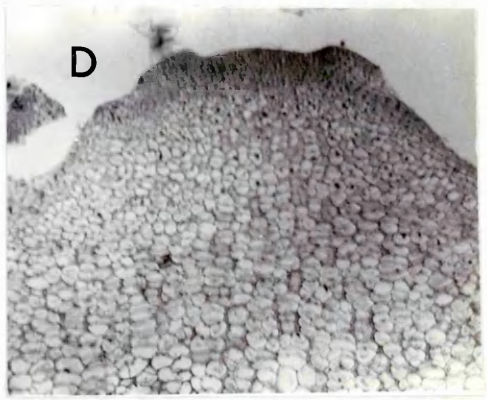
Figure 3.4 Photographic representation of apical development prior to shooting in *Nicotiana plumbaginifolia*

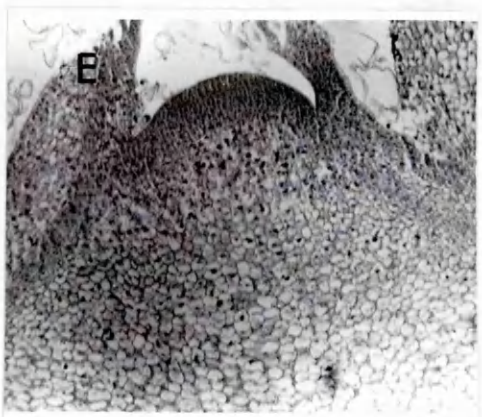
Apices from *Nicotiana plumbaginifolia* plants randomly selected from a population grown as described in section 2.3.1 were harvested as described in section 2.3.5 and sections were taken (section 2.3.5) and examined by light microscopy. The figure photographically depicts the appearance of apex tissue harvested at time points in days after sowing including: (A) 33 (B)35 (C)37 (D) 39 (E) 41 (F) 43 (G) 45

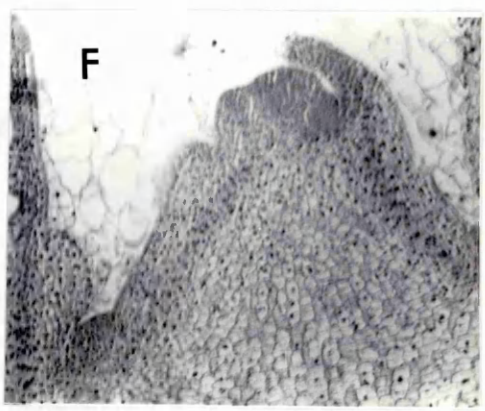

A

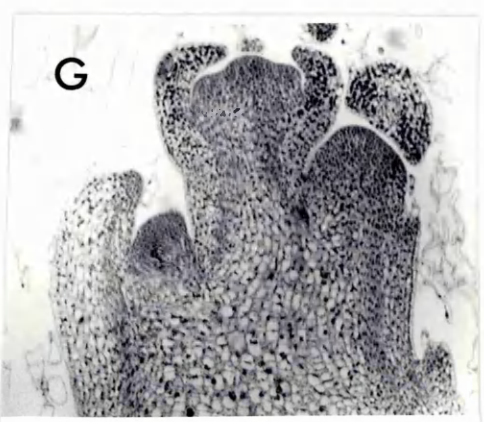

B


C


D


E


F


G

Throughout this thesis, unless otherwise indicated, *in vitro* translations were carried out using a cell free extract prepared from wheat germ (General Mills) in the laboratory (section 2.5.2). The wheat germ extract was prepared without subsequent micrococcal nuclease treatment, and when incubated at 30 ° C without any exogenous mRNA in the presence [^{35}S] - L- methionine, a single translation product with a molecular mass of approximately 85 kD was synthesized as indicated in Figure 3.5.

In vitro translation analysis of an initial experiment which will be referred to as experiment #1 is shown in figure 3.6. The largest leaves of each plant sample were selected for total RNA extraction and *in vitro* translation analysis since there was no other obvious basis for selection, and it was impractical to test all of the leaves of each plant in the first instance. Figure 3.6 shows that at least one mRNA, which synthesized *in vitro* a protein with a molecular mass of approximately 36.5 kD, appeared to be differentially regulated during the course of sampling. In the first experiment, the expression of this mRNA was apparent in samples harvested seven and two days prior to the initiation of shooting (figure 3.6, lanes b and c) but not eleven days prior to shooting (figure 3.6, lane a) or at the time of shooting (figure 3.6, lane d) or three days after the beginning of shooting (figure 3.6, lane e). Figure 3.7 shows that the expression of this mRNA was not limited to the leaves initially assayed but was seen in a majority of leaves harvested from the plant sample and was not influenced by leaf age or ontogeny. Although *in vitro* translations of total RNA from all of the leaves of plant samples which did not produce the 36.5 kD protein were not done, total RNA from at least three leaves from each plant was translated and examination of the products showed that synthesis of the protein was consistently not apparent or much reduced among the

Figure 3.5 ***In vitro*** **translation reaction with wheat germ extract and water only**

The wheat germ translation system was tested with water only and no exogenous mRNA. The figure depicts an autoradiogram of a 12 % one-dimensional SDS-PAGE gel containing: lane (a) resulting product from incubation of the wheat germ extract with water only, lane (b) ^{14}C - methylated protein molecular mass markers with sizes (in kD) indicated to the right. The single product with a molecular mass of 85 kD is indicated with an arrow on the left.

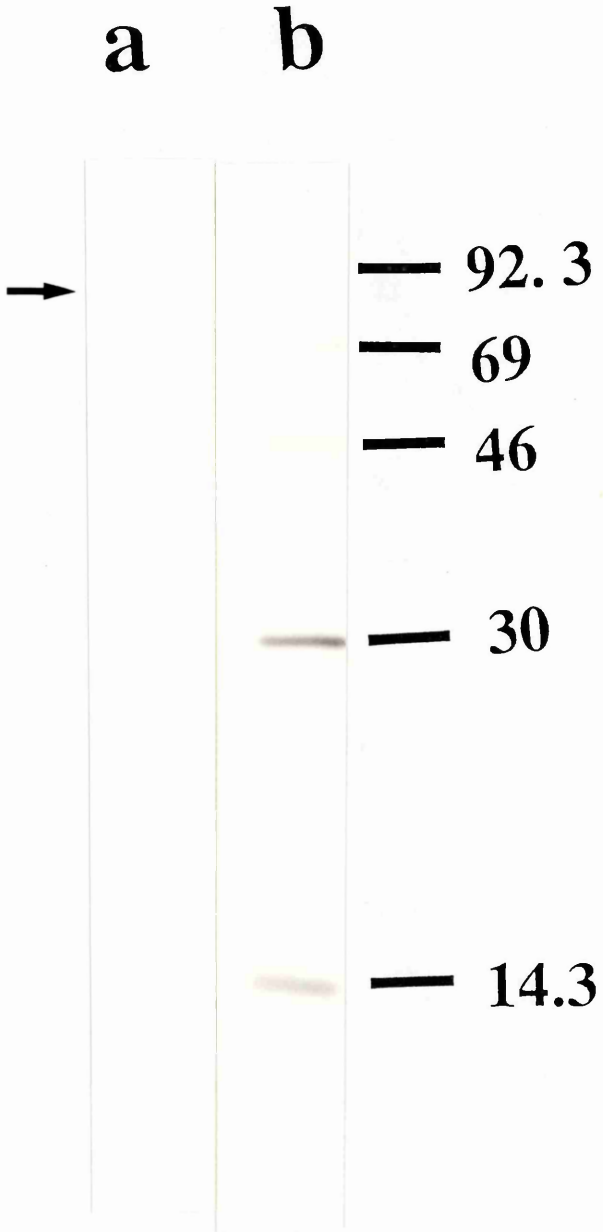

a
b
92. 3
69
46
30
14.3

Figure 3.6 *In vitro* translation analysis of *Nicotiana plumbaginifolia* gene expression in leaves during development using one-dimensional SDS-PAGE - experiment #1

Plants were harvested as described in section 2.3.1 over a time course beginning 34 days after seeds were sown and extending until plants were shooting (45 days after seeds were sown). Total RNA was extracted from individual leaves of plant samples as described in section 2.4.1 and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9).

The figure shows an autoradiogram depicting a 12% one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Nicotiana plumbaginifolia* mRNA extracted from: **(lane a)** leaf #6, 11 days prior to shooting, **(lane b)** leaf #7, 7 days prior to shooting, **(lane c)** leaf #7, 2 days prior to shooting, **(lane d)** leaf #8, the day of visible shooting, **(lane e)** leaf #9, 3 days after shooting began. Molecular mass markers (in kD) are indicated on the right. The region of the gel containing the 36.5 kD product of interest is indicated with a small arrow on the left of the gel.

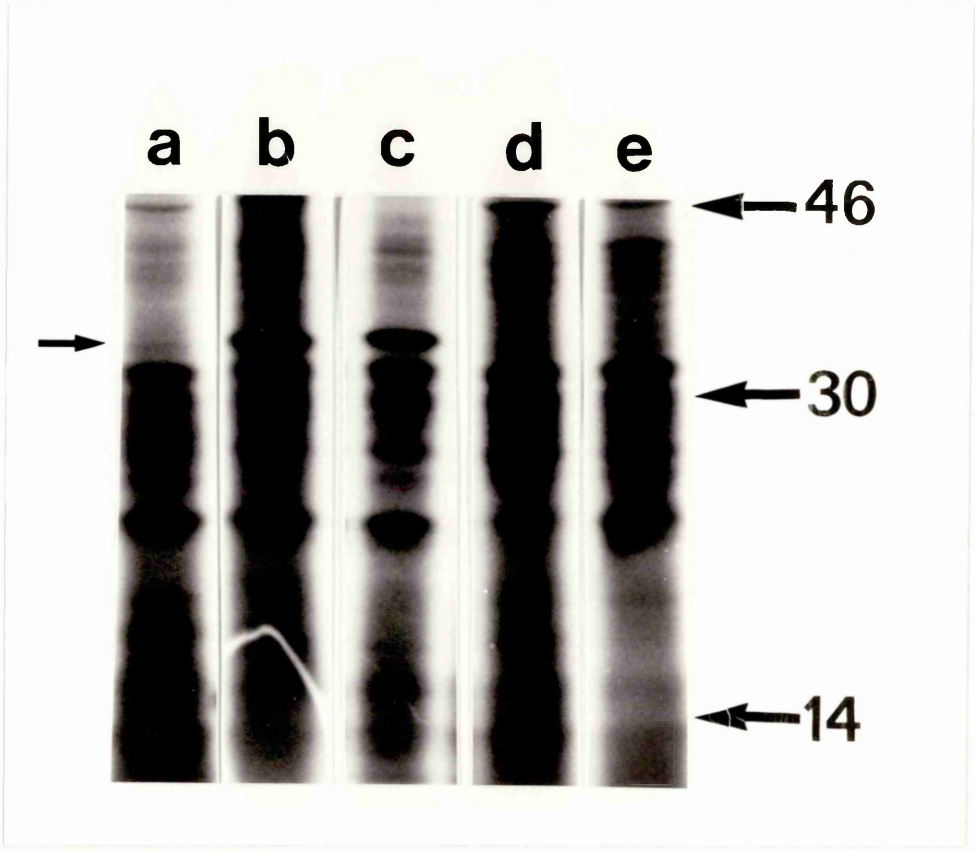

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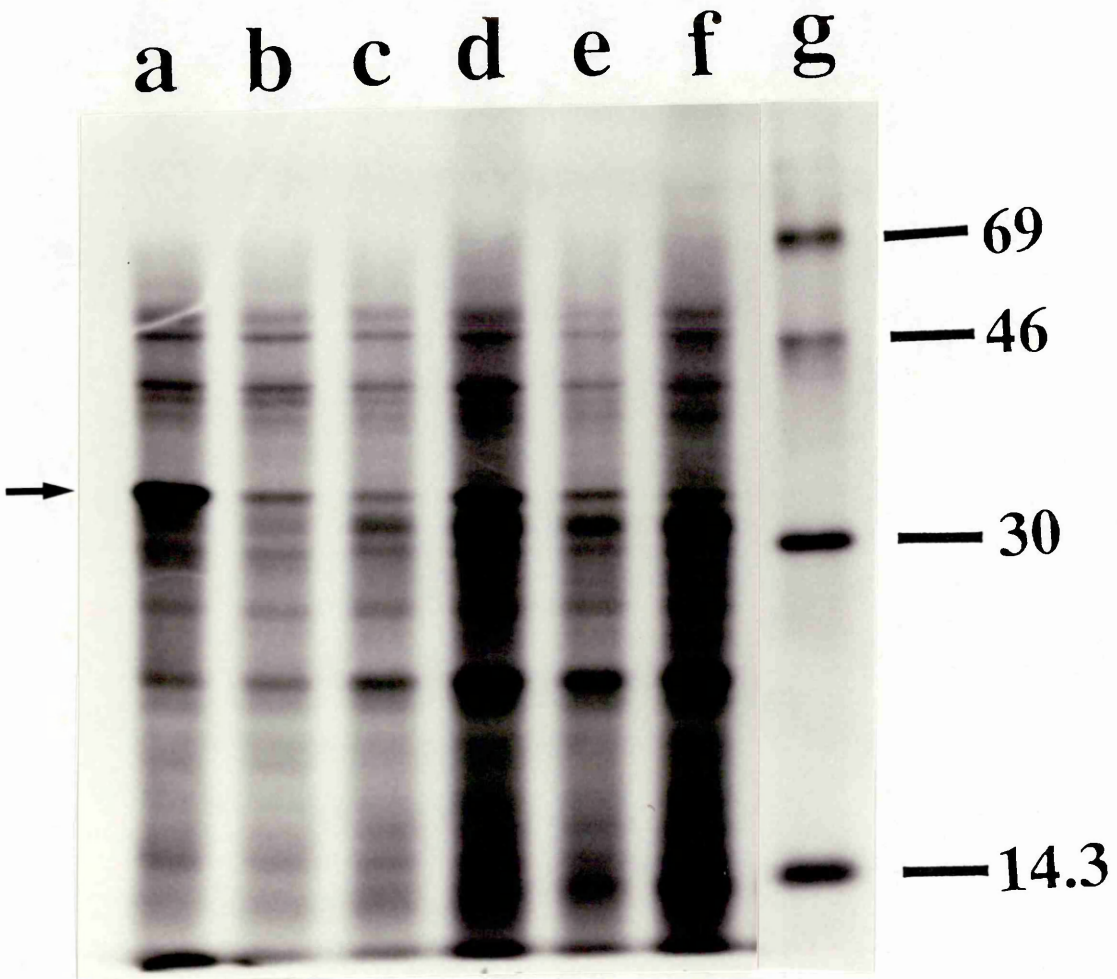
Figure 3.7 *In vitro* translation analysis of *Nicotiana plumbaginifolia* gene expression in a selection of the leaves from a plant sampled 7 days prior to shooting using one-dimensional SDS-PAGE - experiment #1

Plants were harvested as described in section 2.3.1 over a time course beginning 34 days after seeds were sown and extending until plants were shooting (45 days after seeds were sown). Total RNA was extracted from individual leaves of plant samples as described in section 2.4.1 and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9).

The figure shows an autoradiogram depicting a 12 % one dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Nicotiana plumbaginifolia* mRNA extracted from the following leaves which were all taken from the plant sample harvested 7 days prior to shooting: **(lane a)** leaf #3, **(lane b)** leaf #4, **(lane c)** leaf #8, **(lane d)** leaf #9, **(lane e)** leaf #10, **(lane f)** leaf #11. **Lane g** contains ^{14}C - methylated protein molecular mass markers with sizes (in kD) indicated to the right. The region of the gel containing the 36.5.kD product of interest is indicated with a small arrow on the left.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three.

· The results from experiment #1 were encouraging, but when further experiments where conducted, similar results were not obtained. Figure 3.8 shows that a protein of the same molecular mass as the 36.5 kD protein seen in the initial experiment was alsoseen in another experiment, referred to as experiment #2, but at a different time point (at the beginning of the course of sampling as opposed to a time closer to shooting). Figure 3.9 shows yet another experiment, referred to as experiment #3 where at no time point is there any indication of the synthesis of a prominent translation product protein with a molecular weight of 36.5 kD.

Figure 3.8 ***In vitro*** **translation analysis of** ***Nicotiana plumbaginifolia*** **gene expression during development using one-dimensional SDS-PAGE - experiment #2**

Plants were harvested as described in section 2.3.1 over a time course beginning 20 days after seeds were sown and extending until plants were shooting (42 days after seeds were sown). Total RNA was extracted from individual leaves of plant samples as described in section 2.4.1 and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9).

The figure shows an autoradiogram depicting a 12 % one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Nicotiana plumbaginifolia* mRNA extracted from:

(lane a) leaf #3, 22 days prior to shooting, **(lane b)** leaf #4, 20 days prior to shooting, **(lane c)** leaf #5, 18 days prior to shooting, **(lane d)** leaf #6, 16 days prior to shooting, **(lane e)** leaf #7, 14 days prior to shooting, **(lane f)** leaf #7, 11 days prior to shooting, **(lane g)** leaf #7, 10 days prior to shooting, **(lane h)** leaf #7, 9 days prior to shooting. **Lane i** contains *in vitro* translation products synthesized from pea mRNA and is included as a control for the efficacy of the translation reagents. **Lane j** contains ^{14}C - methylated protein molecular mass markers with sizes (in kD) indicated to the right. The region of the gel containing the 36.5 kD protein of interest indicated with a small arrow on the left .

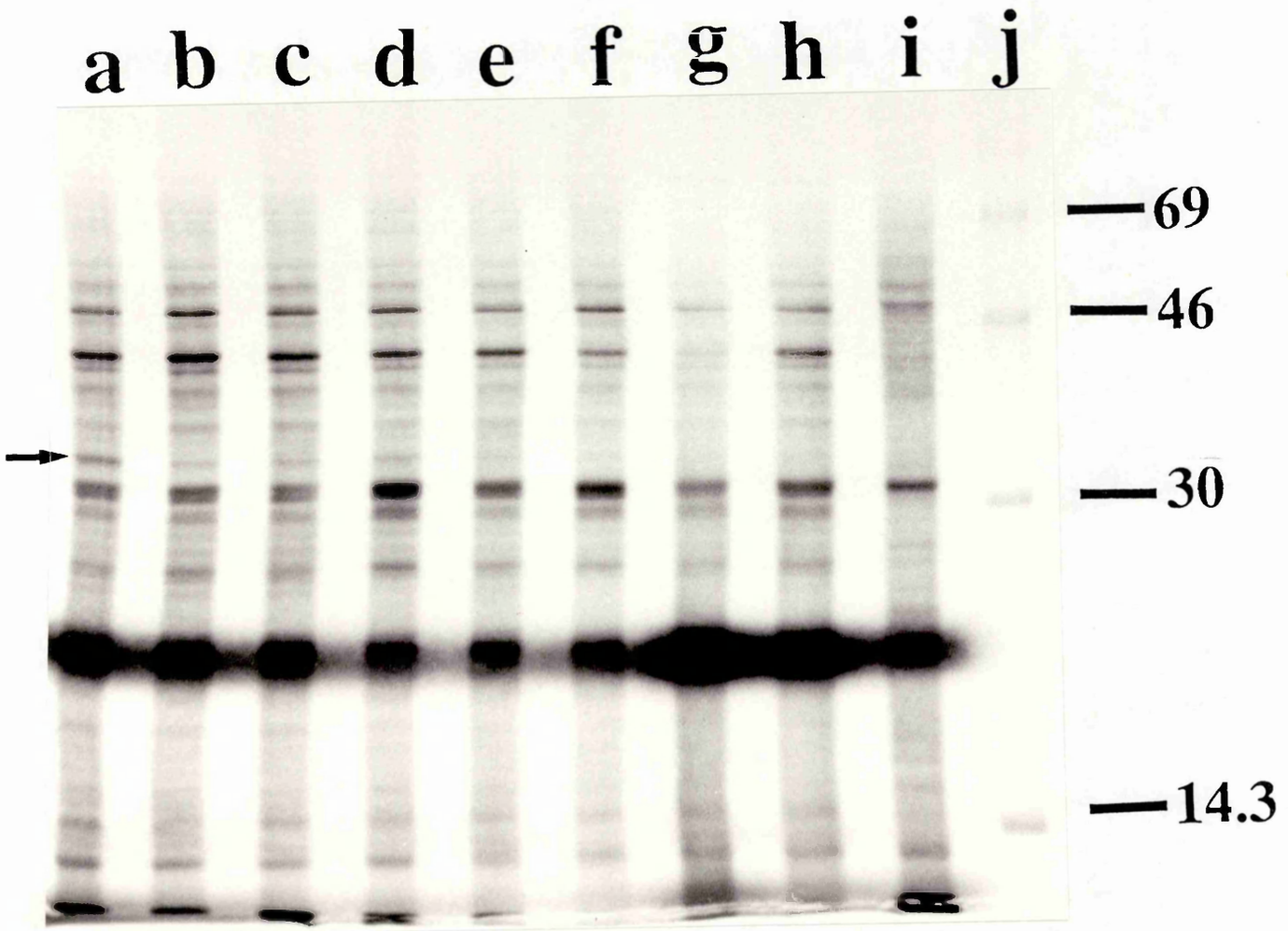
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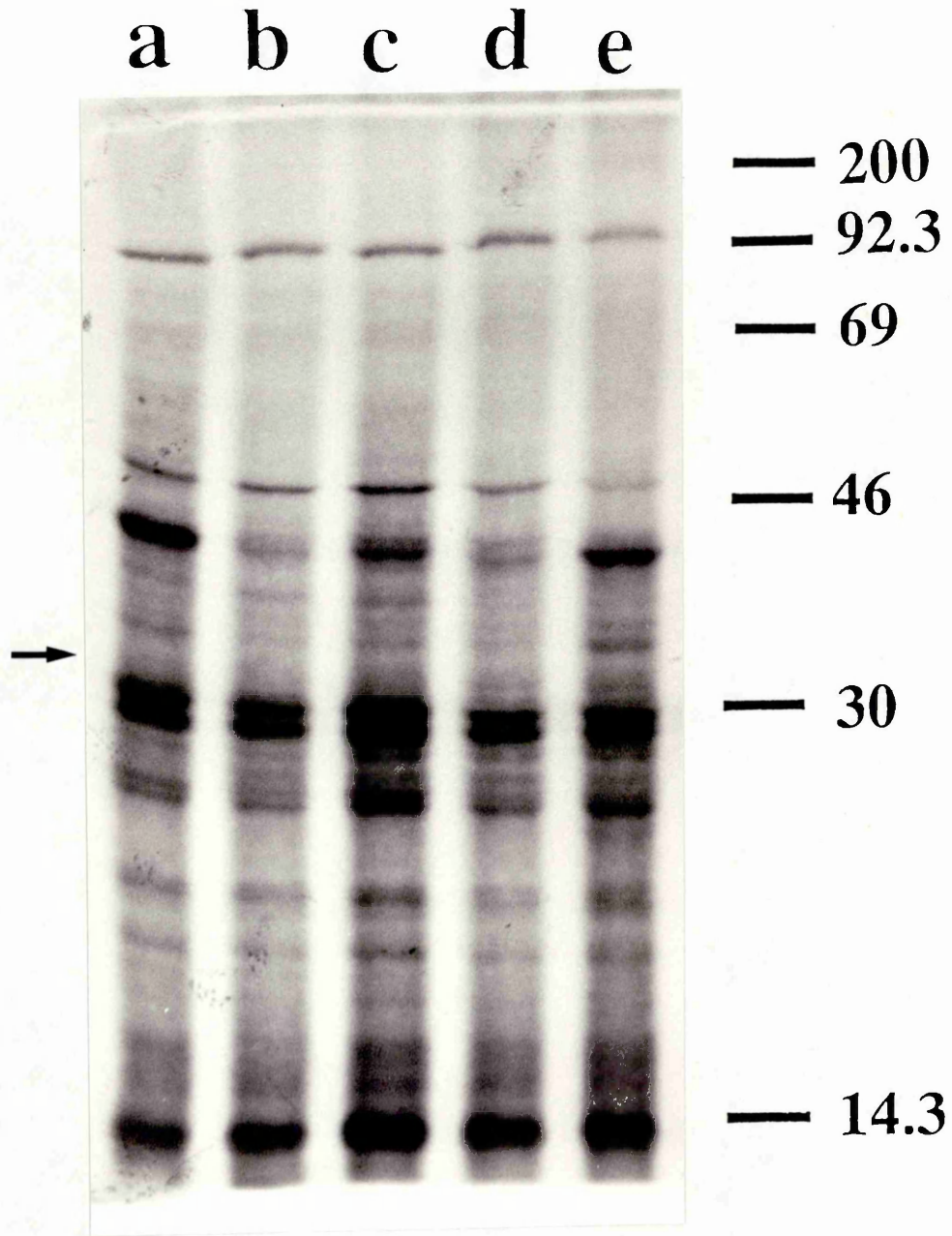
Figure 3.9 ***In vitro*** **translation analysis of** ***Nicotiana plumbaginifolia*** **gene expression during development using one-dimensional SDS-PAGE - experiment #3**

Plants were harvested as described in section 2.3.1 over a time course beginning 22 days after seeds were sown and extending until plants were shooting (44 days after seeds were sown). Total RNA was extracted from individual leaves of plant samples as described in section 2.4.1 and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9).

The figure shows an autoradiogram depicting a 12 % one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Nicotiana plumbaginifolia* mRNA extracted from: (**lane a**) leaf #4, 21 days prior to shooting, (**lane b**) leaf #4, 18 days prior to shooting, (**lane c**) leaf #6, 13 days prior to shooting, (**lane d**) leaf #6, 11 days prior to shooting, (**lane e**) leaf #6, 8 days prior to shooting. Protein molecular mass sizes (in kD) are indicated to the right. The region of the gel containing the 36.5 kD protein of interest in indicated with a small arrow on the left.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3.3 Discussion

The *in vitro* translation experiments presented here initially showed promising results depicted in figure 3.6. A protein of an approximate molecular mass of 36.5 kD appeared highly abundant in *in vitro* translation products synthesized from mRNA extracted from leaves 7 and 2 days prior to shooting but was not notably synthesized by mRNA extracted from leaves 11 days prior to shooting or on the day of shooting and thereafter. Experiments #2 and #3 show *in vitro* translation products synthesized from mRNA extracted at time points which do not overlap with experiment #1, and therefore, it cannot be concluded that the results of experiment #1 were not repeatable. However, in three other experiments which were not shown but where the time points do overlap, the 36.5 kD translation product was seen at a high relative abundance at time points other than those shown in experiment #1. These results can be summarized as follows :

1. 36.5 kD product seen on the day of shooting but not 7 days prior to shooting
2. 36.5 kD product seen 13 and 14 days prior to shooting but not 6 days prior to shooting
3. 36.5 kD product seen 7, 9, 11, and 13 days prior to shooting

Although these other experiments which were not shown seem to provide further evidence for random expression of the gene coding for the 36.5 kD translation product, no attempt was made to determine whether this product which appeared in the different experiments was the same. This could have been investigated in a number of ways. In the first instance, total *in vitro* translation products from two samples which appear to synthesize the 36.5 kD protein could be mixed and applied to one lane of an SDS-PAGE gel which would be compared to products of the individual samples on separate lanes. If the bands represent different products then two proteins of close molecular mass

would appear in the mixed sample lane. The problem with this approach is that it would depend a great deal on the resolving ability of the gel which might require adjustment by alteration of the ratio of bis-acrylamide to acrylamide for detailed analysis of proteins in the molecular mass range between 30 and 40 kD; otherwise, proteins whose molecular masses differ by only 1 to 2 kD may not be resolved, and if the proteins were the same molecular mass the procedure would not work at all.

Alternatively, isoelectric focussing or two-dimensional SDS-PAGE of the *in vitro* translation products could have been carried out to allow for comparison of samples. Also, peptide mapping of excised protein bands presumed to be the same 36.5 kD protein in separate samples (from separate experiments) would be another method to determine whether the protein bands were identical.

None of the above mentioned approaches involve trivial procedures and optimization of the techniques would have been required if they were to be useful. Therefore, use of any of the approaches may not have been practical. Also, the results would not allow for an explanation of the inconsistencies seen in the experiments overall.

Possible explanations for the inconsistent results must take into account the fact that plant gene expression may be influenced by a number of environmental conditions that are difficult to detect or control such as fluctuations in temperature, humidity, and air flow. Therefore, the need to harvest so many samples over a long period of time, as was done in this project, is undesirable because it increases that likelihood of such environmental factors having an effect and may have been the cause of the inconsistencies in the work discussed here. Also, it should be mentioned that as *Nicotiana plumbaginifolia* plants increased in size, more frequent watering was required and although watering was done at the same time of day, the effects of watering on gene

expression were never determined. Watering of the plants as they increased in size necessitated mechanical manipulation to avoid splashing of the water on the leaves which would cause burn spots. Such handling of the plants may have caused 'touch induced' genes to be expressed as recently described by Braam *et al.*, 1990.

These studies using *Nicotiana plumbaginifolia* would have greatly benefitted from preliminary work done to determine the nature of floral induction in the leaves of this plant so that the number of samples harvested could be reduced to a minimum. Inorder to do this, grafting experiments would have to be done using a photoperiodically induced receptor plant (i.e. plants that could be kept in the vegetative state) like, for example, *Nicotiana sylvestris*. Data collected from such work might show that *Nicotiana plumbaginifolia* leaves were induced three weeks after sowing, and therefore, samples would only be collected up until that point. It might also be discovered that only certain leaves become induced and overall sampling and mRNA extractions could be reduced to a minimum. However, grafting studies such as these would not have been practical for this project and would have required independent effort.

In conclusion, it seemed that choosing a selection of plants which were strictly photoperiodic and induced by a minimum number of cycles would allow for more rigorous sampling and increase the chances of identifying differentially expressed genes in which regulation can be reasonably associated with photoperidic treatment only.

Chapter 4 - Floral Induction Studies on *Amaranthus caudatus* and *Silene coeli-rosa*

4.1 Introduction

Because of the confusing results obtained with *Nicotiana plumbaginifolia*, it was decided that it would be preferable to search for and develop a plant system which would allow for the study of a floral induction process that occurs over a very short period of time. To satisfy this altered approach, plants with well documented flowering physiology as a first priority were considered. Suitability for genetic or reverse genetic studies was clearly no longer a primary advantage for this project although the plants selected for the continuation of the work were all dicot species and might eventually allow for reverse genetic techniques where ploidy is not a limitation (e.g. anti-sense RNA approaches).

This chapter concerns studies which were conducted on the plant species *Amaranthus caudatus* and *Silene coeli-rosa*. Although these plants are not induced by exposure to a single photoperiodic cycle, there is an indication from previous studies that both plants might yield positive results (see sections 1.9.2 and 1.9.3). *Amaranthus caudatus* seemed to be an appropriate choice to introduce to the study since previous studies demonstrated significant changes in protein composition in the leaves of plants after they were transferred from a 24 hour photoperiod to an 8 hour photoperiod (Kohli *et al.*, 1980). The contradictory evidence for the photoperiodic requirements, as discussed in section 1.9.2, was initially a concern. It was thought, however, that if the conditions for induction were duplicated as described in the experiments by Kohli *et al.*

(1980), the reward would be worth the minimal time investment.

Although full flower induction in *Silene coeli-rosa* is not achieved by a particularly small number of cycles [seven cycles are required for complete induction (Lyndon, 1985)], studies on apex activity indicate that a response to the spectral transition, from high intensity light (fluorescent and tungsten) to the dim light extension light (tungsten only) used for induction (see section 2..3.3), is rapid. A significant increase in nuclear DNA replication has been detected 30 minutes after the transition, and this is believed to be mediated by a leaf generated stimulus (or stimuli) since shading of the leaves suppresses the response (Ormrod and Francis,1986). This increase in nuclear DNA replication brings about a shortening of the cell cycle and a transient accumulation of cells in the G2 phase of the cell cycle. This G2 increase occurs 3 hours after the spectral transition on each the three cycles which follow and it is thought that a change in apex sensitivity to the floral stimulus occurs during these first four cycles of induction (Francis, 1987). Shading of the leaves but not the apex will suppress this activity and the process demonstrates "far red/red reversibility" (see section 1.2.3).

It would be interesting to see if changes in gene expression can be visualised at some point during the first four LD cycles which can independently bring about threshold induction. It might be possible to associate these changes with a particular event occuring at the apex. The investigation could also be extended to look for changes which might occur after four LD cycles and up to seven LD cycles. Appearence of sepal primordia is reported to occur nine days days after the start of the seven day inductive treatment, and this implies that the transmission of the necessary stimuli is complete by this point. Changes in gene expression which may occur after this time point would not

be involved with induction.

4.2 Results

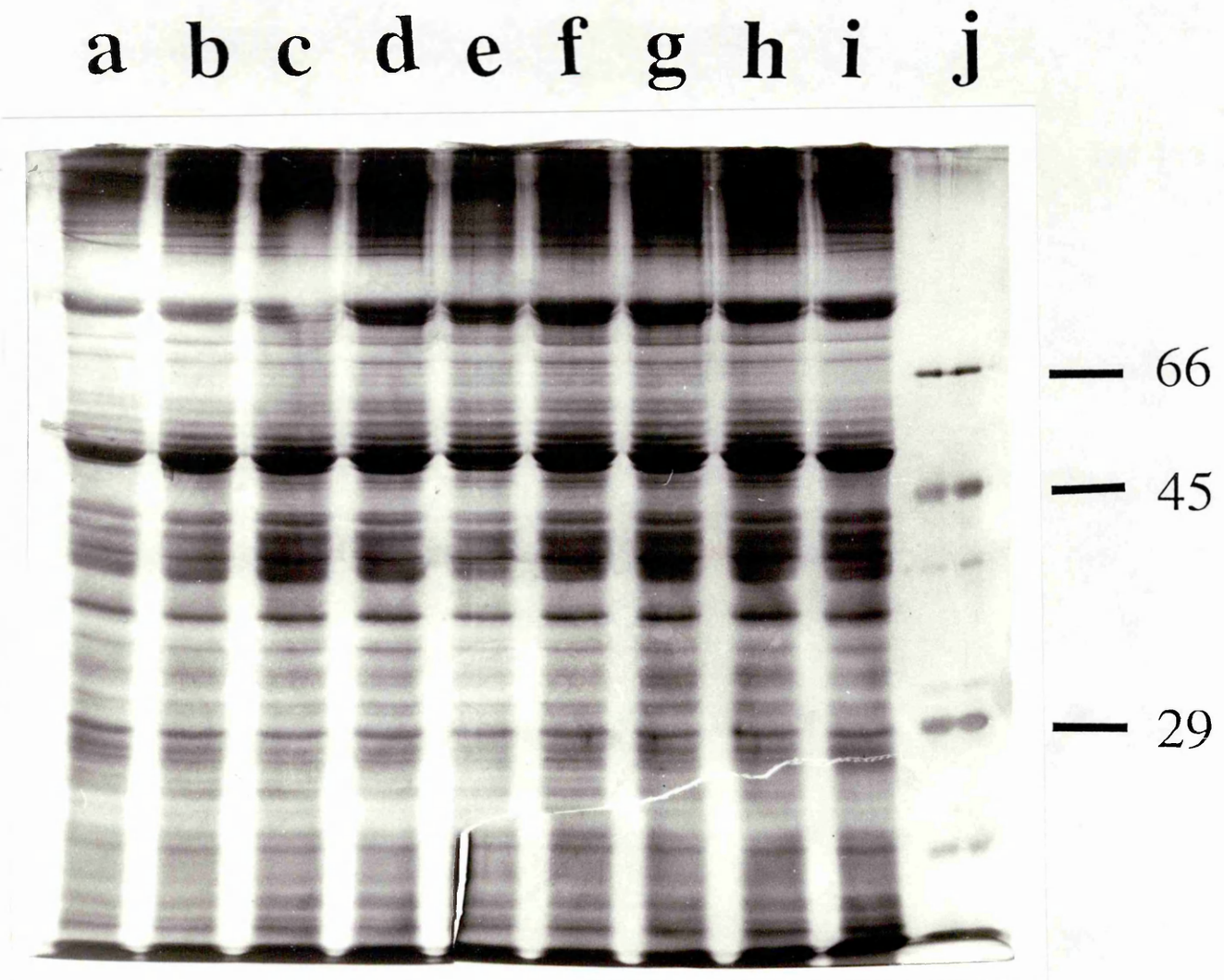
4.2.1 Studies on *Amaranthus caudatus*

Initial experiments done to investigate changes in gene expression at the protein level with *Amaranthus caudatus* showed that the results obtained by Kohli *et al*. (1980) were not repeatable. Figure 4.1 shows the total protein extracted from the leaves of plants that had been transferred to a growth room where they received the number of cycles of short day light exposures indicated. Even after five cycles of short day exposure, there is no apparent difference in the protein pattern produced on a one-dimensional SDS-PAGE gel. Figure 4.2 shows that total RNA extracted from the same plants and translated *in vitro* also showed no apparent differences. There is one protein band at approximately 38 kD, that appears in lane c of figure 4.2; however, when *in vitro* translations were repeated using the same total RNA preparations used in lanes a through c, the products run on a separate SDS-PAGE gel showed no difference in the molecular mass region between 30kD and 46 kD. It must therefore be assumed that this band was simply artifactual.

No further experiments were carried out using this species because it was discovered that, contrary to previous reports by Kholi *et al.* (1980) flowering proceeded in continuous light that was composed of daily exposure to either 24 hours of high intensity light or 8 hours of high intensity light followed by 16 hours of dim light. Flowering behaviour in long day light conditions was different from that which was demonstrated by plants that had been transferred to short days: flower initiation became apparent later

Figure 4.1 Analysis of protein extracted from *Amaranthus caudatus* leaves during photoperiodic induction using one-dimensional SDS-PAGE

Total protein was extracted (2.6.1) from *Amaranthus caudatus* leaves harvested from plants growing in continuous light and at time points after transfer to short day inducing conditions (harvesting, growth, and induction conditions as described in section 2.3.2). Proteins were analysed on a 12 % one-dimensional SDS-PAGE gel (2.7.1). Lanes **a** through **d** show total protein extracted from leaves of control plants growing in continuous light at time points 1, 2, 3, and 4 days, respectively, after the test plants were transferred to short day conditions. Lanes **e** through **i** show protein extracted from the leaves of plants that had been transferred to short day conditions for 1, 2, 3, 4, and 5 days, respectively. Lane **j** contains protein molecular mass markers with sizes (in kD) indicateed to the right. The proteins in the gel in the figure were stained with silver as described in section 2.10.2.


a b c d e f g h i j
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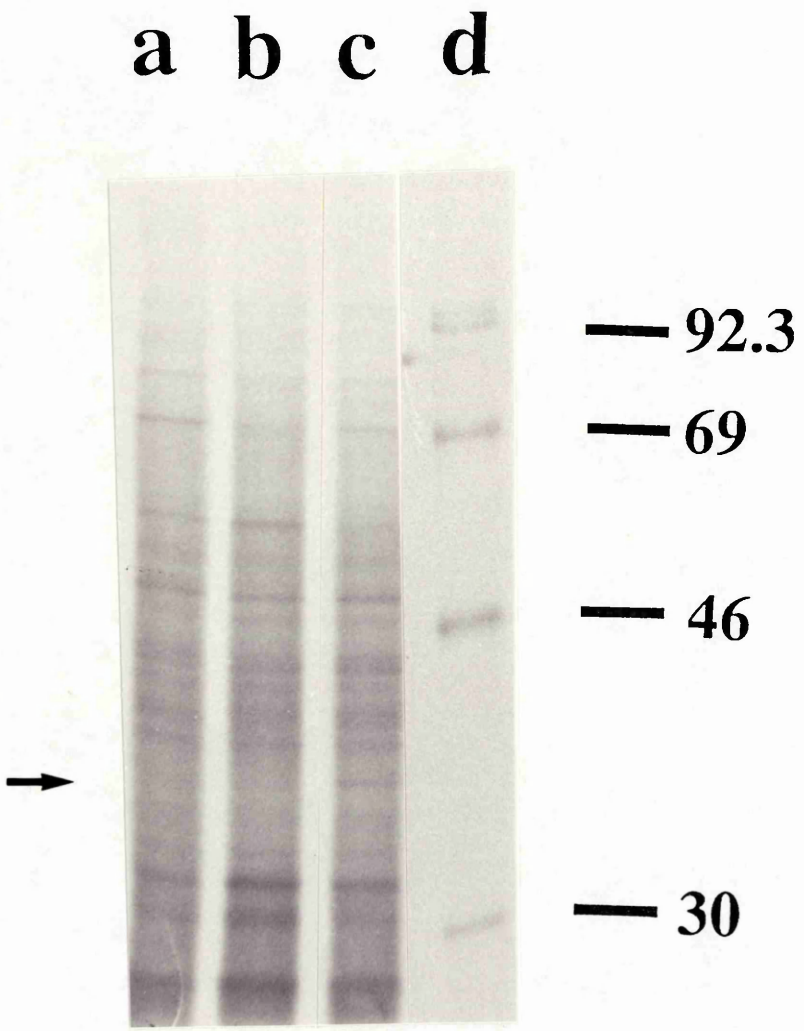
29

Figure 4.2 *In vitro* translation analysis of *Amaranthus caudatus* gene expression during photoperiodic floral induction using one-dimensional SDS-PAGE

Plants were grown under long day conditions and induced under short day conditions as described in section 2.3.2. Leaf samples were harvested as described in section 2.3.2, and total RNA was extracted from the pooled leaf samples of *Amaranthus caudatus* as described in section 2.4.2 and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using EN^{3}HANCE™ (section 2.8.2) and autoradiography (section 2.9). The figure shows an autoradiogram depicting a 12 % one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Amaranthus caudatus* mRNA extracted from: (**lane a**) one short day cycle, (**lane b**) two short day cycles, (**lane c**) four short day cycles. **Lane d** contains ^{14}C - methylated protein molecular mas markers with sizes (in kD) indicated to the right. The 38 kD product discussed in the text is indicated with a small arrow to the left.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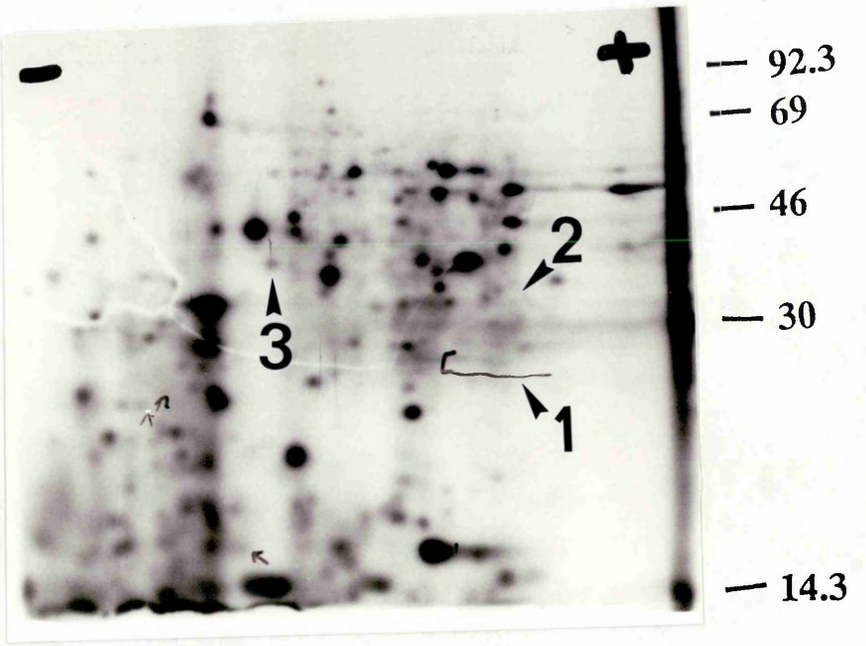
(by approximately one week) and the flower structures formed did not acquire thecharacteristic red coloring which is featured by plants transferred to growth in short day conditions. It also became obvious, in further attempts to grow and induce this species, that the leaves of the individual plants became spotted with brown lesions (indicating possible pathogen infection or an unknown physiological problem) while other types of plants in the same room appeared normal. These observations (or problems) made this species unsuitable for further investigation.

4.2.2 Studies on *Silene coeli-rosa*

Initial experiments were done to ensure that this species exhibited the flowering behaviour that was previously documented (Lyndon, 1985). All plants given seven cycles of long days, which included eight hours of high intensity light extended to twenty four hours with dim light, flowered while plants maintained in short day conditions never flowered when observed for a period of four months. It was thought when initiating work with this species that translation products would be more suitably analysed by two-dimensional SDS-PAGE. The difficulty in using this technique became apparent when after many attempts, it was not possible to obtain duplicate sets of results. Figure 4.3 shows three autoradiograms which depict examples of two dimensionally electrophoresed *in vitro* translation products synthesized from total RNA extracted from leaves of plants growing in short days (figures 4.3 A and 4.3 B) and from leaves of plants which have been exposed to three long day cycles (figure 4.3 C). There appears to be one major difference indicated as protein #1 and one minor difference indicated as #2, but further attempts at obtaining a complete set of duplicate translations of duplicate samples from separate experiments would be necessary to determine whether these

Figure 4.3 *In vitro* translation analysis of *Silene coeli-rosa* gene expression during photoperiodic floral induction using two-dimensional SDS-PAGE

Leaves were harvested from *Silene coeli-rosa* plants (section 2.3.3) growing in short day conditions (section 2.3.3) and from plants exposed to three long days (section 2.3.3). Total RNA was extracted from the leaves (section 2.4.2) in duplicate and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 1.5 x 10^6 precipitable cpm of each sample onto the first dimension of a two-dimensional SDS-PAGE gel (section 2.7.2). Completion of the second dimension was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9). The figure shows an autoradiogram depicting a 12% two-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Silene coeli-rosa* mRNA extracted from: (A and B) short day control leaves, (C) leaves exposed to three long days. The basic and acidic ends of the gel are indicated with a + and a - , respectively. Proteins of interest are indicated with arrows and numbers.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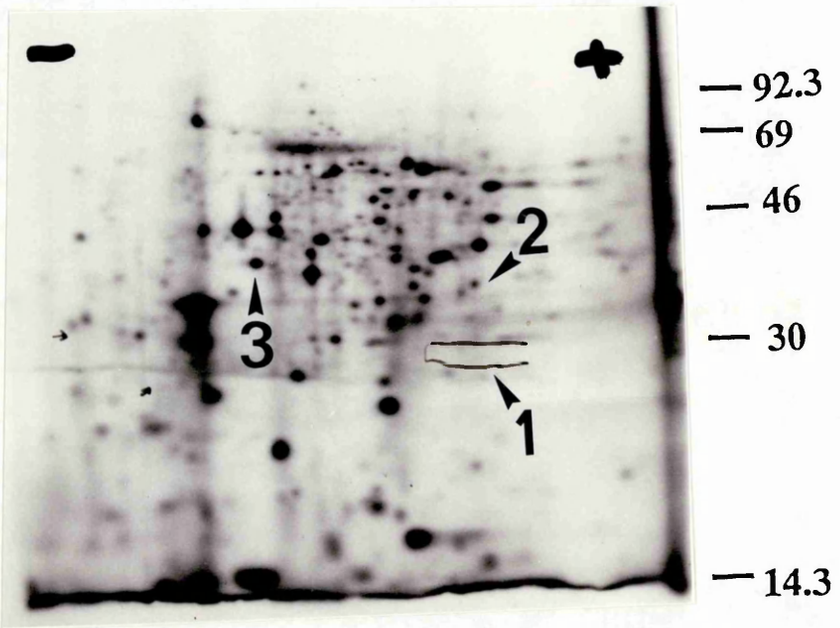
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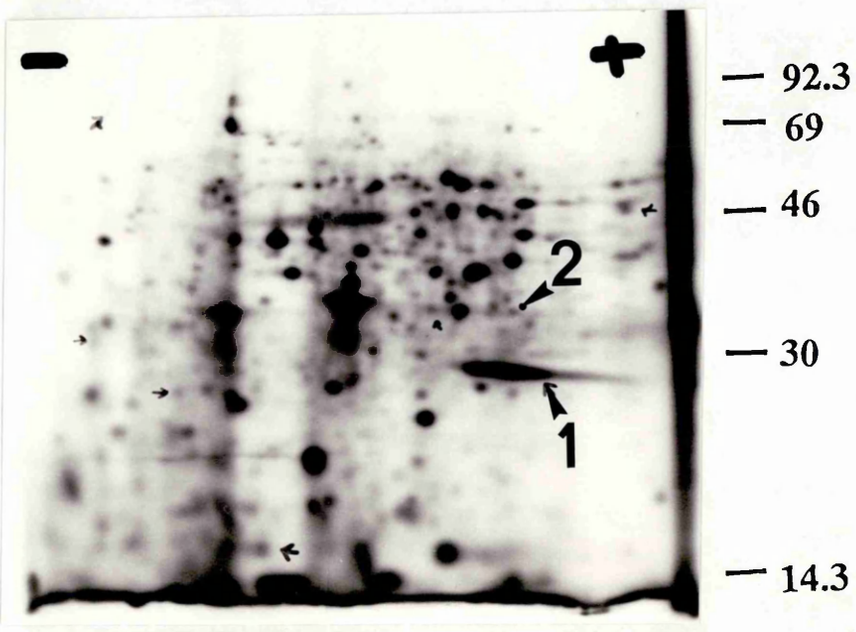
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differences are true. Protein # 3 is a good example of the differences that can arise when comparing two samples which should be identical and thus establishes the need for duplication in these experiments.

No further experiments were conducted with *Silene coeli-rosa* because of the need to generate a large number of two-dimensional SDS-PAGE gels to allow for a proper comparison of the samples to be made. The plants also were particularly susceptible to infestation by insects and it was thought that this might yield further problems in the future.

4.3 Discussion

The results obtained for *Amaranthus caudatus* do not support findings of another group where the species was described as a qualitative short day plant and *de novo* synthesis of two proteins had been noted three days after transfer from long days to short days (Kohli *et al.*, 1980). The photoperiodic behaviour of *Amaranthus caudatus* described by Kohli *et al.* (1980) is questioned since it did not display the same behaviour here. This was not suprising since contradictory reports on photoperiodic floral induction in this species have been noted (Fuller, 1949; Zabka, 1961; Kigel and Rubin, 1985), but the pure line seeds used for growing the plants in the experiments described here were provided by the Royal Botanic Gardens at Kew and were identical to those used by Kohli *et al.* (1980). Clearly, the role of light and photoperiod in the control of flowering in this species is not straightforward.

The data reported by Kohli *et al.* (1980) were presented in the form of diagramatic illustrations of proteins separated by the Ornstein-Davis electrophoresis method (Ornstein, 1964; Davis, 1964) and stained with Coomassie blue stain as opposed to photographs of the actual gels. Also, the illustrations showed that only 15 bands could be visualised with the techniques used (Ornstein-Davis electrophoresis system (Ornstein, 1964; Davis, 1964) as opposed to the more current Laemmli SDS-PAGE electrophoresis system (Laemmli, 1970). The extraction, electrophoresis and staining procedures used in the experiment described here allowed for the resolution of a greater number of proteins (both water-soluble and water-insoluble) and it was thought that if the changes in protein composition were previously validly described, the same changes as well as possible others would be visualised once again. As this was not the case, and again

considering the identical seed source, the previously described results should be considered with caution.

The results obtained with *Silene coeli -rosa* were not entirely unpromising, but due to the difficulties which arose in producing two-dimensional separations of *in vitro* translation products of consistent quality and the problem with insect infestation, further work with this plant could not be feasibly incorporated into this project given time constraints.

Chapter 5 - Studies on the Floral Induction of *Anagallis arvensis*

5.1 Introduction

The results obtained with *Amaranthus caudatus* and *Silene coeli-rosa* indicated that the remaining time alloted to the project would be best spent analyzing molecular aspects of floral induction in *Anagallis arvensis* . *Anagallis arvensis* provides the ideal type of induction protocol by being induced by a single long day (see section 1.9.4). Results obtained from comparing rapidly induced plants with uninduced controls using the *in vitro* translation technique or direct protein analysis would initially determine if the floral inducing treatment of a single long day dim light extension (see section 2.3.4) caused any changes in gene expression as measured at the mRNA or protein level. If none were noted, a more careful regime of sampling could be carried out which would include harvesting tissue during the dim light night extension. Sampling could continue after the plants had been returned to short day light exposures (at least until transmission of the stimulus is complete, i.e. 48 hours from the beginning of the inducting treatment - see section 1.9.4). This would not be as labour intensive as would be necessary to study induction in *Silene coeli-rosa* , since in that species at least 96 hours of sampling would be required as opposed to 48 hours for *Anagallis arvensis*. Once genes are identified which demonstrate altered expression as a result of the dim light extension, attempts could be made to isolate cDNA clones that could be used as probes to analyse expression in a more sensitive manner.

5.2 Results

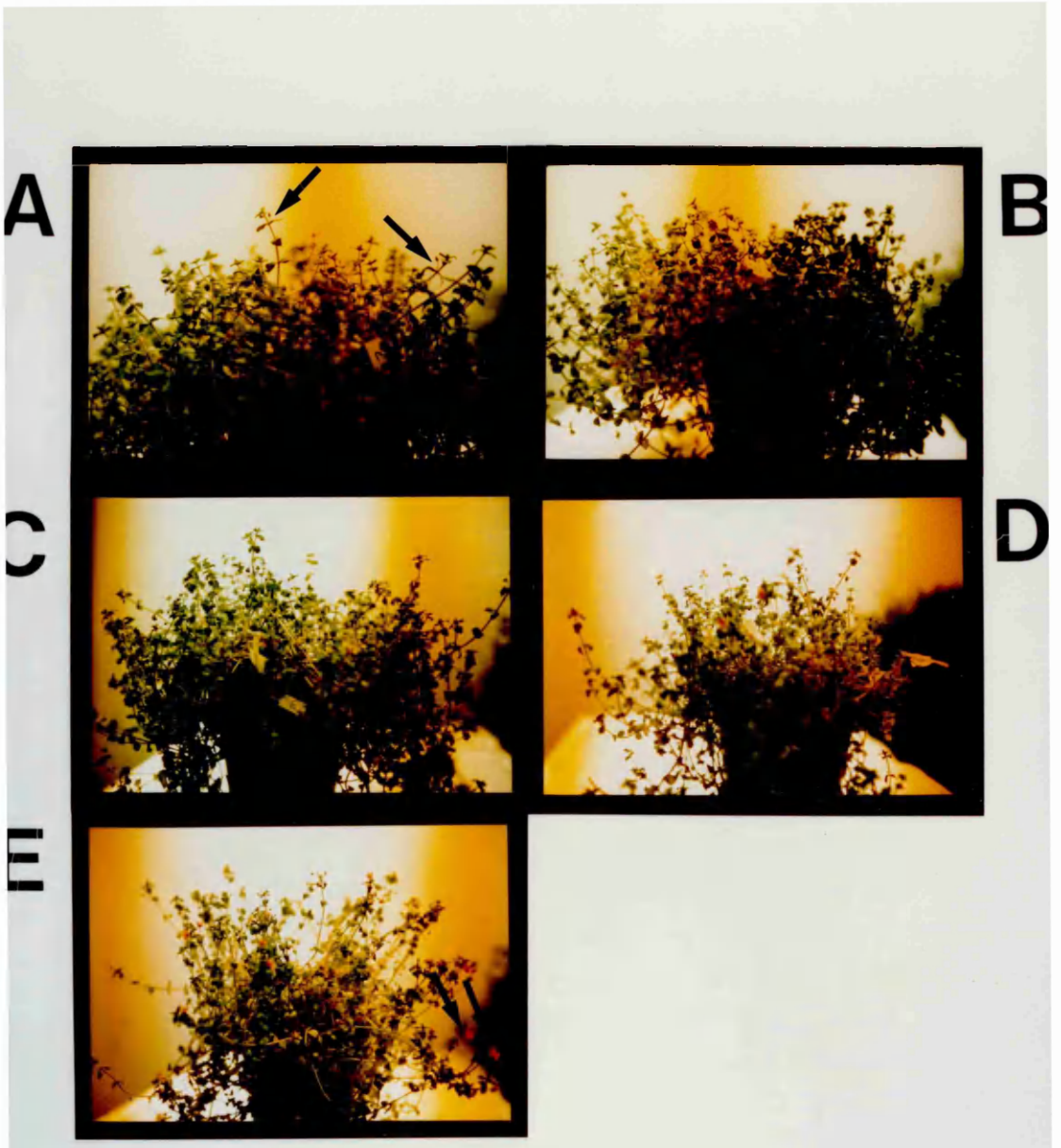
5.2.1 Response to photoperiodic treatment

Initial induction experiments demonstrated that *Anagallis arvensis* could be induced to flower by one long day cycle made up of eight hours of high intensity light and extended to twenty four hours with dim light. Figure 5.1 shows the response of the plant to exposures of one to five long day cycles. The plants in this experiment resumed growth in short days after treatment until flowering was complete. It is clear that all treatments result in the production of flowers (indicated in figure 5.1 with small arrows); however, an increasing number of flowers resulted by increasing the number of long day cycles to which the plants were exposed. Plants exposed to one long day cycle produced flowers at the next node after the node present during induction, and those exposed to five long day cycles produced flowers for up to four nodes after the node present at the time of induction. It is also apparent from comparing figures 5.1 A through 5.1 E that the development of the flowers was more rapid in plants given a greater number of long day cycles; for example, the flowers indicated with arrows in figure 5.1 A are not yet opened while the flowers indicated in figure 5.1E are fully opened and displaying the bright salmon colored flowers.

The response at the apex in plants exposed to one cycle of long days (followed by placement back into short days) as revealed by light microscopy is depicted in figure 5.2. Figure 5.2 shows that flowers produced by plants given a single long day cycle were microscopically visible 112 hours (on the fifth day) from the beginning of the dim light

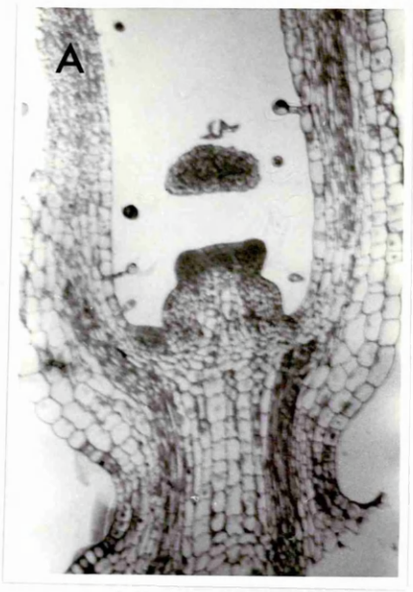
Figure 5.1 Photographic representation of *Anagallis arvensis* plants exposed to 1 through 5 long days

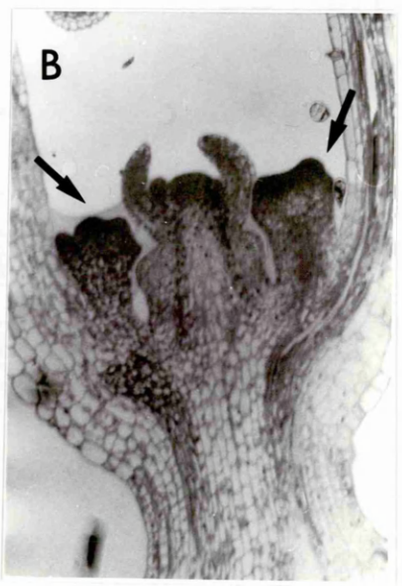
Photographs were taken of flowering *Anagallis arvensis* plants which were grown in short day conditions (section 2.3.4) and then transferred to long day conditions (section 2.3.4) for the following number of cycles : (A) 1 (B) 2 (C) 3 (D) 4 (E) 5. Arrows indicate representative flowers possessed by plants given 1 and 5 cycles of long days.


A
B
C
D
E

5.2 Apical tissue from *Anagallis arvensis* 112 hours after treatment with one long day

An *Anagallis arvensis* plant was exposed to one long day (section 2.3.4) and then returned to short day conditions (section 2.3.4). Apical tissue was removed from the induced plant (2.3.5) as well as from a plant which was exposed to short days only. Sections of the apices were examined by light microscopy and photographed. The figure depicts *Anagallis arvensis* apex tissue from a plant exposed to: (A) short days only, (B) one long day. Arrows indicate the developing flowers.


A


B

extension period. Developing flowers were macroscopically visible 156 hours (on theseventh day) from the beginning of the dim light extension period (data not shown).

5.2.2 *In vitro* Translation analysis of total RNA (one-dimensional SDS-PAGE)

Attempts to characterise changes in gene expression during floral induction in *Anagallis arvensis* at the mRNA level employed the use of the *in vitro* translation technique as described in section 1.8.1.1. In the first instance, *in vitro* translation products were analysed using one-dimensional SDS-PAGE, since this technique is a significantly less difficult procedure than two-dimensional SDS-PAGE.

Figure 5.3 shows an illustration of an *Anagallis arvensis* stem and the three leaf pairs which were harvested and pooled from both induced and control in all of the experiments prior to total RNA extraction. When preparing the total RNA in all experiments, a small aliquot of leaves was removed from a pooled leaf stock by pouring the leaves from the stock directly into the mortar and pestal used for grinding the tissue. Since false results might arise from an unintentional bias in the proportions of leaves present in any given aliquot of leaf tissue sample used in a given total RNA preparation (for example, one aliquot might have more leaves from pair #1 than another), it was important to check that no differences could be seen when comparing *in vitro* translation products synthesized from mRNA extracted from individual leaf pairs. Figure 5.4 shows that, indeed, no difference could be detected when *in vitro* translation products from individual leaf pairs were analysed using one-dimensional SDS-PAGE. Therefore,

Figure 5.3 Illustration of an *Anagallis arvensis* stem indicating the leaf pairs which were harvested

Thie figure shows an illustration of the appearance of an *Anagallis arvensis* stem and the leaf pairs which were harvested in all experiments for RNA or protein analysis.

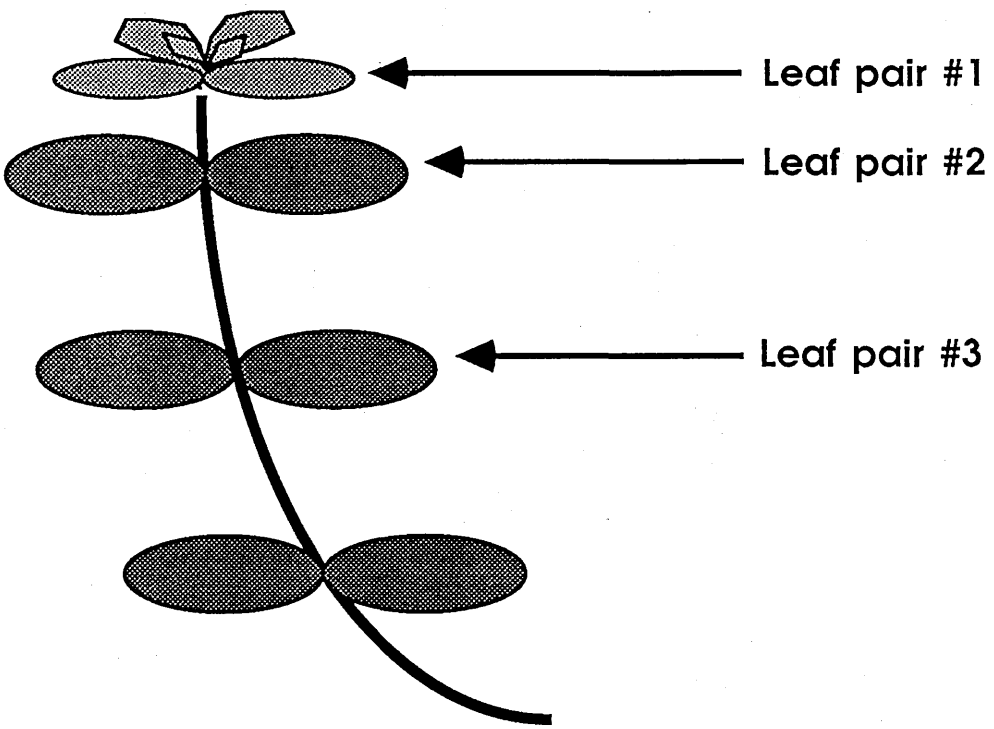
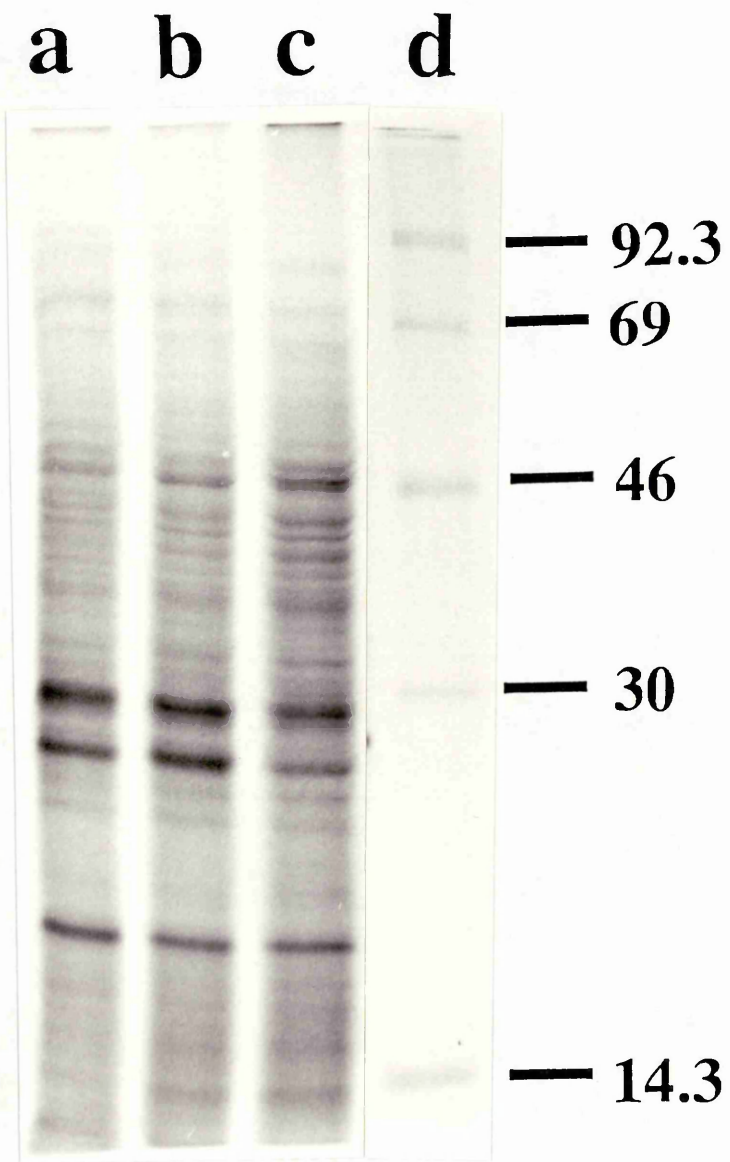

Leaf pair #1
Leaf pair #2
Leaf pair #3

Figure 5.4 *In vitro* translation analysis of *Anagallis arvensis* gene expression in individual leaf pairs using one-dimensional SDS-PAGE

The individual leaf pairs indicated in figure 5.3 from plants grown in short day lengths (section 2.3.4) were harvested from *Anagallis arvensis* plants (section 2.3.4) and total RNA from the individually pooled pairs was extracted (section 2.4.2) and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9). The figure shows an autoradiogram depicting a 12 % one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Anagallis arvensis* mRNA extracted from: (lane a) leaf pair 1, (lane b) leaf pair 2, (lane c) leaf pair 3. Lane d contains ^{14}C - methylated protein molecular mass markers with sizes (in kD) indicated to the right.


a
b
c
d
92.3
69
46
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14.3

it could be assumed that when long day induced and short day control leaf RNA was analysed, results would be indicative of the treatments only and not result from anunintentional bias in the amounts of the individual leaf pairs (at least when comparing one-dimensional SDS-PAGE).

Figure 5.5 shows *in vitro* translation products synthesized by mRNA extracted from leaves directly after one exposure to the long day extension period (figure 5.5 A, lane **b**) and from leaves taken from a short day control plant on the same day (figure 5.5 A, lane **a**). There are no apparent differences. There were also no differences seen when comparing *in vitro* translation products synthesized from mRNA extracted from leaves of plants exposed to two inducing cycles (figure 5.5 B, lane **a**) or three inducing cycles (figure 5.5 B, lane **c**) to *in vitro* translation products synthesized from mRNA extracted from leaves of short day control plants (figure 5.5 B, lane **b**). It may seem that the amount of a translation product of approximately 28 kD is slightly greater in the induced samples (figure 5.5 A, lane b and figure 5.5 B, lanes a and c) as compared to the two control lanes (figure 5.5 A, lane a and figure 5.5 B lane b), but in other experiments the apparent amount of this product was seen to fluctuate regardless of the type of sample (data not shown) and is, therefore, presumably a translational artefact.

5.2.3 *In vitro* translation analysis of total RNA (two-dimensional SDS-PAGE)

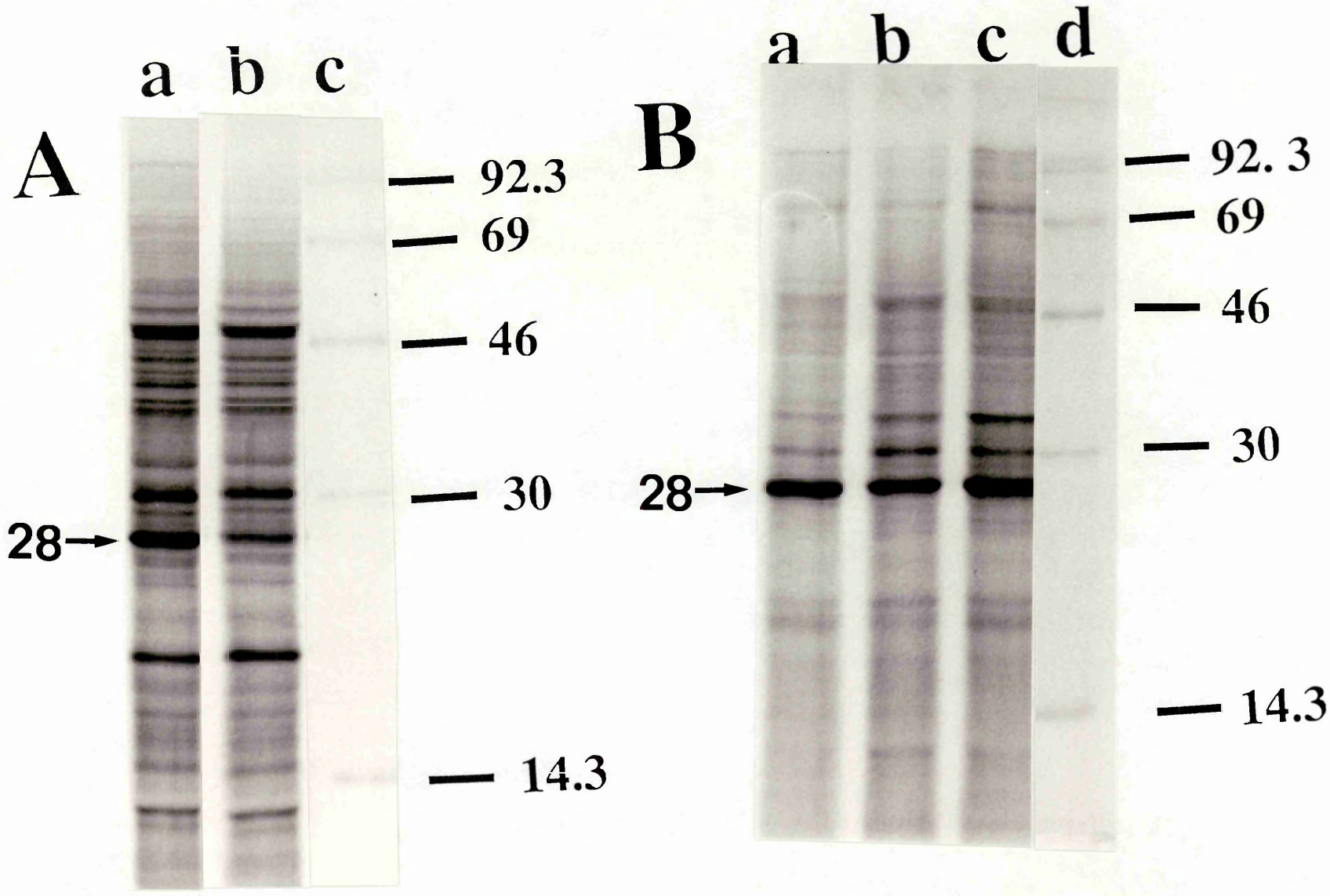
Since a greater number of *in vitro* translation products could be visualised using two-dimensional SDS-PAGE, it was thought that an attempt should be made to employ

Figure 5.5 A *In vitro* translation analysis of *Anagallis arvensis* gene expression during photoperiodic floral induction by one long day using one-dimensional SDS-PAGE

Leaves were harvested from *Anagallis arvensis* plants (section 2.3.4) growing in short day conditions (section 2.3.4) and from leaves of plants exposed to one one day (section 2.3.4) Total RNA was extracted from the leaves (section 2.4.2) and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using PPO (section 2.8.1) and autoradiography, (section 2.9). The figure shows an autoradiogram depicting a 12 % one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Anagallis arvensis* mRNA extracted from: (lane a) short day control leaves, (lane b) leaves exposed to one long day. Lane c contains ^{14}C - methylated protein molecular mass markers with sizes (in kD) indicated to the right.

Figure 5.5 B *In vitro* translation analysis of *Anagallis arvensis* gene expression during photoperiodic floral induction by two and three long days using one-dimensional SDS-PAGE

Leaves were harvested from *Anagallis arvensis* plants (2.3.4) growing in short day conditions (2.3.4) and from plants exposed to one and three cyles of long days (section 2.3.4) . Total RNA was extracted (section 2.4.2) and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9). The figure shows an autoradiogram depicting a 12 % one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Anagallis arvensis* mRNA extracted from: (lane a) leaves exposed to two cycles of long days, (lane b) short day control leaves (lane c) leaves exposed to three cycles of long days. Lane d contains ^{14}C - methylated protein molecular mass markers with sizes (in kD) indicated to the right.


A
a
b
c
92.3
69
46
30
28
14.3
B
a
b
c
d
92. 3
69
46
30
28
14.3

this technique before concluding that no changes in gene expression could be noted when using the *in vitro* translation technique. When two-dimensional SDS-PAGE was carried out on *in vitro* translation products synthesized by mRNA extracted from leavesof plants exposed to one cycle of long day treatment and from leaves of short day control (figure 5.6), there is at least one protein spot of molecular mass of 14 - 15 kD seen on the autoradiogram showing the 'induced' products (figure 5.6 A, protein spot #1) which is not present on the autoradiogram displaying control products (figure 5.6 B).

When the experiment was repeated in duplicate, however, the protein product spot patterns and quality were not comparable to the first experiment (figure 5.7). This difference can be partially attributed to the fact that the wheat germ translation system used in the duplicate experiments (figure 5.7) was a commercial preparation obtained from Amersham International, plc as opposed to the wheat germ extract prepared in the laboratory which had been used throughout this project (see section 3.2.2). One-dimensional analysis of translation products synthesized from the same mRNA used in wheat germ *in vitro* translation systems from two different companies (Amersham and Promega) shows the difference in performance which can result when using a given lot of *Anagallis arvensis* mRNA (figure 5.8).

It is not possible to conclusively note any significant differences between the autoradiograms shown in figure 5.7 because the duplicates themselves are not comparable. This difficulty, similar to the problems associated with work on *Silene coeli-rosa*, indicated that a great deal of time and effort would be required toresolve these problems and allow for routine production of quality autoradiograms.

Figure 5.6 ***In vitro*** **translation analysis of** ***Anagallis arvensis*** **gene expression during photoperiodic floral induction by one long day using two-dimensional SDS-PAGE**

Leaves were harvested from *Anagallis arvensis* plants (section 2.3.4) growing in short day conditions (section 2.3.4) and from plants exposed to one long day (section 2.3.4). Total RNA was extracted (section 2.4.2) and used in a wheat germ extract *in vitro* translation reaction (section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 1.5 x 10^6 precipitable cpm of each sample onto the first dimension of a two-dimensional SDS-PAGE gel (section 2.7.2). Completion of the second dimension was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9). The figure shows an autoradiogram depicting a one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Anagallis arvensis* mRNA extracted from: (A) leaves exposed to one long day, (B) short day control leaves. Protein molecular mass sizes (in kD) are indicated to the right. The basic and acidic ends of the gel are indicated with a + and a - , respectively. The protein of interest is indicated by an arrow and the number 1.

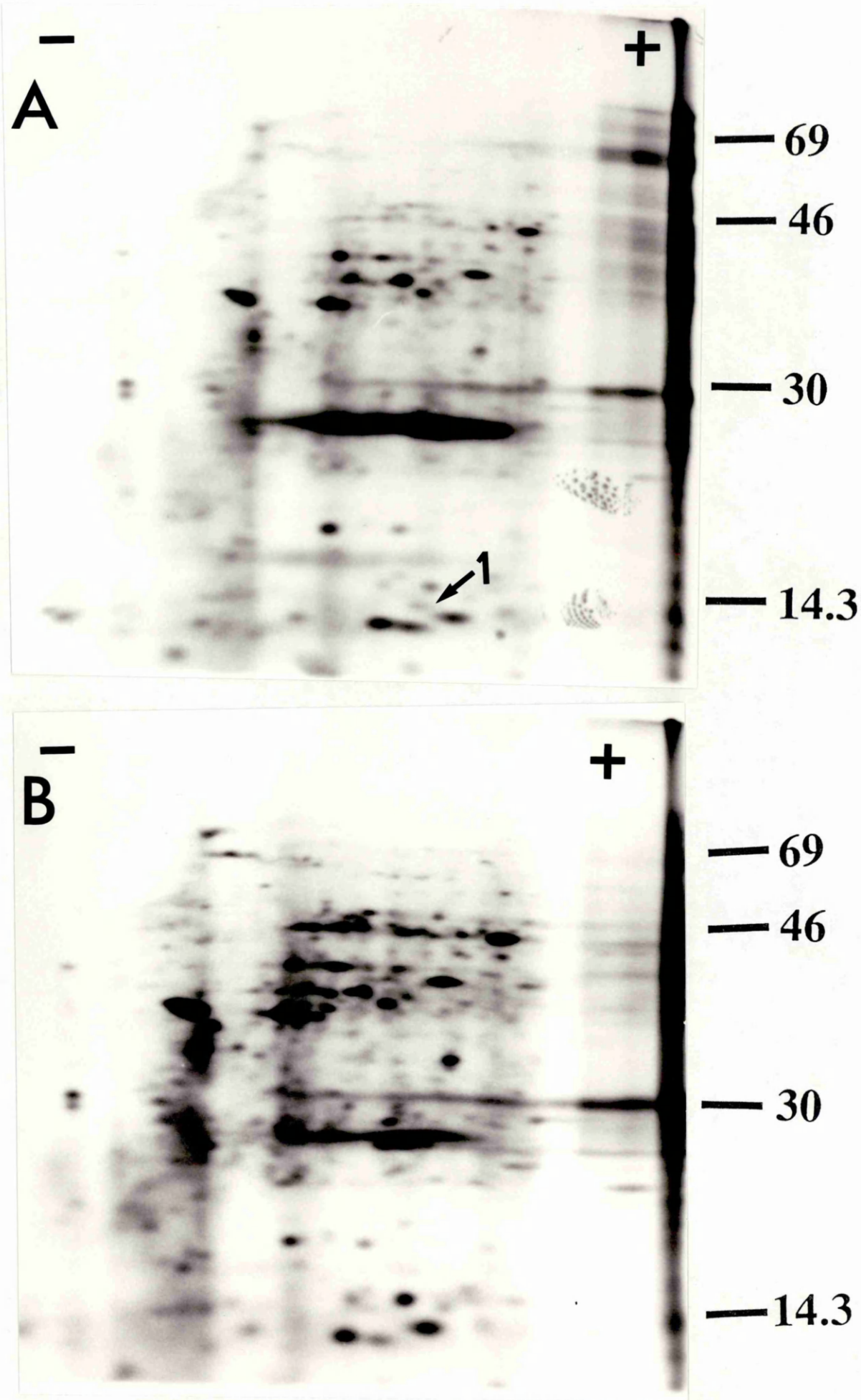

A
−
+
69
46
30
1
14.3
B
−
+
69
46
30
14.3

Figure 5.7 *In vitro* translation analysis of *Anagallis arvensis* gene expression during photoperiodic floral induction by one long day using two-dimensional SDS-PAGE of translation products synthesized from duplicate mRNA samples

Leaves were harvested from *Anagallis arvensis* plants (section 2.3.4) growing in short day conditions (section 2.3.4) and from plants exposed to one long day (section 2.3.4). Total RNA was extracted (section 2.4.2) in duplicate and used in a wheat germ extract *in vitro* translation reaction (wheat germ extract from Amersham, International, plc - section 2.5.3). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 1.5 x 10^6 precipitable cpm of each sample onto the first dimension of a two-dimensional SDS-PAGE gel (section 2.7.2). Completion of the second dimension was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9). The figure shows 4 autoradiograms depicting 12 % two-dimensional SDS-PAGE gels of *in vitro* translation products synthesized from *Anagallis arvensis* mRNA extracted from: (A and C) leaves exposed to one long day, (B and D) short day control leaves. The basic and acidic ends of the gel are indicated with a + and a - , respectively.

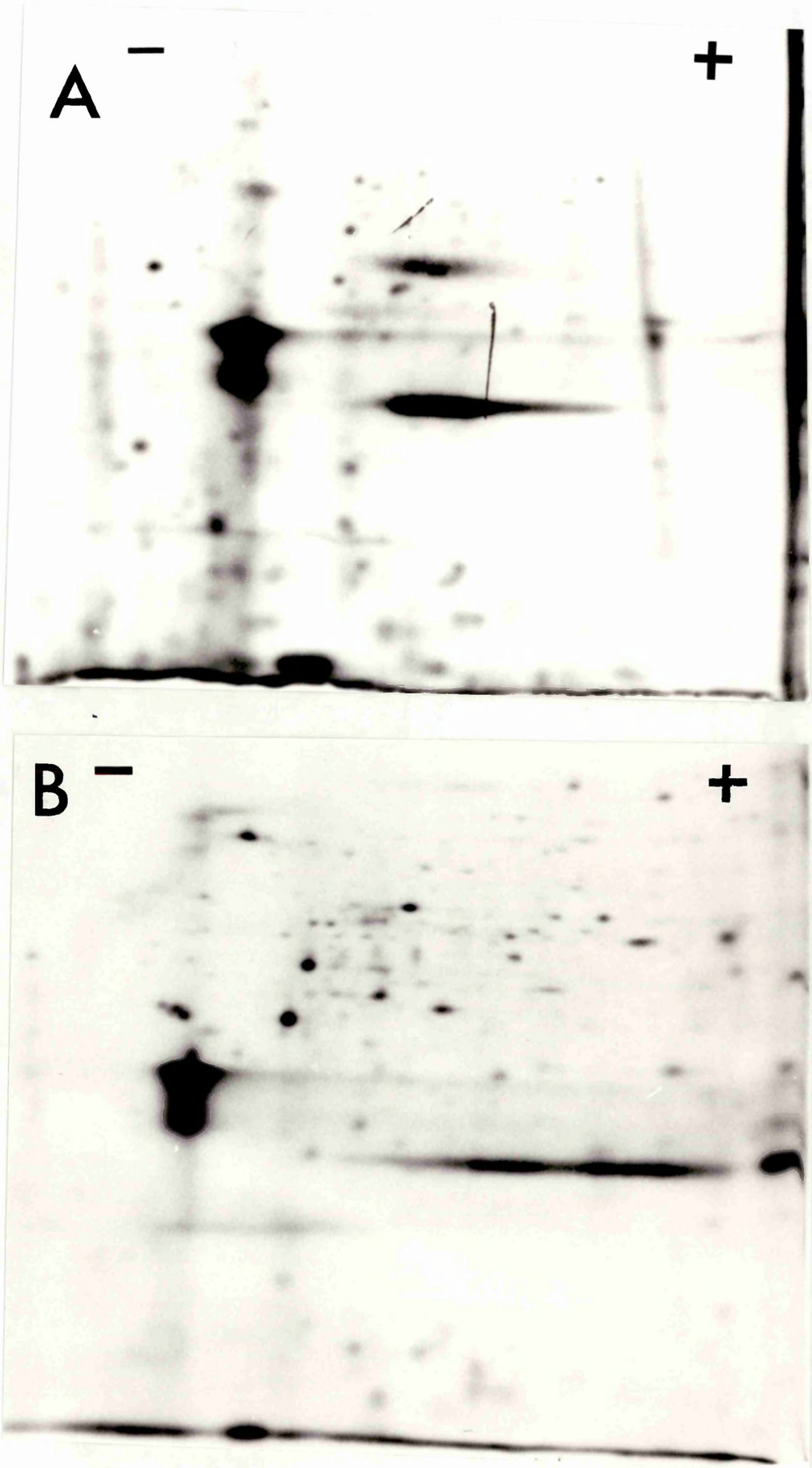
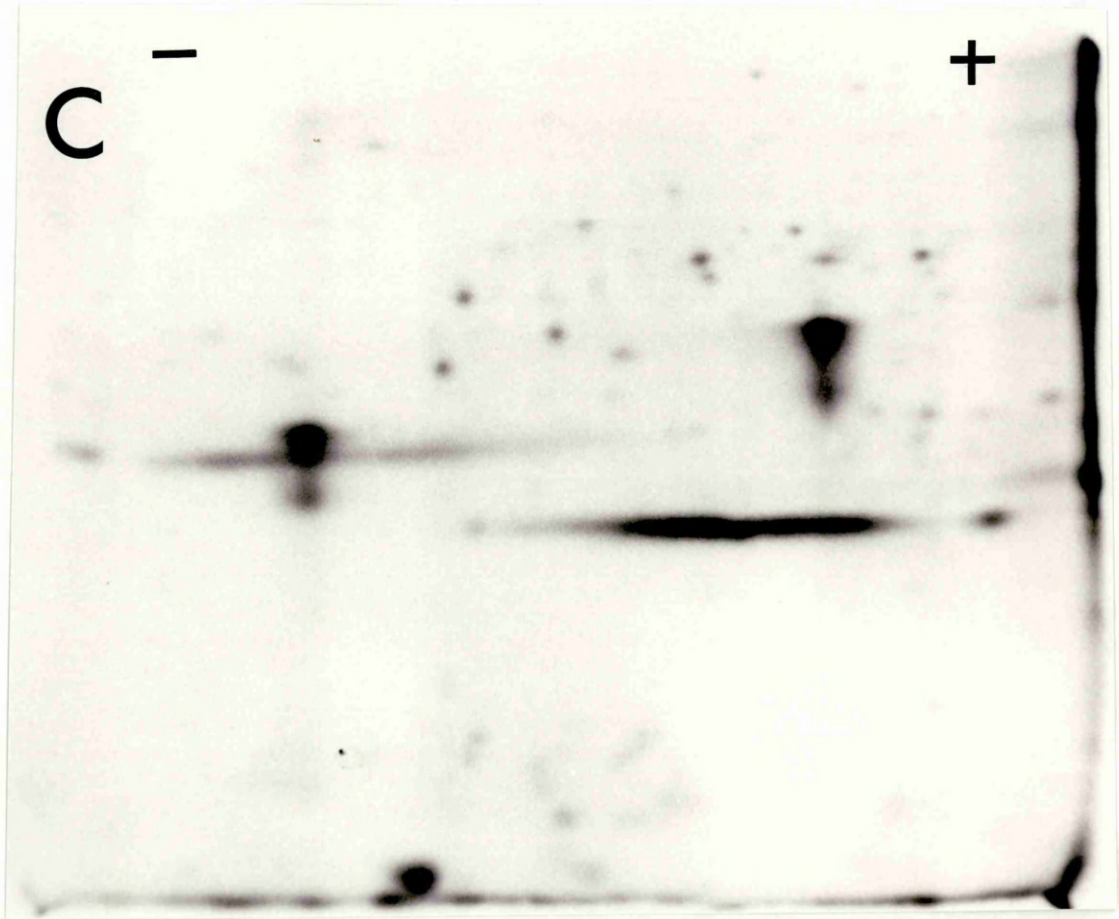

A
−
+
B
−
+

Figure 5.7 (continued)


C
−
+

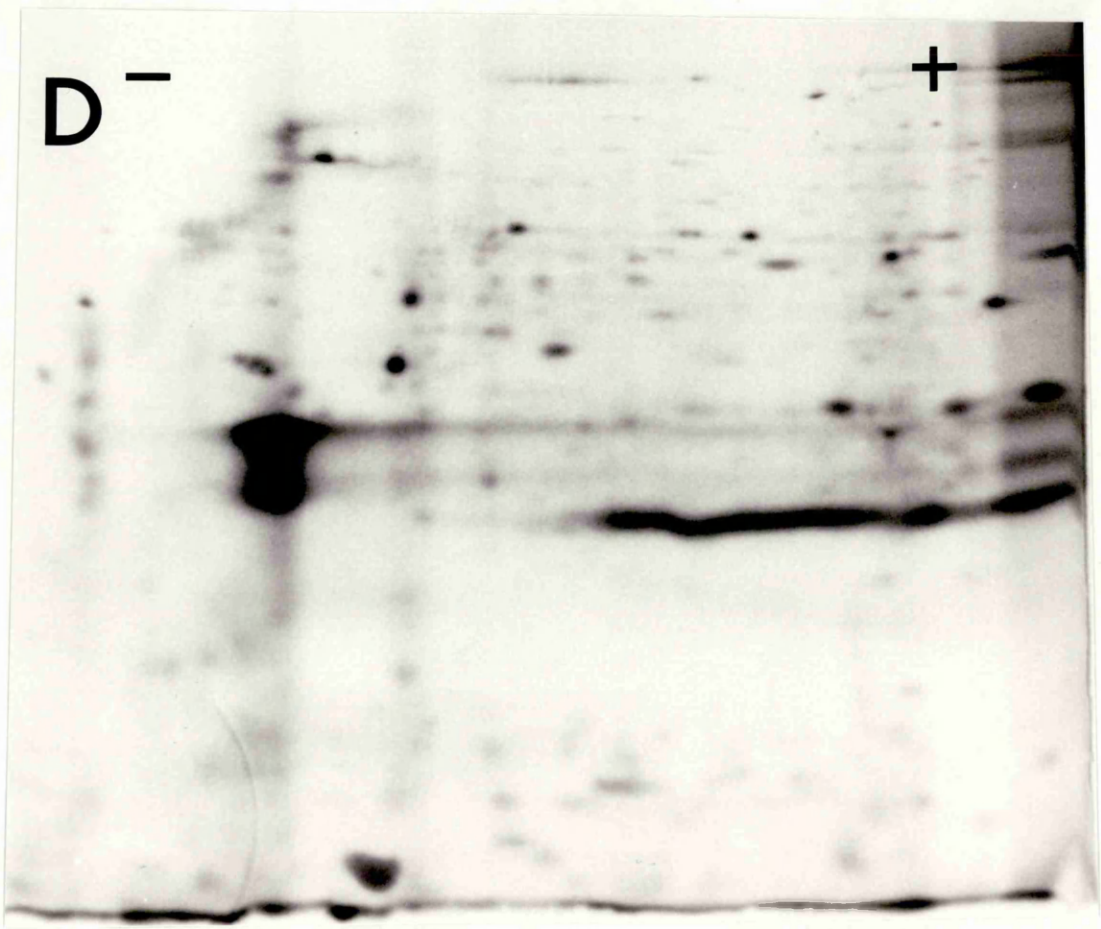
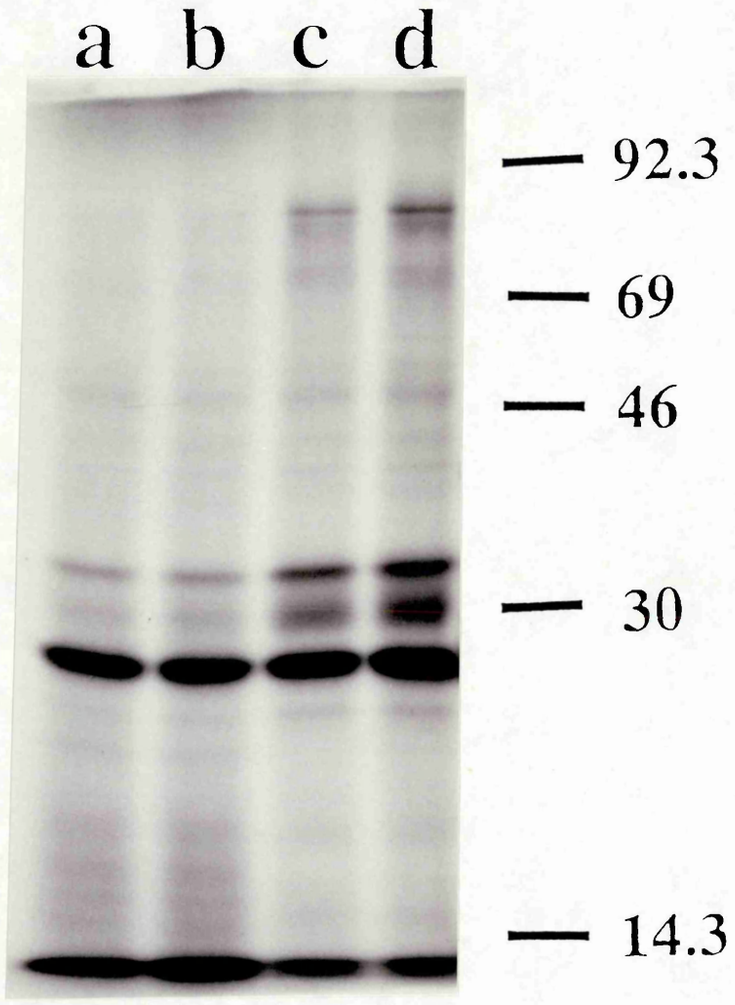

D
−
+

Figure 5.8 Comparison of *in vitro* translation products synthesized by wheat germ extracts purchased from different companies using one-dimensional SDS-PAGE

Leaves were harvested from *Anagallis arvensis* (section 2.3.4) exposed to one long day and total RNA was extracted (section 2.4.2) and used in duplicate *in vitro* translation reactions with wheat germ extract from Amersham International, plc (section 2.5.4) or Promega (section 2.5.5). The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using EN^{3}HANCE™ (section 2.8.1) and autoradiography (section 2.9). The figure shows an autoradiogram depicting a 12 % one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from *Anagallis arvensis* mRNA extracted from leaves exposed to one long day using wheat germ translation systems purchasd from: (lanes a and b) Amersham International plc (lanes c and d) Promega Corporation. Protein molecular mass sizes (in kD) are indicated to the right.


a
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d
92.3
69
46
30
14.3

5.2.4 One-dimensional SDS-PAGE analysis of total protein

The difficulty encountered in using the *in vitro* translation technique and the desire to investigate gene expression at both the mRNA and protein levels necessitated theinvestigation of total protein present during induction. Figure 5.9 shows that when comparing total protein extracted from leaves of plants immediately after receiving one long day (8 hours of high intensity light followed by 16 hours of dim light) and total protein extracted from short day (8 hours of high intensity light) control plants on one-dimensional SDS-PAGE, no difference in protein composition can be detected when either a Coomassie blue stain (figure 5.9, lanes a and b) or a more sensitive silver stain procedure (figure 5.9 c and d) is used.

5.2.5 Two-dimensional SDS-PAGE analysis of total protein

As with the *in vitro* translation approach, the need to visualise as many proteins as possible was obvious before concluding that there were no apparent differences in protein composition in induced and control leaves. It was found that two-dimensional SDS-PAGE was more efficiently and reliably carried out on total protein samples as opposed to *in vitro* translation products. Figure 5.10 shows a set of six two-dimensional SDS-PAGE gels representing two duplicate extractions of total protein from leaves of plants growing in short days (figures 5.10 A and 5.10 D), induced by one long day (figures 5.10 B and 5.10 E), or induced by three cycles of long days (figures 5.10 C and 5.10F). There are at least three differences that can be noted when comparing the gels of short day control protein with the two sets of induced protein.

Figure 5.9 Analysis of total protein extracted from leaves of *Anagallis arvensis* during photoperidic floral induction by one long day using one-dimensional SDS-PAGE

Protein extracts were made from leaves (section 2.6.1) of *Anagallis arvensis* plants growing in short day conditions (section 2.3.4) and from plants exposed to one long day (section 2.3.4). The figure shows a silver stained (section 2.10.2) 12 % one-dimensional SDS-PAGE gel (section 2.7.1) of *Anagallis arvensis* total protein extracted from short day control leaves (lanes a and c) and from leaves exposed to one long day (lanes b and d). Lanes a and b are stained with Coomassie blue and lanes c and d are stained with silver. Lane e contains protein molecular mass markers with sizes (in kD) indicated to the right.

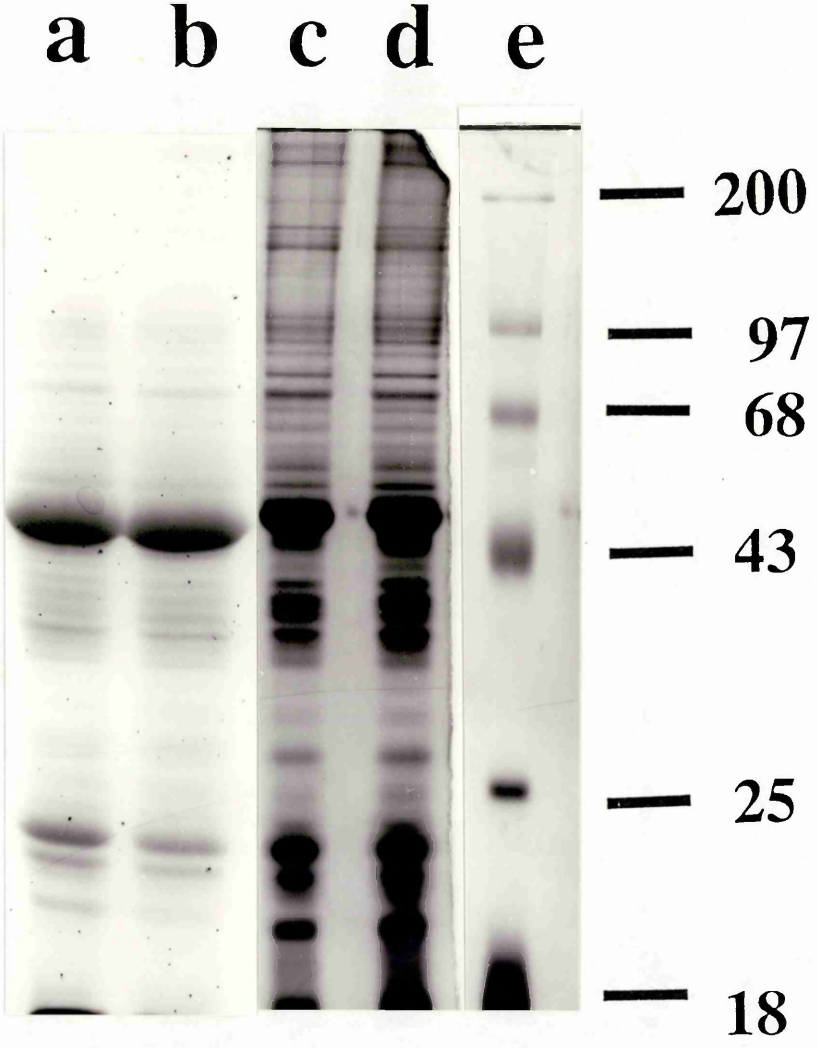
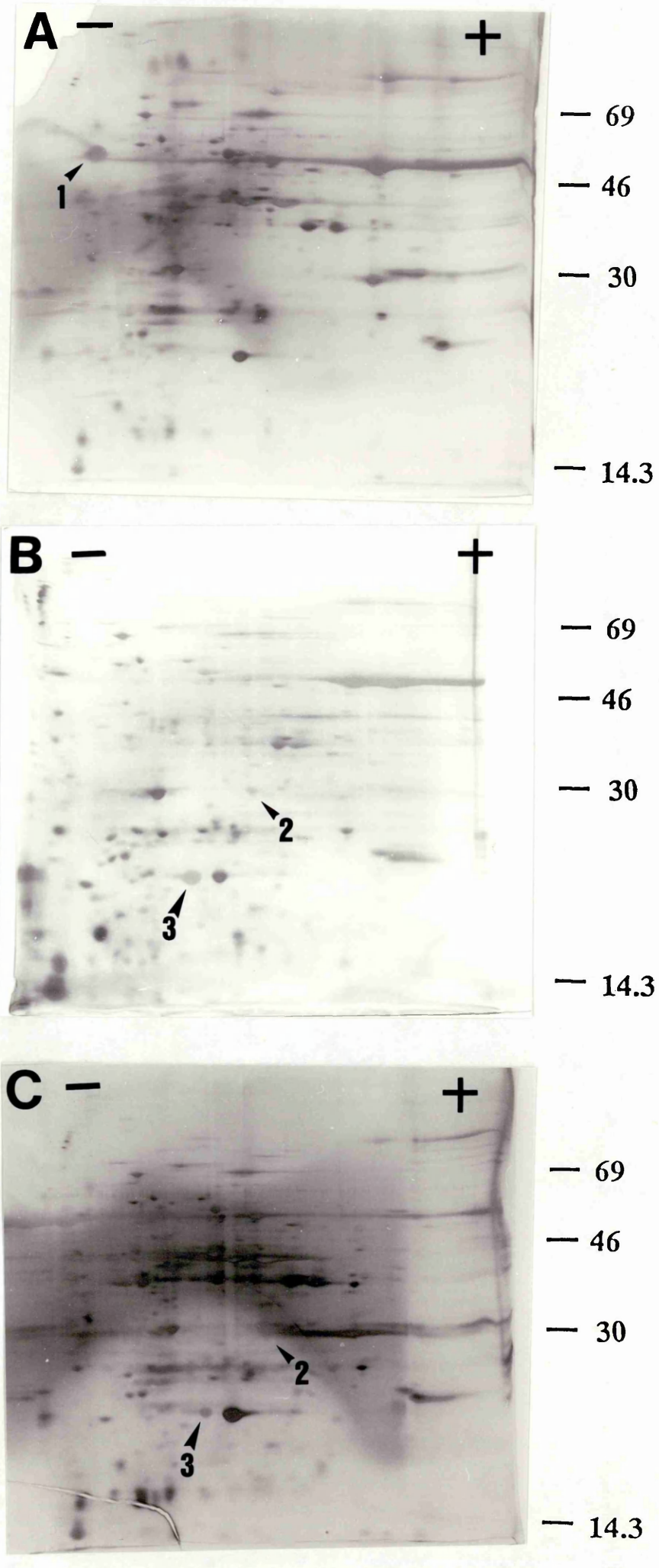

a
b
c
d
e
200
97
68
43
25
18

Figure 5.10 Analysis of total protein extracted from leaves of *Anagallis arvensis* during photoperiodic floral induction by one or three long days using two-dimensional SDS-PAGE

Duplicate protein extracts were made from leaves (section 2.6.2) of *Anagallis arvensis* plants growing in short day conditions (section 2.3.4) and from plants exposed to one long day (section 2.3.4) or three long days. The figure shows silver stained (section 2.10.2) two-dimensional SDS-PAGE gels (section 2.7.2) of duplicate samples from: (A and D) short day control leaves, (B and E) leaves exposed to one long day, (C and F) leaves exposed to three long days. Protein molecular mass sizes (kD) are indicated to the right. Proteins of interest are indicated with arrows and numbers. The basic and acidic ends of the gel are indicated with a + and a - , respectively.


A
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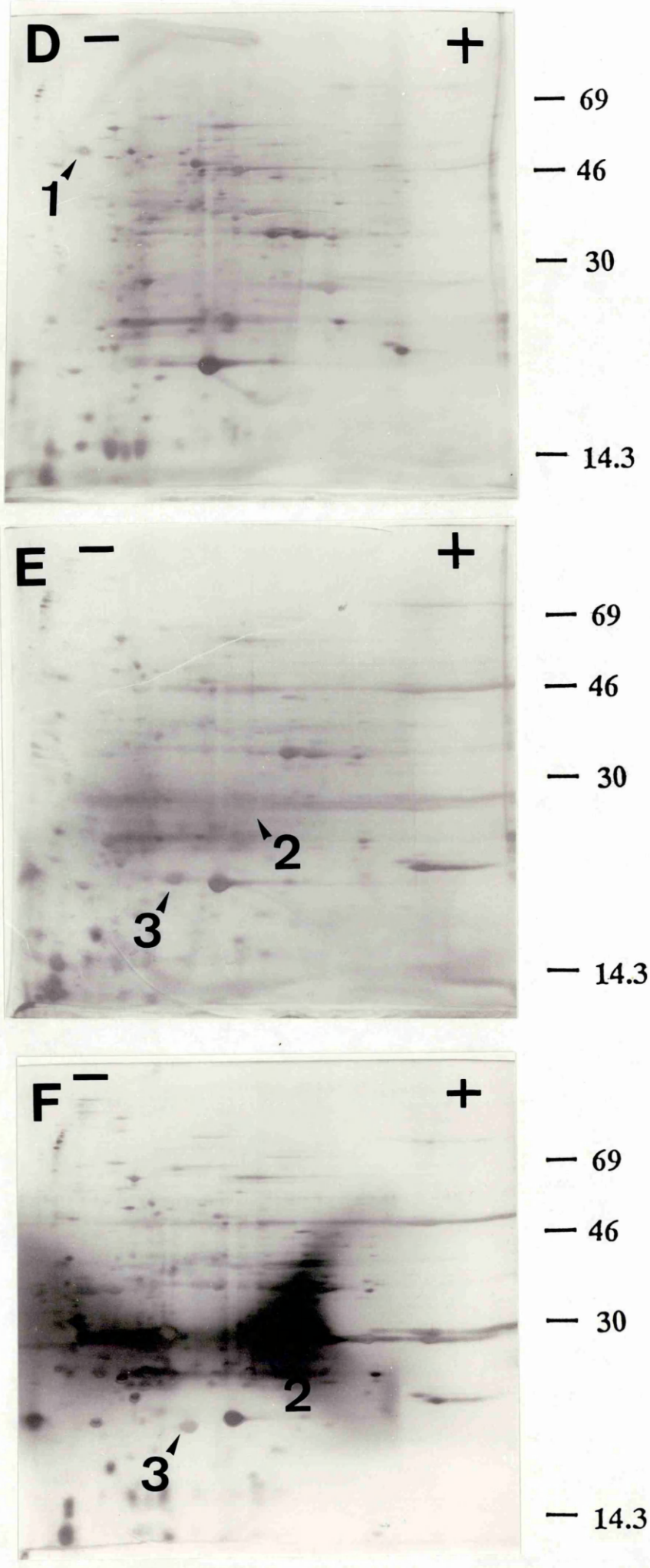
+
1
69
46
30
14.3
B
−
+
2
3
69
46
30
14.3
C
−
+
2
3
69
46
30
14.3


D
−
+
1
69
46
30
14.3
E
−
+
2
3
69
46
30
14.3
F
−
+
2
3
69
46
30
14.3

Figures 5.10 A and 5.10 D show a high molecular mass protein of approximately 57.5 kD (protein # 1) which is present significantly in the short day control protein and not apparent in either of the induced sets of protein (figures 5.10 B, C, E, and F). There are also two proteins with molecular masses of approximately 33 kD and 21.5 kD (proteins # 2 and # 3, respectively) which are represented in significant amounts in protein extracts from induced plants but negligible in protein extracts from short day control plants.

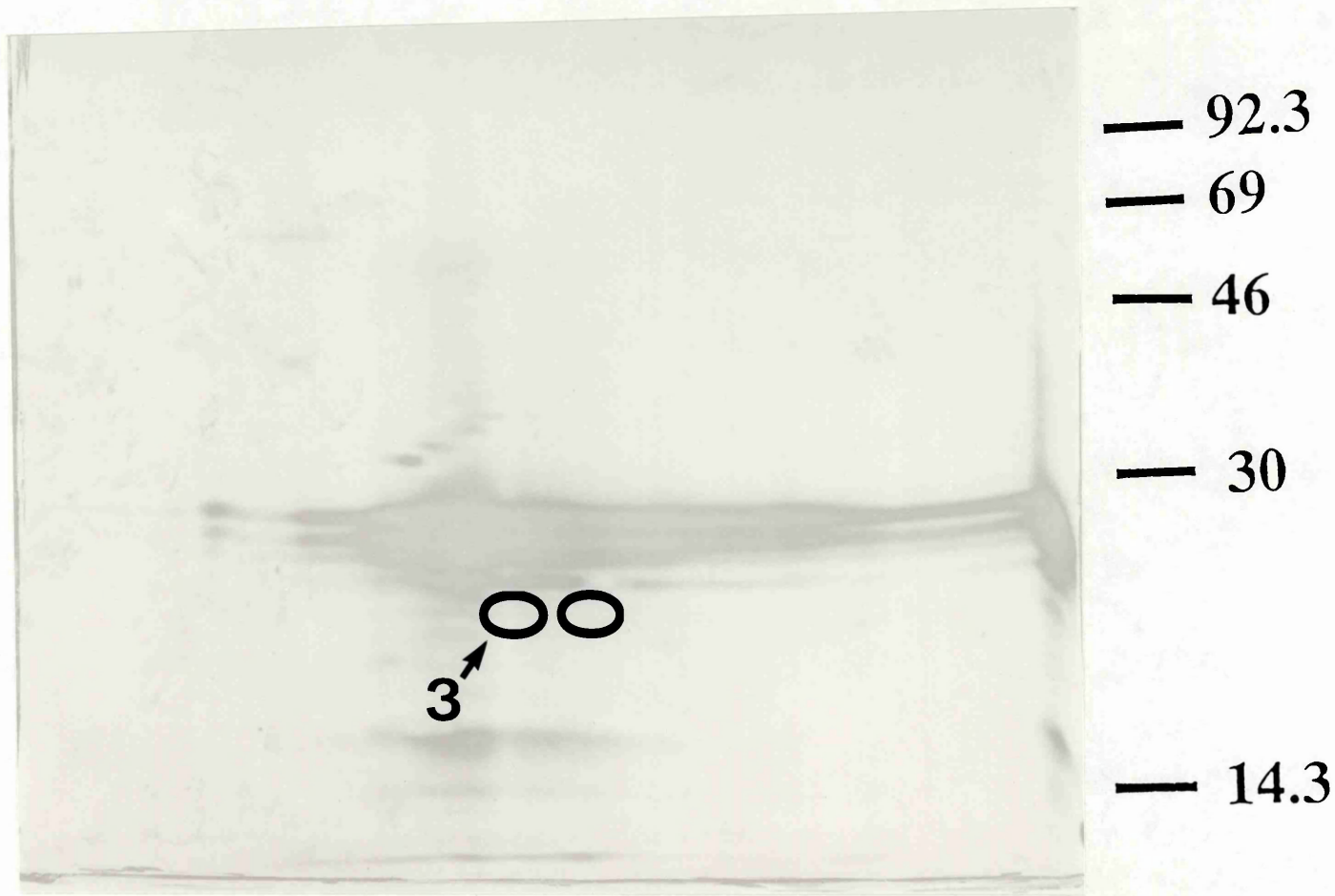
5.2.6 Western blotting with an antiserum against chlorophyll a/b binding protein

Light-harvesting chlorophyll a/b binding protein is the major component of light harvesting complex II in the chloroplast thylakoid membranes. This protein can become phosphorylated under certain light conditions (Bennett *et al.*, 1980), and it was thought that protein #3 (figures 5.10 B, C, E, and F) might represent the phosphorylated protein with the unphosphorylated form appearing directly to the right (in the positive direction). Figure 5.11 shows the activity of the chlorophyll a/b binding protein antiserum against a nitrocellulose blot of a two-dimensional SDS-PAGE gel containing total protein extracted from leaves exposed to one long day. The region of the blot which should contain protein #3 and the protein just to the right of it is indicated with open circles. It can therefore be concluded that neither protein #3 or the protein directly to the right of it (figures 5.11 B, C, E, and F) are forms of the light harvesting chlorophyll a/b binding protein.

5.2.7 Preparation of polyclonal antiserum against total protein from

Figure 5.11 Analysis of chlorophyll a/b binding protein antiserum activity against total protein extracted from *Anagallis arvensis* leaves exposed to one long day using two-dimensional SDS-PAGE and western blotting procedures

A protein extract was made from leaves (section 2.6.1) of *Anagallis arvensis* plants exposed to one long day (section 2.3.4). Proteins were separated on a 12 % two-dimensional SDS-PAGE gel (section 2.7.2) and then transferred to a nitrocellulose membrane (section 2.13.1). The nitrocellulose membrane was incubated with antiserum (2.13.2) against the chlorophyll a/b binding protein and binding of the antiserum was detected as described in section 2.13.2 using orthodianisidine as a chromogen. The area on the blot which would contain protein #3 and the protein to the right of it (in the positive direction) is indicated with two adjacent circles. An arrow and number indicate which circle represents protein #3. Protein molecular mass sizes (in kD) are indicated to the right.


92.3
69
46
30
14.3
3

induced plants

. The data presented in section 5.2.6 demonstrated using duplicate protein extracts that exposure to one or three long days results in the accumulation of at least two proteins in the leaves of *Anagallis arvensis*. These observations justified consideration of experiments designed to facilitate the isolation of cDNA clones corresponding to the proteins which appear in induced leaves (proteins #2 and #3, figures 5.10 B, C, E, and F), and of the approaches detailed in section 1.8.1.3, the immunoadsorption and screening procedure was deemed appropriate in the attempt to satisfy this goal. This technique would hopefully produce an antiserum which specifically recognized proteins #2 and #3 and could be used in the screening of a cDNA expression library.

The first step in this technique is to raise an antiserum in rabbits (see section 2.11) against a total protein extract containing the proteins of interest. Prior to injection into rabbits, it was necessary to check that the extract preparation was not degraded in any way. Figure 5.12 shows that the two extracts, one from leaves of plants induced by one cycle of long days and the other from leaves of plants induced by three cycles of long days, appear normal and not visibly degraded on a one-dimensional silver stained SDS-PAGE gel.

After the serum was obtained from the rabbits, it was used to probe a nitrocellulose blot of a one-dimensional SDS-PAGE gel with lanes containing total protein from the leaves of short day control plants and total protein obtained from the leaves of plants induced by one long day cycle. Figure 5.13 shows that MB-1 recognizes a full range of

Figure 5.12 Analysis of total protein preparations used in MB-1 antiserum production using one-dimensional SDS-PAGE

Protein extracts were prepared from the leaves of *Anagallis arvensis* for injection into rabbits (section 2.11.1) The figure shows a silver stained (section 2.10.2) 10% one-dimensional SDS PAGE gel (section 2.7.1) of *Anagallis arvensis* total protein extracted from: **(lane a)** leaves exposed to one long day, **(lane b)** leaves exposed to three long days. **Lane c** contains protein molecular mass markers with sizes (in kD) indicated to the right.

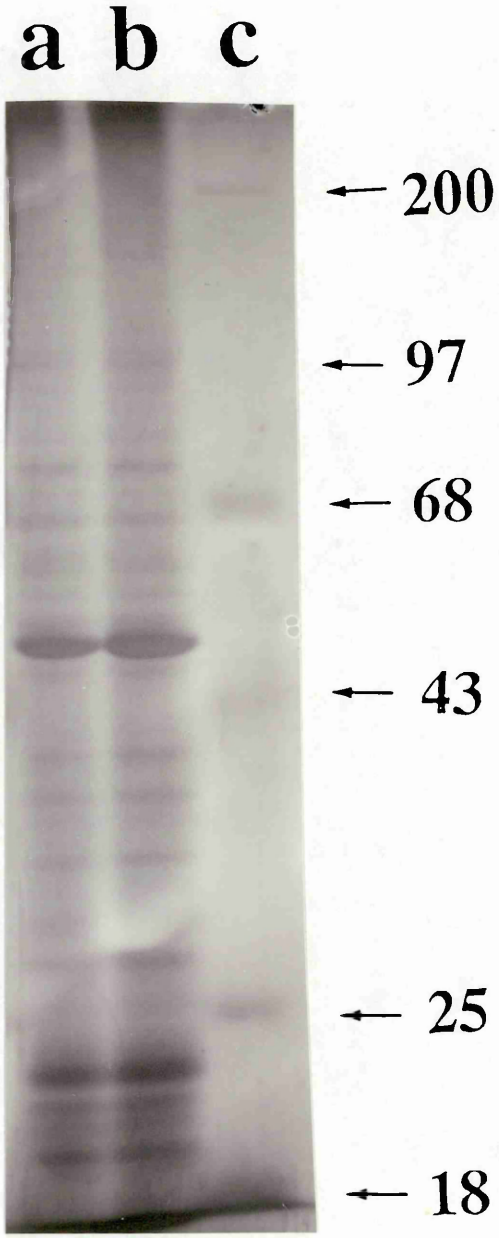
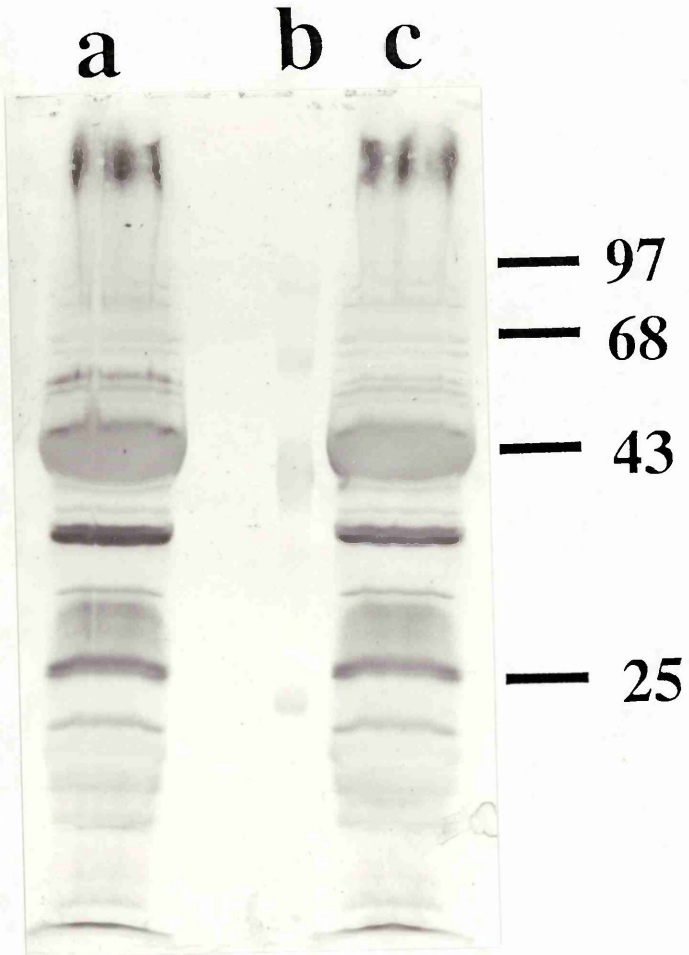

a b c
200
97
68
43
25
18

Figure 5.13 Analysis of MB-1 antiserum activity against protein from leaves exposed to one long day and protein from leaves of short day control plants using one-dimensional SDS-PAGE and western blotting procedures

Protein extracts were made from leaves (section 2.6.1) of *Anagallis arvensis* plants growing in short day conditions (section 2.3.4) and from plants exposed to one long day (section 2.3.4). Proteins were separated on a one-dimensional SDS-PAGE gel (section 2.7.1) and then transferred to a nitrocellulose membrane (section 2.13.1). The nitrocellulose membrane was incubated with MB-1 antiserum and binding of the antiserum to proteins was detected as described in section 2.13.2 using orthodianisidine as a chromogen. The figure shows the resulting western blot using MB-1 antiserum of a 12 % one-dimensional SDS-PAGE gel containing total protein extracted from leaves of *Anagallis arvensis* exposed to: (lane a) short days only, (lane c) one long day. Lane b contains protein molecular weight markers with sizes (in kD) indicated to the right.


a
b
c
97
68
43
25

proteins from both control leaves (figure 5.13, lane 2) and induced leaves (figure 5.13 lane 3).

It was also important to see that there was activity against the proteins of interest. Figure 5.14 shows a section of the gel displayed in figure 5.10 D which had been blotted onto nitrocellulose and probed with the serum made against 'one cycle induced' proteins (this serum will be referred to as MB-1). It is clear that MB-1 contains antibodies against protein #3 but it is difficult to demonstrate reactivity against protein #2.

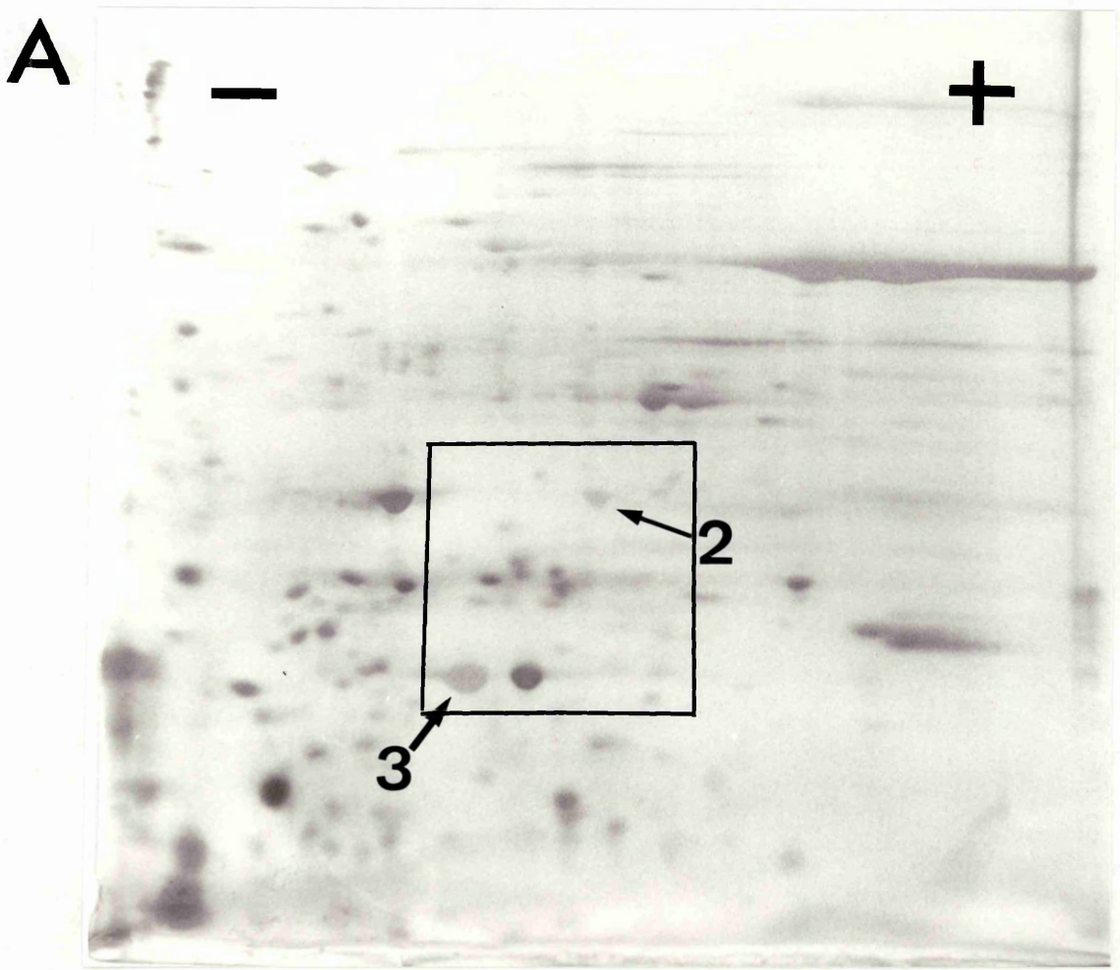
5.2.8 Probing Western blots of one-dimensional SDS-PAGE gels with immunoadsorbed MB-1 using the protocol described by Choi *et al.* (1987)

Using the immunoadsorption protocol described by Choi *et al.* (1987) which involved incubation of the antiserum with strips of nitrocellulose saturated with protein from short day control plants, it was not possible to remove very much of the background immunoactivity. Figure 5.15 shows that when MB-1 was immunoadsorbed by several incubations with nitrocellulose saturated with total protein from the leaves of short day control plants, many of the protein bands seen with unadsorbed MB-1 are still readily visible.

5.2.9 Probing Western blots of one-dimensional SDS-PAGE gels with immunoadsorbed MB-1 using an improved protocol

Figure 5.14 A and B Analysis of MB-1 antiserum activity against 'induced specific' proteins using two-dimensional SDS-PAGE and western blotting procedures

(A) An actual size version of the same two-dimensional SDS-PAGE gel depicted in figure 5.11 B is shown here. The section of the gel containing proteins #2 and #3 (indicated with arrows and numbers) which was removed and transferred to a nitrocellulose membrane (section 2.13.1) is indicated with a box. (B) The nitrocellulose membrane representing the section of the gel containing proteins #2 and #3 which was incubated with MB-1 antiserum (section 2.13.2) is shown here. Binding of MB-1 was detected as described in section 2.13.2 using orthodianisidine as a chromogen. Activity against protein #3 is indicated with an arrow and the number 3.


A
–
+
2
3

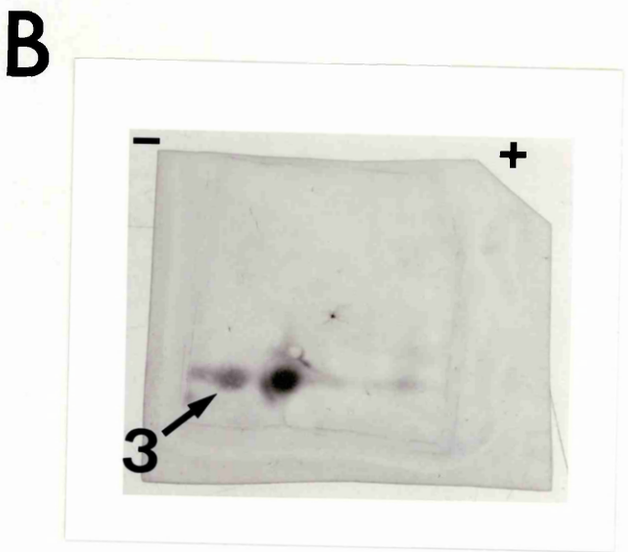
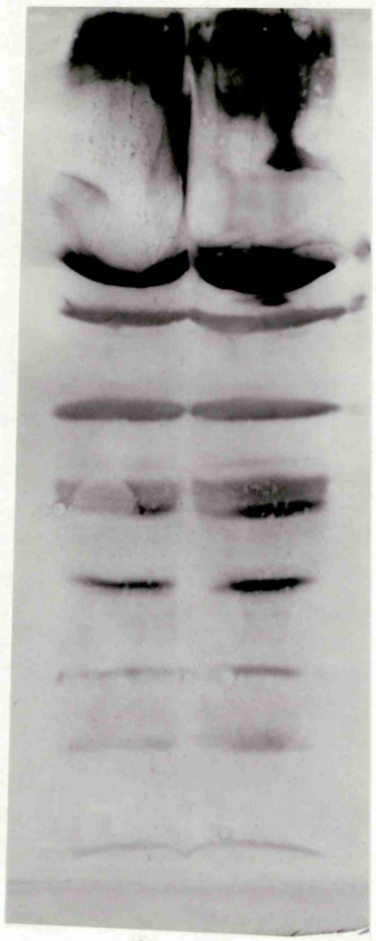

B
–
+
3

Figure 5.15 Analysis of immunoadsorbed MB-1 antiserum activity against protein from leaves exposed to one long day and protein from leaves of short day control plants using one-dimensional SDS-PAGE and western blotting procedures - protocol according to Choi *et al.* (1987)

Protein extracts were made from leaves (section 2.6.1) of *Anagallis arvensis* plants growing in short day conditions (section 2.3.4) and from plants exposed to one long day (section 2.3.4). Proteins were separated on a one-dimensional SDS-PAGE gel (section 2.7.1) and then transferred to a nitrocellulose membrane (section 2.13.1). The nitrocellulose membrane was incubated with immunoadsorbed MB-1 antiserum (section 2.12.1) and binding of the antiserum to proteins was detected as described in section 2.13.2 using orthodianisidine as a chromogen. The figure shows athe resulting western blot using MB-1 antiserum of a 12 % one-dimensional SDS-PAGE gel containing total protein extracted from leaves of *Anagallis arvensis* exposed to: (**lane a**) short days only, (**lane b**)one long day.

a b



The MB-1 serum was not useful using the cited immunoadsorption procedure, so attempts to improve the technique were made by employing the properties of protein A sepharose as described in section 2.12.2. Unfortunately, when the experimental conditions for this technique were optimized, there was still background activity from proteins of high abundance as displayed in figure 5.16. Also, no differences could be seen between the short day control protein (lane b) and the one cycle induced protein (lane c). Figure 5.17 shows that incubation of the MB-1 serum with protein A sepharose alone does not result in a loss of activity. No further efforts were devoted towards further improvement of the immunoadsorption technique because it was felt that doing so would not be profitable and the time remaining would be more wisely spent constructing and differentially screening a cDNA library.

5.2.10 cDNA library construction

5.2.10.1 Synthesis of cDNA and cloning into λgt10

The lack of success in visualising changes in gene expression using the *in vitro* translation approach and the indication that some genes are likely to be differentially expressed as indicated by the total protein analysis implied that the use of a more sensitive and direct approach would need to be taken. Construction and screening of a cDNA library not only provides much more sensitivity for showing differential expression of genes but also yields clones of the genes of interest.

The cDNA library constructed in this work was made using polyadenlyated mRNA

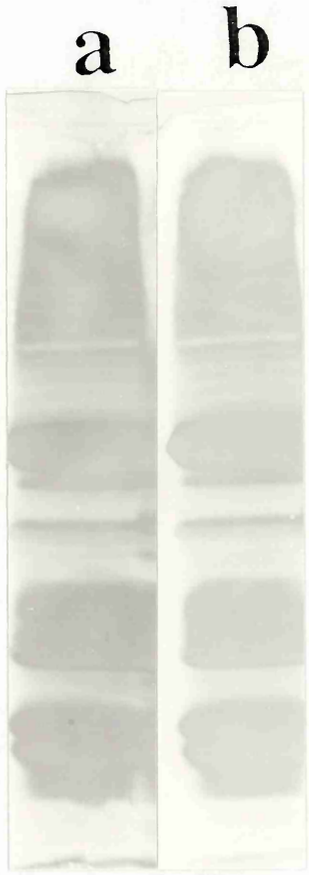
Figure 5.16 Analysis of immunoadsorbed MB-1 antiserum activity against protein from leaves exposed to one long day and protein from leaves of short day control plants using one-dimensional SDS-PAGE and western blotting procedures - improved protocol

Protein extracts were made from leaves (section 2.6.1) of *Anagallis arvensis* plants growing in short day conditions (section 2.3.4) and from plants exposed to one long day (section 2.3.4). Proteins were separated on a one-dimensional SDS-PAGE gel (section 2.7.1) and then transferred to a nitrocellulose membrane (section 2.13.1). The nitrocellulose membrane was incubated (section 2.13.2) with immunoadsorbed MB-1 antiserum (section 2.12.2) produced by incubation with proteins from short day control plants followed by precipitation with protein A-Sepharose®. Binding of the antiserum to proteins was detected as described in section 2.13.2 using orthodianisidine as a chromogen. The figure shows the resulting western blot using immunoadsorbed MB-1 antiserum of a 12 % one-dimensional SDS-PAGE gel containing total protein extracted from leaves of *Anagallis arvensis* exposed to: (lane a) short days only, (lane b) one long day.

a b

Figure 5.17 Effect of incubating MB-1 antiserum with protein A - Sepharose® alone

Protein extracts were made from leaves (section 2.6.1) of *Anagallis arvensis* plants exposed to one long day (section 2.3.4). Proteins were applied in duplicate to a 12 % one-dimensional SDS-PAGE gel (section 2.7.1) and then transferred to a nitrocellulose membrane (section 2.13.1). The nitrocellulose membrane was sectioned into two duplicate portions such that one portion was incubated (section 2.13.2) with untreated MB-1 antiserum or MB-1 antiserum which had been mixed with protein A-Sepharose® without previous immunoadsorption to short day control proteins (2.12.2). The figure shows the resulting western blots incubated with: **(lane a)** untreated MB-1 antiserum, **(lane b)** MB-1 antiserum incubated with protein A - Sepharose® only.


a
b

that was selected on oligo-dT cellulose from total RNA extracted from leaves of plants immediately after exposure to the long day dim light extension period. Prior to use in library construction, the integrity of the polyadenylated mRNA was tested by translating it *in vitro* and the resulting products were analysed by one-dimensional SDS-PAGE. Figure 5.18 shows that 0.5 μg of the polyadenylated mRNA translates with the same efficiency as 5 μg of total RNA and produces a very similar pattern of products.

The mRNA was used to synthesize double stranded cDNA and construct a cDNA library using synthesis and cloning kits purchased from Amersham International, plc. Figure 5.19 shows small aliquots of the [α-^{32}P]dCTP labelled first strand and second strand cDNA synthesis products (yielded from the polyadenylated mRNA) that were used in ligation reactions with the λgt10 cloning vector. The cDNA synthesis reactions produced 1.8 μg of double stranded cDNA from 5 μg of polyadenylated mRNA.

5.2.10.2 cDNA library quality analysis : titration of recombinants

Ligation reactions of the double stranded cDNA with the λgt10 cloning vector were prepared as displayed in table 5.1. The results from the titration experiments are presented in table 5.2. In analyzing theses results, values were compared to those recommended in Amersham kit instruction manuals. The titration results on the L87 *E. coli* strain for reaction #1 , which is recommended to be close to 1 x 10^7, indicates that the ligation conditions were effective and the packaging extracts used worked efficiently. The results also demonstrate that the biological selection of the NM514 *E. coli* strain is

Figure 5.18 *In vitro* translation analysis of total and poly $(A)^+$ mRNA extracted from leaves of *Anagallis arvensis* plants exposed to one long day

Leaves were harvested (section 2.3.4) from *Anagallis arvensis* plants exposed to one long day (2.3.4) and total RNA was extracted from them (section 2.4.2) and used to isolated poly$(A)^+$ mRNA . Both total and poly$(A)^+$ mRNA were used individually in wheat germ extract *in vitro* translation reactions. The *in vitro* translation products were analysed by first determining the TCA precipitable cpm per 2µl of reaction (section 2.5.6) and then by loading 2 x 10^5 precipitable cpm of each sample onto a one-dimensional SDS-PAGE gel (section 2.7.1) which was followed by fluorography using PPO (section 2.8.1) and autoradiography (section 2.9). The figure shows an autoradiogram depicting a 12 % one-dimensional SDS-PAGE gel of *in vitro* translation products synthesized from: (lane a) 5 µg of total RNA , (lane b) 0.5 µg of poly $(A)^+$ mRNA. Lane c contains ^{14}C - methylated protein molecular mass marker with sizes (in kD) indicated to the right.

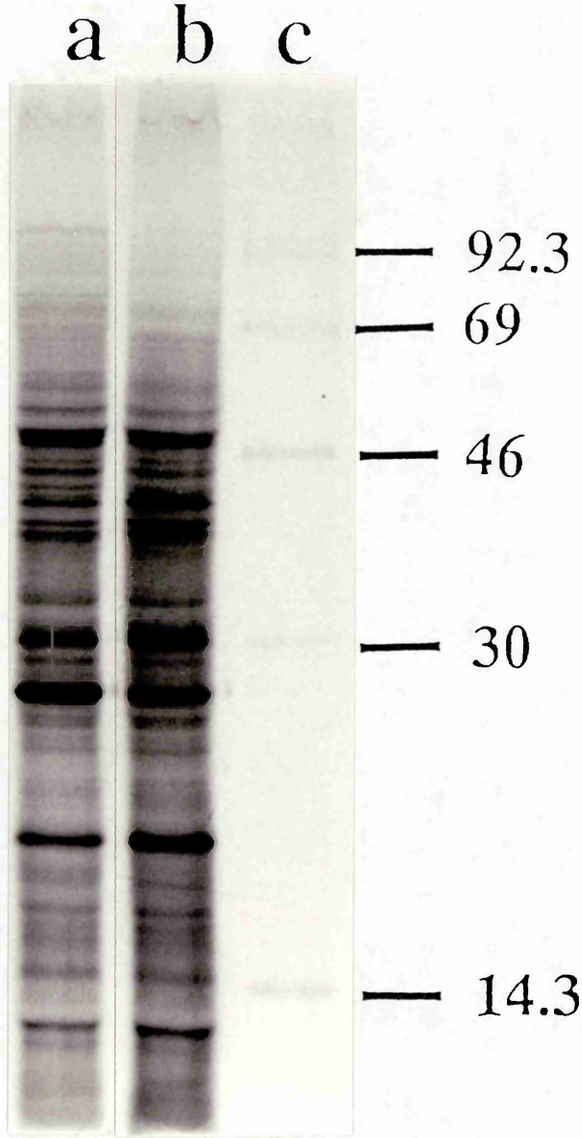

a
b
c
92.3
69
46
30
14.3

Figure 5.19 Visualization of cDNA synthesis products

Anagallis arvensis polyadenylated mRNA was used in a first strand cDNA synthesis reaction as described in section 2.14.2.1. The products of the first strand reaction were used in a second strand reaction as described in section 2.14.2.1. Aliquots of each reaction were purified (section 2.14.2.2) and prepared for electrophoresis (section 2.14.2.4.3) on a 1.5 % alkaline agarose gel (2.14.2.4.4). The figure shows a 1.5 % alkaline agarose gel electrophoresis of cDNA synthesis products resulting from: **(lane a)** the first strand reaction, **(lane b)** the second strand reaction. Molecular size markers (in base pairs) are indicated to the right.

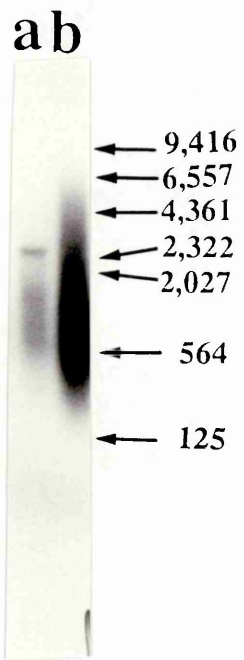

a b
9,416
6,557
4,361
2,322
2,027
564
125

Table 5. 1 Ligation reactions for cDNA library construction

The table presents the amounts of of double stranded cDNA and the amount of λgt10 vector arms present in each ligation reaction. Ligation conditions were as described in section 2.14.3.

Reaction #	Contents
1	1 µg λGT10 arms
2	1 µg λGT10 arms + 50 ng cDNA
3	1 µg λGT10 arms + 100 ng cDNA
4	1 µg λGT10 arms + 150 ng cDNA

Table 5.2 cDNA library titration results

The table presents data collected from plating out dilutions (see section 2.14.4.3) of packaged phage onto the two strains of *E.coli* - L87(wild type) and NM514 (hfl^+). See section 5.2.10.2 for the analysis of the data presented here.

Reaction #	L87 titre pfu/ml	NM514 titre pfu/ml	L87:NM514 ratio	True Background pfu	Total Recombinants pfu	% Recombinants on NM514	Recombinants per µg cDNA pfu
1	8.1×10^6	1.1×10^4	736.4				
2	9.5×10^6	1.2×10^5	79.2	1.3×10^4	1.1×10^5	89	2.14×10^6
3	1.4×10^7	2.1×10^5	66.7	1.9×10^4	1.9×10^5	90	1.91×10^6
4	2.1×10^7	3.8×10^5	55.3	2.8×10^4	3.5×10^5	92	2.35×10^6

effective as indicated by the L87:NM514 ratio for reaction #1 which should be >100 and close to 1000. In determining the efficiency of cDNA reactions #2 through #4, a true background figure was obtained by dividing the L87 titre for the given reaction by the L87:NM514 ratio derived from results in reaction #1. The true background was then subtracted from the NM514 titre to obtain the number of total recombinants in each library (reactions 2 through 4) expressed as pfu (plaque forming units) per ml. It should be noted, however, that the total volume of the libraries was 0.5 ml. Therefore, the number of recombinants that could be obtained from pooling the three libraries would total 3.25×10^5.

5.2.10.3 Physical analysis of cDNA library (reaction #4)

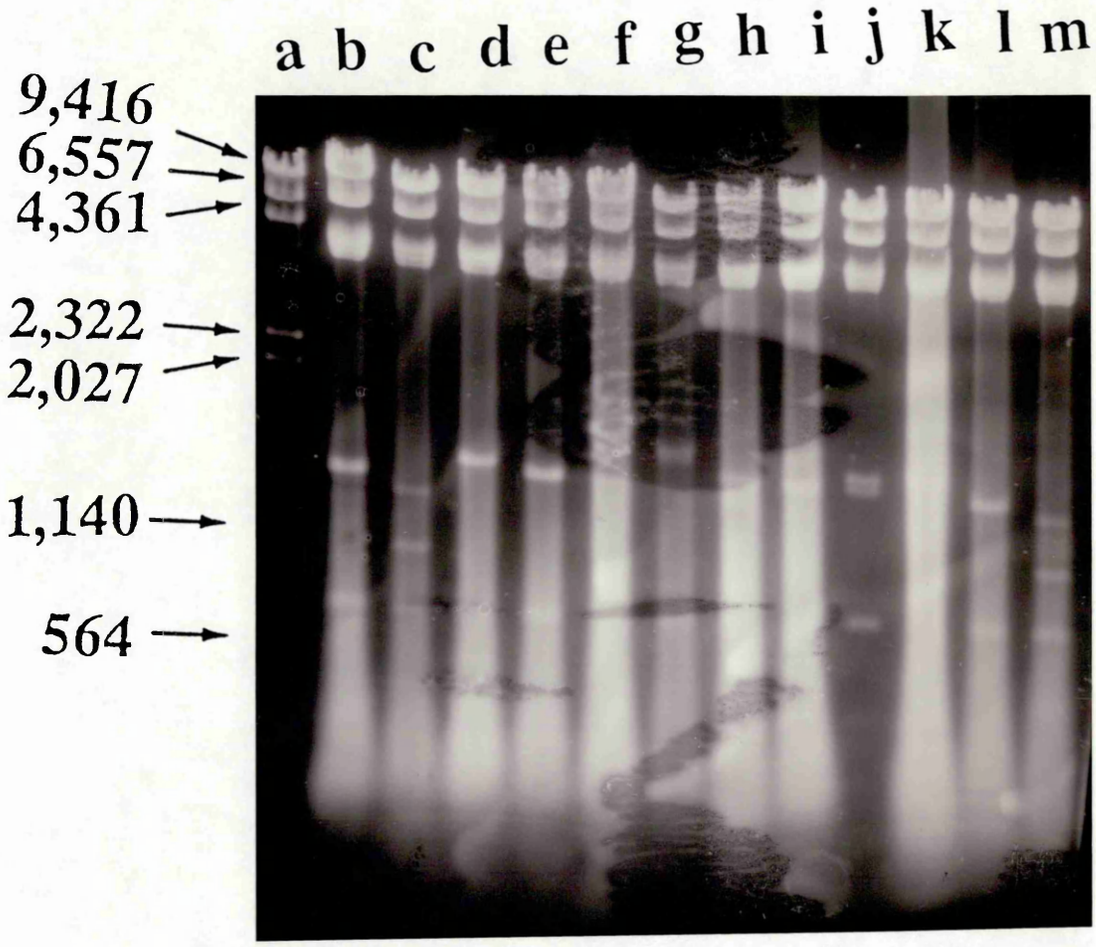
The cDNA library produced from reaction #4 was selected for further analysis based on its superior titration results. Figure 5.20 shows the insert sizes of 10 randomly picked plaques from which DNA was extracted and digested with the restriction enzymes Bgl II and Hind III. The double digest releases a 1.14 kb DNA fragment from the λgt10 vector plus the insert attatched. The size of 1.14 kb DNA fragment will therefore be seen to increase according to the size of the insert. The plaques selected all appear to have an insert ranging in size from 235 bp to 1860 bp.

5.2.11 Differential screening of the *Anagallis arvensis* cDNA library

5.2.11.1 Test of hybridization conditions

Figure 5.20 Physical analysis of recombinants from the cDNA library prepared from *Anagallis arvensis* mRNA extracted from leaves exposed to one long day

Tris-borate agarose gel electrophoresis (section 2.14.4.6) of Bgl II/Hind III double digested λgt10 DNA (section 2.14.4.5) from randomly selected plaques of the *Anagallis arvensis* cDNA library. **Lanes b** through **l** contain the digests of the DNA from the randomly selected plaques, **lane a** contains phage λDNA (Sigma) digested with Hind III producing DNA markers of the sizes (in base pairs) indicated to the left, and **lane m** contains control λgt10 DNA without an insert and therefore displays the 1.14 kb fragment that is released as discussed in the text. Please note that the DNA digests contained in **lanes c** and **m** were accidently mixed and therefore both lanes contain the 1.14 kb fragment and an additional fragment containing the insert seen directly above it.


a b c d e f g h i j k l m
9,416
6,557
4,361
2,322
2,027
1,140
564

Prior to attempting to perform differential screening procedures on the cDNA library, the conditions for the hybridisation were tested. Figure 5.21 shows the results of hybridising a nylon plaque lift filter taken from a plate containing ~2000 plaques with a [α-^{32}P]dCTP labelled cDNA probe derived from a first strand synthesis reaction using either oligo-dT primer (figure 5.21 A) or random hexanucleotide primer (figure 5.21 B). Hybridisation with the oligo-dT primed probe yielded good clean signals of varying intensities (figure 5.21 A). This demonstrates that the hybridisation conditions were satisfactory and nonspecific hybridisation which would have produced equivalent signals from all of the recombinant plaques was sufficiently blocked by the addition of polyadenylic acid. The results produced from hybridisation with the random hexanucleotide primed cDNA probe were poor and, therefore, indicated that this method of probe synthesis should be avoided here.

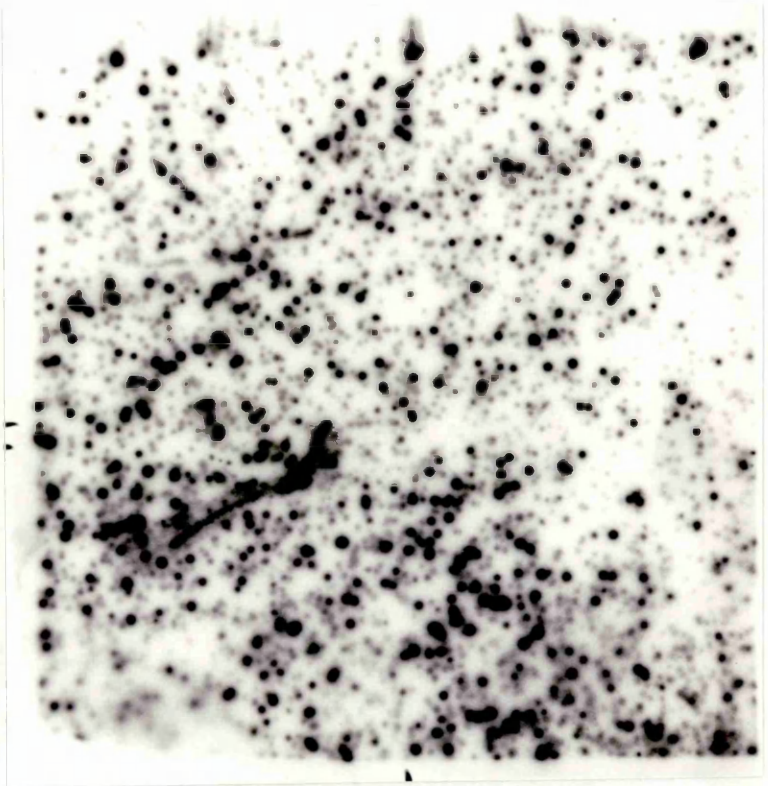
5.2.11.2 Differential screening and selection of positives from the cDNA library

One experiment using differential screening involved making four identical plaque lift filters from each of eight L-agarose plates containing 1500 plaques such that two of the filters were incubated with 'control' probe (single stranded cDNA synthesized from mRNA extracted from the leaves of short day control plants) and the other two filters were incubated with 'induced' probe (single stranded cDNA synthesized from mRNA extracted from the leaves of plants induced with one long day). Comparison of autoradiograms produced by all four filters allowed for the selection of 13 positive plaques which displayed a significantly greater signal on filters that were hybridised

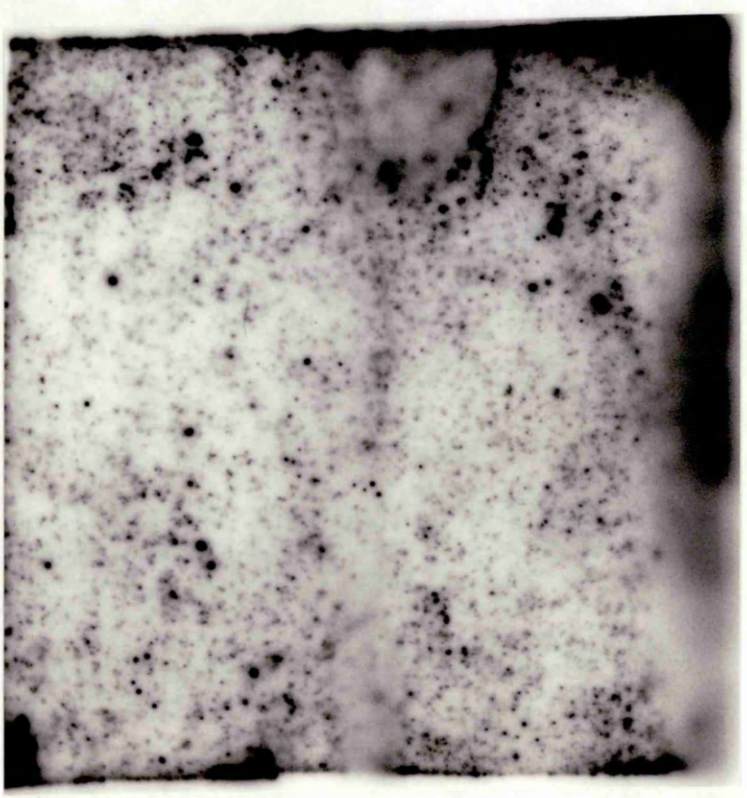
Figure 5.21 Comparison of hybridisation efficiency using cDNA probes prepared with oligo-dT or random hexanucleotide primers.

Nylon membranes were used to take duplicate plaque lift filters from a plate containing 2000 plaques from the *Anagallis arvensis* cDNA library as described in section 2.15.1. Hybridisations were carried out as described in section 2.15.3 of the plaque lift filters with cDNA probes prepared as described in section 2.15.2 using: (A) oligo-dT primer, (B) random hexanucleotide primer.

A



B



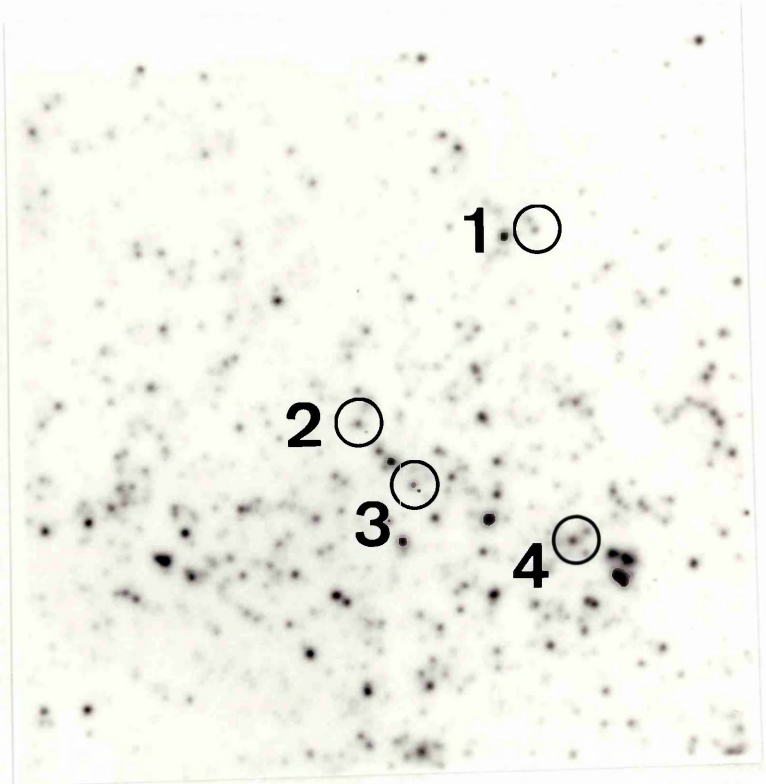
with the 'induced' probe. Figures 5.22 through 5.25 show autoradiograms of the plaque lift filters (from four of the eight plates) indicating the positives which were selected (the duplicates are not shown nor are the other autoradiograms of the plaque lift filters from the other four plates which did not display any differential signals).

The positive plaques were isolated and replated onto individual L-agarose plates from which were taken four identical plaque lift filters to use in secondary screening. However, because of background difficulties which were inexplicably encountered with secondary hybridisations and time constraints, five of the best positives (positives #3, 4, 7, 9, 10) were pooled on one plate and the plaque lift filters were screened with cDNA probes synthesised from the remaining polyadenylated mRNA. This probe was also used to screen plaque lift filters taken from a plate containing plaques obtained from the positive #7 considered to give the strongest differential signal in the first screen. The results of this screen of mixed positives and positive #7 are shown in figures 5.26 and 5.27, respectively. Unfortunately, neither filters showed any plaques displaying a differential signal when comparing the duplicate incubated with induced probe with the duplicate incubated with short day control probe.

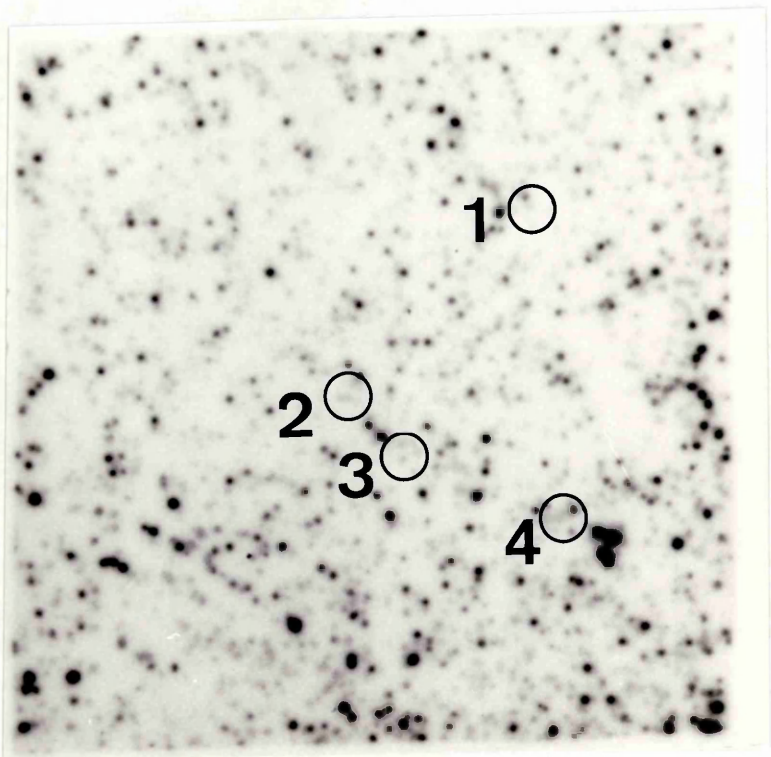
Figures 5.22 through 5.25 Differential screening of *Anagallis arvensis* cDNA library prepared from 'induced' mRNA

1500 pfu were plated onto each of four plates as described in section 2.14.4.2, and four plaque lift filters were prepared from each plate as described in section 2.15.1. Two of the four filters from each plate were incubated with cDNA probe prepared from polyadenylated mRNA extracted from leaves exposed to one long day, and the other two plaque lift filters were incubated with cDNA probe prepared from polyadenylated mRNA extracted from short day control leaves. Probe synthesis was as described in section 2.15.2 and hybridisation conditions were as described in section 2.15.3. Each figure shows an autoradiogram with plaque-specific signals from the filters after hybridisation was complete using cDNA probe prepared from: (A) mRNA extracted from leaves exposed to one long day, (B) mRNA extracted from short day control leaves. Plaques that were selected as positives are circled and numbered.

A



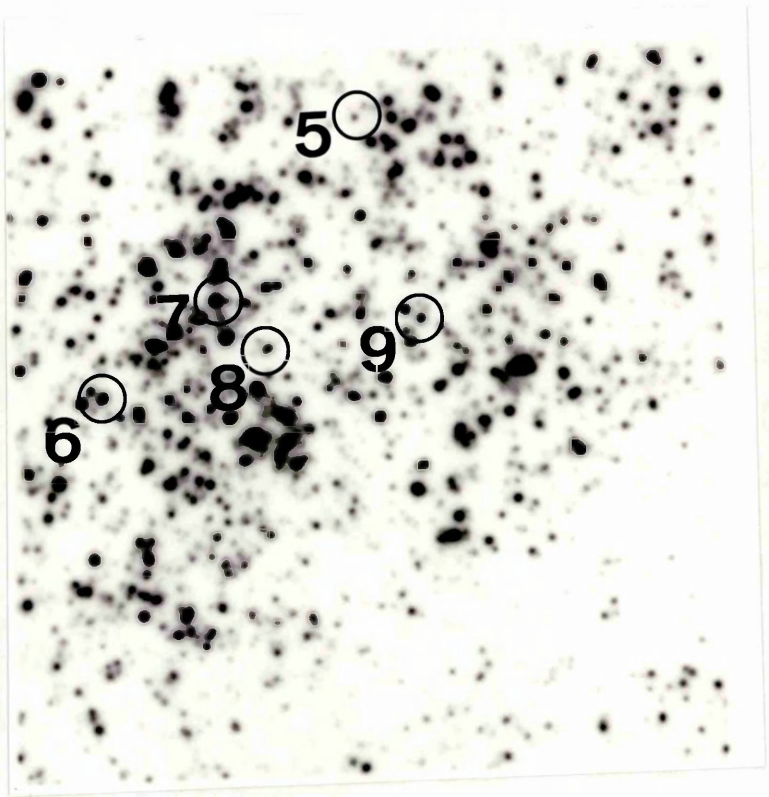
B



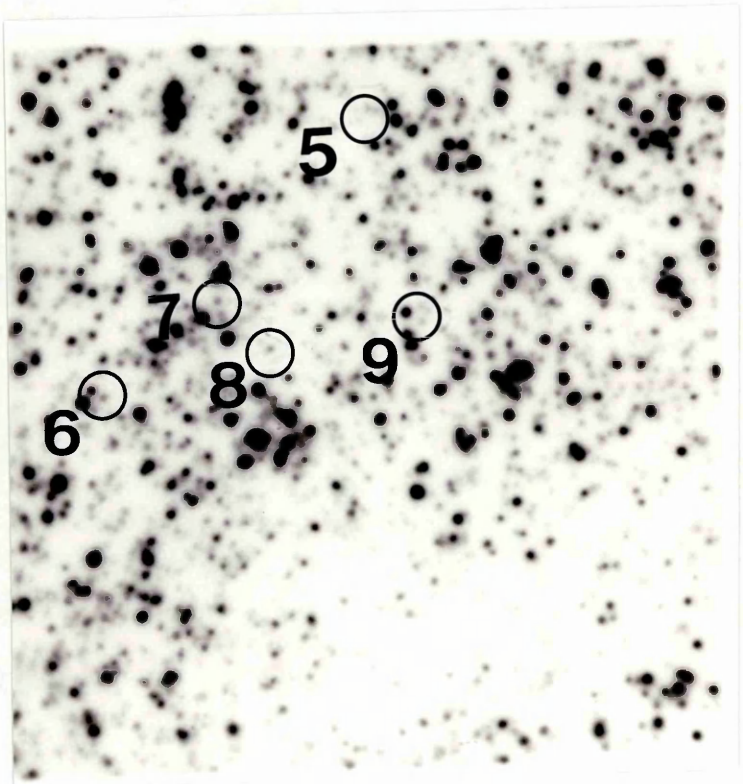
Figures 5.22 through 5.25 Differential screening of *Anagallis arvensis* cDNA library prepared from 'induced' mRNA

1500 pfu were plated onto each of four plates as described in section 2.14.4.2, and four plaque lift filters were prepared from each plate as described in section 2.15.1. Two of the four filters from each plate were incubated with cDNA probe prepared from polyadenylated mRNA extracted from leaves exposed to one long day, and the other two plaque lift filters were incubated with cDNA probe prepared from polyadenylated mRNA extracted from short day control leaves. Probe synthesis was as described in section 2.15.2 and hybridisation conditions were as described in section 2.15.3. Each figure shows an autoradiogram with plaque-specific signals from the filters after hybridisation was complete using cDNA probe prepared from: (A) mRNA extracted from leaves exposed to one long day, (B) mRNA extracted from short day control leaves. Plaques that were selected as positives are circled and numbered.

A



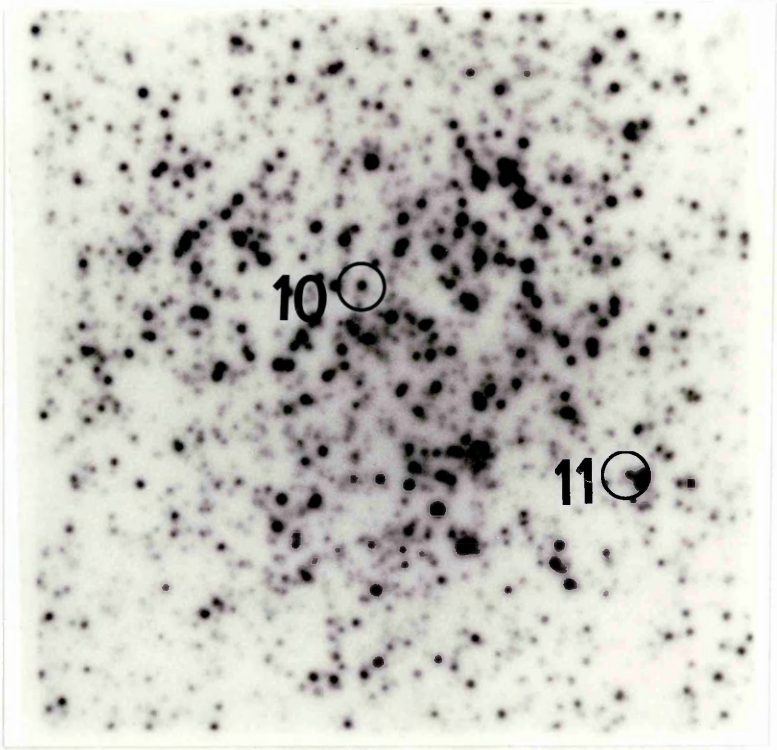
B



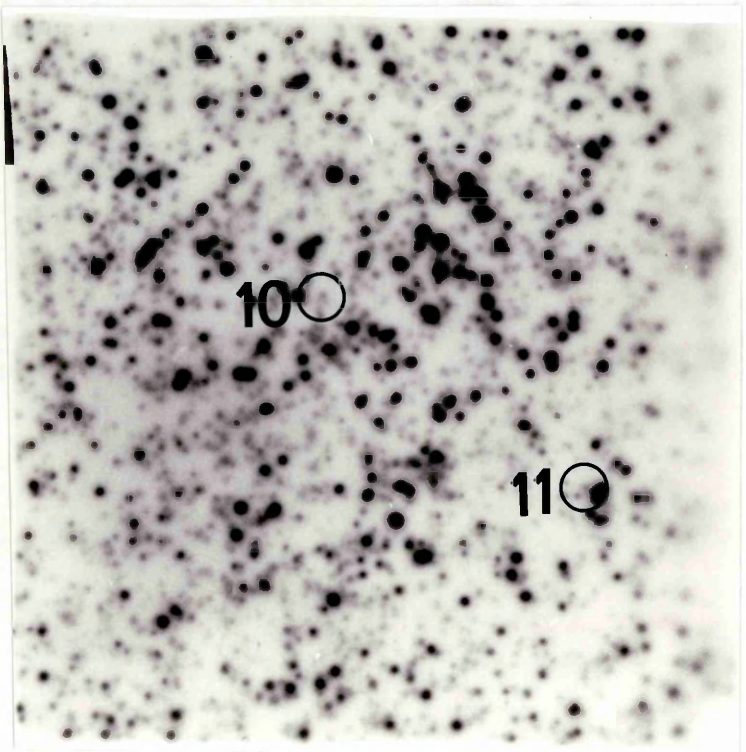
Figures 5.22 through 5.25 Differential screening of *Anagallis arvensis* cDNA library prepared from 'induced' mRNA

1500 pfu were plated onto each of four plates as described in section 2.14.4.2, and four plaque lift filters were prepared from each plate as described in section 2.15.1. Two of the four filters from each plate were incubated with cDNA probe prepared from polyadenylated mRNA extracted from leaves exposed to one long day, and the other two plaque lift filters were incubated with cDNA probe prepared from polyadenylated mRNA extracted from short day control leaves. Probe synthesis was as described in section 2.15.2 and hybridisation conditions were as described in section 2.15.3. Each figure shows an autoradiogram with plaque-specific signals from the filters after hybridisation was complete using cDNA probe prepared from: (A) mRNA extracted from leaves exposed to one long day, (B) mRNA extracted from short day control leaves. Plaques that were selected as positives are circled and numbered.

A



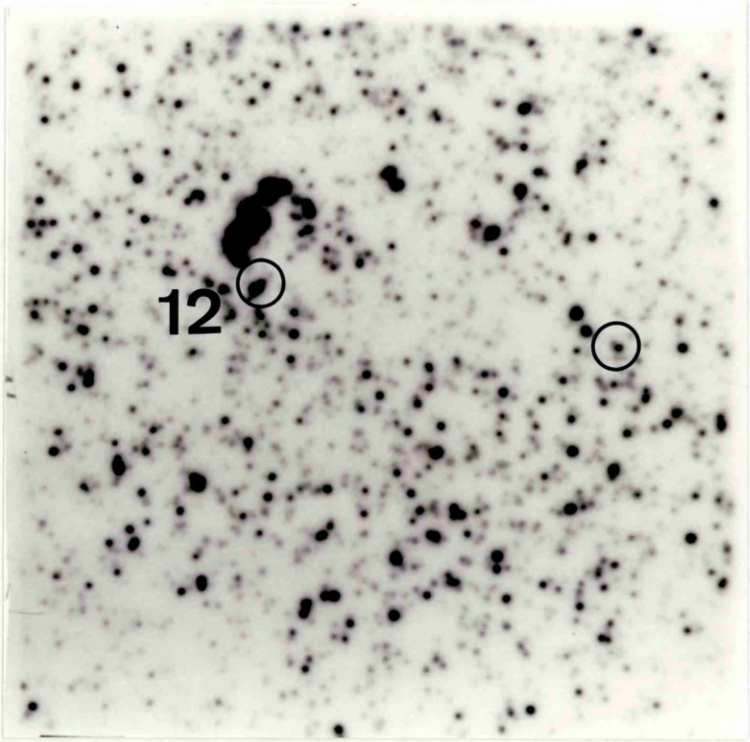
B



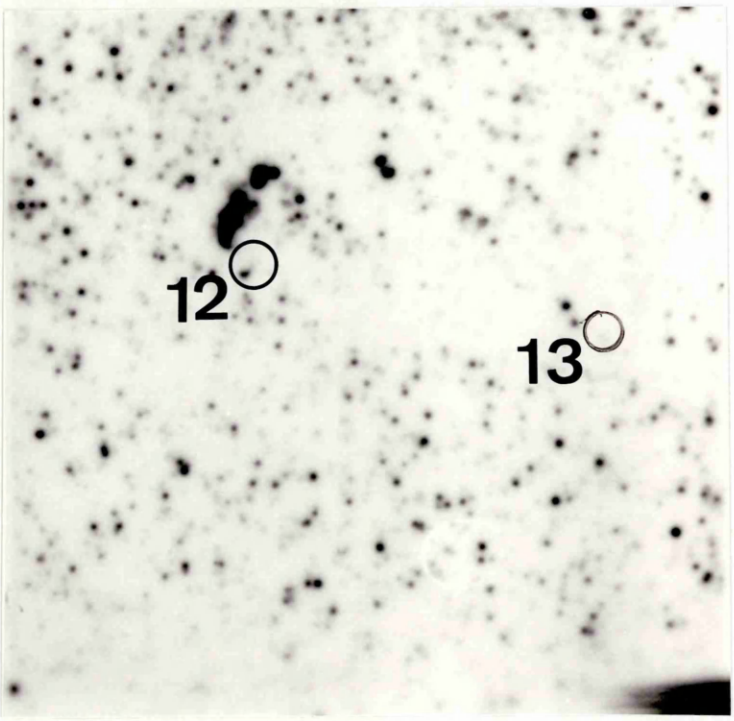
Figures 5.22 through 5.25 Differential screening of *Anagallis arvensis* cDNA library prepared from 'induced' mRNA

1500 pfu were plated onto each of four plates as described in section 2.14.4.2, and four plaque lift filters were prepared from each plate as described in section 2.15.1. Two of the four filters from each plate were incubated with cDNA probe prepared from polyadenylated mRNA extracted from leaves exposed to one long day, and the other two plaque lift filters were incubated with cDNA probe prepared from polyadenylated mRNA extracted from short day control leaves. Probe synthesis was as described in section 2.15.2 and hybridisation conditions were as described in section 2.15.3. Each figure shows an autoradiogram with plaque-specific signals from the filters after hybridisation was complete using cDNA probe prepared from: (A) mRNA extracted from leaves exposed to one long day, (B) mRNA extracted from short day control leaves. Plaques that were selected as positives are circled and numbered.

A



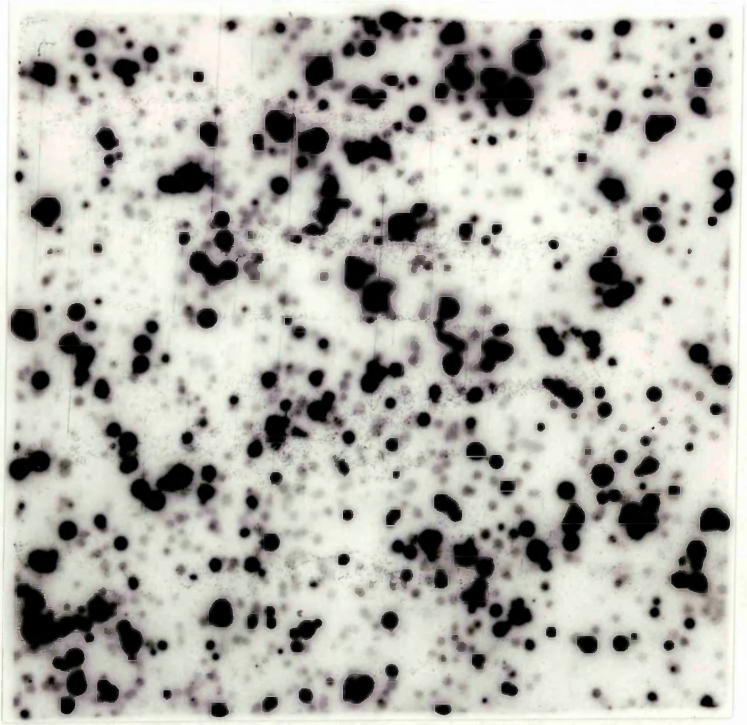
B



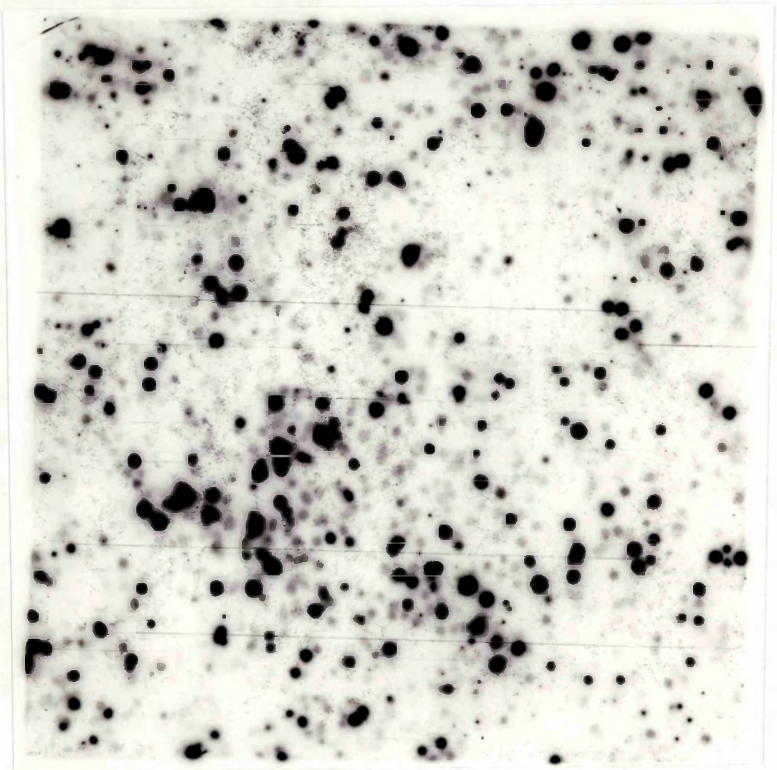
5.26 Differential screening of a mixed pool of positive plaques

Positives #3, 4, 7, 9, and 10 were pooled and 1500 pfu were plated onto plates as described in section 2.14.4.2. Four plaque lift filters were prepared as described in section 2.15.1. Two of the four filters from each plate were incubated with cDNA probe prepared from polyadenylated mRNA extracted from leaves exposed to one long day, and the other two plaque lift filters were incubated with cDNA probe prepared from polyadenylated mRNA extracted from short day control leaves. Probe synthesis was as described in section 2.15.2 and hybridisation conditions were as described in section 2.15.3. Each figure shows an autoradiogram with plaque-specific signals from the filters after hybridisation was complete using cDNA probe prepared from: (A) mRNA extracted from leaves exposed to one long day, (B) mRNA extracted from short day control leaves.

A



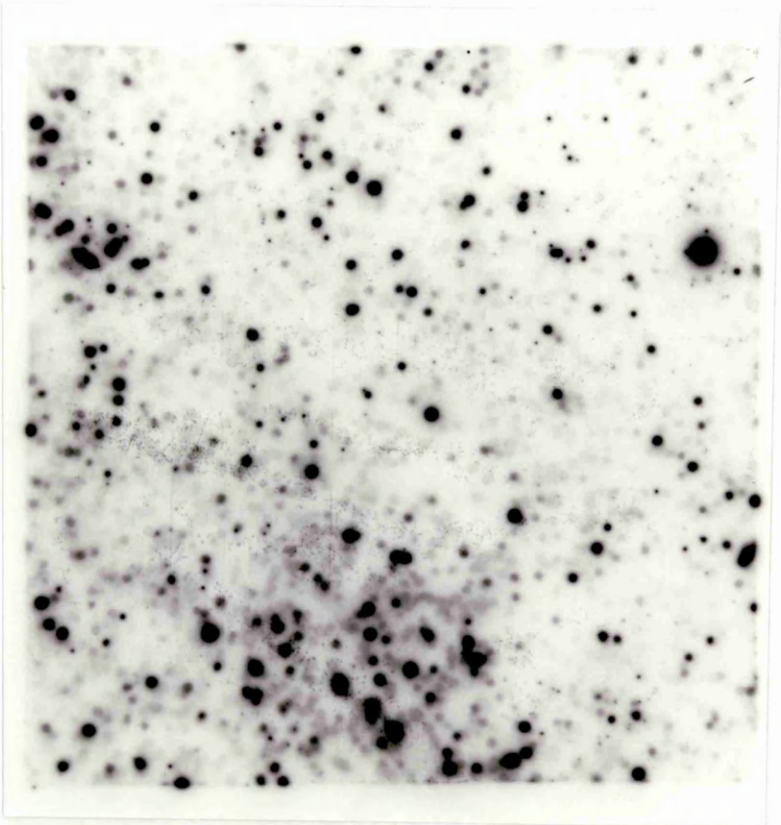
B



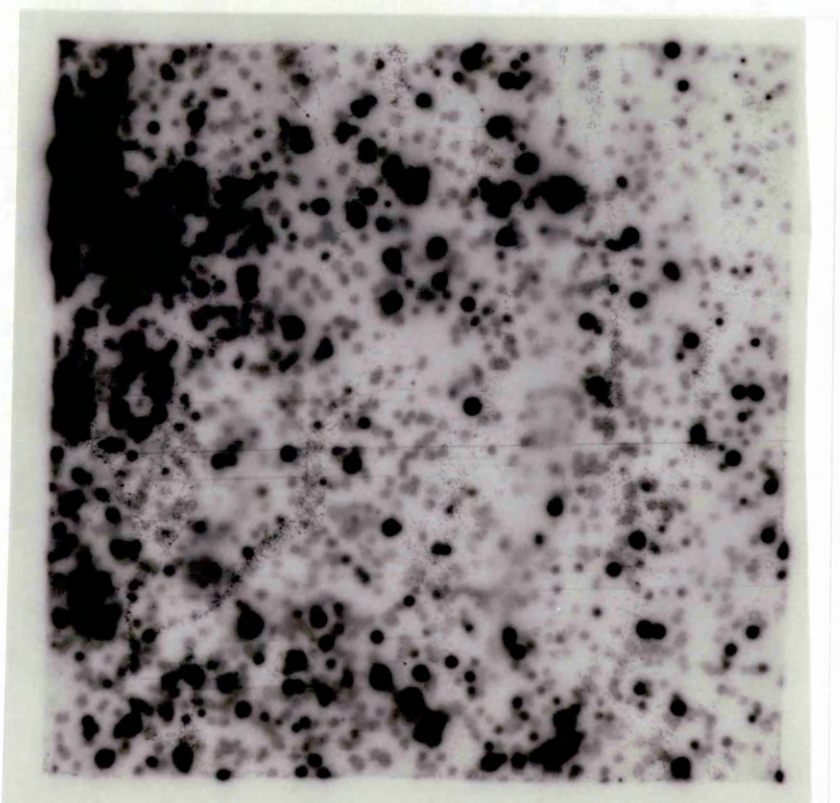
5.27 Differential screening of positive plaque #7

1500 of positive plaque #7 was plated onto plates as described in section 2.14.4.2. Four plaque lift filters were prepared as described in section 2.15.1. Two of the four filters from each plate were incubated with cDNA probe prepared from polyadenylated mRNA extracted from leaves exposed to one long day, and the other two plaque lift filters were incubated with cDNA probe prepared from polyadenylated mRNA extracted from short day control leaves. Probe synthesis was as described in section 2.15.2 and hybridisation conditions were as described in section 2.15.3. Each figure shows an autoradiogram with plaque-specific signals from the filters after hybridisation was complete using cDNA probe prepared from: (A) mRNA extracted from leaves exposed to one long day, (B) mRNA extracted from short day control leaves.

A



B



5.3 Discussion

The initial floral induction treatments verified previous photoperiodic behaviour studies (Ballard, 1969; Brulfert *et al.*, 1985). Induction was possible with one cycle of long day treatment, and the response in producing flowers was rapid but not maintained under short day conditions where the plant eventually reverted to vegetative growth. The gradient seen in the number of flowers produced and the speed of floral development when the number of inducing cycles is increased was predictable because it is known that variation occurs in the response at the apex depending upon the plastochronic stage and the plants used in these experiments represented a mixture of apices at various stages. Brulfert *et al.* (1985) catgorized the plastochronic stage into four groups. These stages numbered I through IV coincide with the development of the youngest leaf primordia around the apex from a precocious phase (I) to an older phase (IV). The apex is most sensitive to stimulation at stages II and III. For example, it has been shown that plants grouped at stage II can yeild fifty percent more flowers than plants grouped at stage IV when both are given an identical inducing treatment (Brulfert *et al.*, 1985). It has also been shown previously that as the number of cycles of induction is increased, so does the number of nodes bearing flowers increase (Ballard, 1969; Brulfert *et al.*, 1985).

The induction data are actually not so easily interpreted and several paradoxes exist. The production of more flowers with more cycles of induction cannot be explained simply. It could be that when inducing a random selection of plants with apices at various plastochronic stages with one long day cycle only a proportion of them will be able to respond during the time when the stimulus is rapidly produced and translocated.

Exposure to more cycles would, presumably, progressively increase the number of apices available to respond overall. Apices of a group of plants given more than three cycles will all have been ready to respond during one of the given cycles since the plastochron for *Anagallis arvensis* is three days (Ballard, 1969; Brulfert *et al.*, 1985). However, some evidence suggests that the flowering stimulus has a relatively long life since when meristems were removed prior to induction, secondary or adventitious meristems became organized and flowering took place 20 days later (Ballard, 1969; Brulfert *et al.*, 1975). If the floral stimulus is long lived, it is curious that flowers are only formed at a small number of nodes.

One of the most interesting aspects of floral induction in *Anagallis arvensis* is the production of 'proliferous flowers' or 'vegetative flowers' which appear under conditions of minimal induction or at the node appearing prior to the reversion to vegetative growth in short days. In the studies described here these atypical flowers were noted occassionally in plants induced by one cycle and appeared mainly as a foliaceous structure. In one experiment, not described in the results, the production of a flower which never fully completed its development was noted. Although these observations are not particularly relevant to the work presented here, they do provoke speculation on the evocation process at the apex, i.e. is commitment to flower a unitary process or do the individual flower parts require their own unique stimulus or amount of stimulus? Also, how might these observations reflect the processes occurring in the leaves which produce the stimulus or stimuli?

Because it has been previously documented that transport of the floral stimulus is completed 48 hours after the beginning of the inducing treatment (Brulfert *et al.*, 1985),

it seemed reasonable to assume initially that changes in gene expression would be apparent immediately after the dim light extension treatment used to induce the plants. The lack of results obtained when *in vitro* translation products synthesized from mRNA extracted from induced leaves were compared to those produced by control mRNA on one-dimensional autoradiograms was not unreasonable. It could only be expected that major product differences would be readily apparent at this level of resolution and minor differences would be hidden. This is presumably the reason for the implementation of two-dimensional SDS-PAGE in other floral induction studies (Warm, 1984; Lay-Yee *et al.*, 1987a; Lay-Yee *et al.*, 1987b; Felsheim and Das, 1988) as well as other work investigating differential gene expression such as the effects of auxin application (Theologis, 1986).

Results obtained here using two-dimensional SDS-PAGE of *in vitro* translation products are not particularly valuable due to the lack of consistency and routine quality. The results shown in figure 5.6, however, should not be completely disregarded since the autoradiograms are extremely comparable, and the translation product which is visible in the induced sample is not visible at all (as opposed to a difference in amount) in the control sample even though more sample is clearly loaded on the control gel (and therefore appears darker).

Unfortunately, the difficulties that can arise when attempting to produce identical two-dimensional SDS-PAGE gels are numerous. Complications and variation encountered in the work presented here occurred (or may have occurred) at all stages of the procedure. These include the RNA preparation itself, the *in vitro* translations results, the isoelectric focussing of the translation products, the running of the second

dimension, and lastly, the fluorography and drying of the complete gels. One primary consideration with regard to complications is reliable equipment. For example, whole experiments are easily destroyed or rendered incomplete due to untimely leakage of the electrophoresis equipment during either dimension or improper function of gel drying apparatus resulting in cracked gels. With regard to variation, the dissimilarities noted in figure 4.3 section 4.2.2 depicting duplicate products of *Silene* control mRNA or of figure 5.7 of *Anagallis* mRNA *in vitro* translation products could be due to natural variation occurring during the RNA extraction procedure (which is not apparent on a one-dimensional SDS-PAGE gel), or to the many problems associated with isoelectric focussing such as inconsistent entrance of proteins into the gel. This latter problem is particularly worth consideration and has been discussed in detail along with other aspects of trouble shooting in two-dimensional SDS-PAGE by Dunbar (1987)

It is clear that if samples are to be compared using two-dimensional analysis, an ample amount of time should be devoted to optimization of the system. It has been suggested that optimization is best carried out using samples that are well studied with well documented protein patterns (Dunbar,1987). If this were done in every laboratory employing two-dimensional techniques, the frequency in the publication of results which are uninterpretable or unreproducible in other laboratories could be reduced. Improvements on the original O'Farrell two-dimensional SDS-PAGE method (O'Farrell, 1975) can also be considered such as using immobilized pH gradients in the first dimension. For example, the successful application of this technique was recently reported by Görg *et al.* (1988) where a high degree of reproducibility and improved resolution was achieved and allowed for genetic analysis of leaf proteins from barley.

Optimization of two-dimensional SDS-PAGE in this work clearly could have been carried a great deal further particularly with analysis of *in vitro* translation products. The time constraints did not allow for this as quite a lot of time was devoted to finding an mRNA extraction procedure to be used with both *Silene* and *Anagallis* that allowed for acceptable yields of biologically active mRNA . Since the unavoidable change in wheat germ extract resulted in such a dramatic change in quality this might have been the first area for investigation, i.e. extracts could have been obtained from many companies and compared on the basis of quality. It is possible that the wheat germ extract purchased from Amersham, International, plc. somehow caused interference with the entrance of particular synthesized proteins into the first dimension. The reason for this speculation is that incorporation rates and protein pattern on one-dimensional SDS-PAGE gels were comparable to results obtained with the home made extract and the difference was only obvious on two-dimensional analysis. The number of aspects of the entire procedure which could be tested is indeed virtually unlimited.

The results obtained with total protein extracts were much more promising and compared to analysis of *in vitro* translation products more easily obtained. Once again, one-dimensional SDS-PAGE was not useful in showing differences in the protein population of induced and control leaves regardless of the method of detection employed, i.e. Coomassie blue or the highly sensitive silver stain. Two-dimensional SDS-PAGE proved, however, to be much less problematic when working with protein samples once a protocol for the preparation of protein extracts was developed. This was not trivial since there are several methods available and these have recently been compared for the extraction of proteins from plants (Granier, 1988). It was important to allow for visualisation of as many proteins as possible so it was necessary to extract

under denaturing conditions which allows for total proteins to be obtained. One technique that has been used is to simply grind the plant tissue directly in the isoelectric focussing buffer but when this was attempted in this work, extremely poor gels with very few protein spots were produced (data not shown). This is possibly due to the transient and even increased activity of certain proteinases in the urea prior to autolysis or denaturation by the urea (Colas des Frances *et al.*, 1985). A more satisfactory procedure was found as described in section 2.6.2 where the extraction was carried out under denaturing conditions which allowed for heating of the preparation. Heating ground plant tissue in the Tris-Cl^- buffer containing ß-mercaptoethanol has been shown to dramatically reduce the appearance of Rubisco large subunit degradation products used as markers for proteinase activity (Colas des Frances *et al.*, 1985) and use of this protocol here allowed for the production of high quality two-dimensional separations of proteins. Heating ground tissue in the isoelectric focussing buffer is not possible as it would potentially destroy the carrier ampholytes and cause carbamylation of the proteins by the urea.

The consideration of protease activity is serious given that it is possible that the proteins seen in figure 5.10 might represent one protein (#1) and its two degradation products (#2 and #3). It seems that this would be unlikely given the choice of the protocol used and the fact that all of the protein extracts were prepared at the same time. Also, the relative amounts of proteins #2 and #3 are unequal, and presumably, this would not be the case if they were degradation products of protein #1 (although the gels in figure 5.10 are stained with silver which does not allow for quantitation, a section of gel containing proteins #2 and #3 was destained and restained with Coomassie blue which also indicated that the proteins were present in unequivalent amounts). However,

it is also possible that protein #3 represented a covalently modified (e.g. by phosphorylation or acetylation) form of the protein which appeared immediately to its right. If the modification was phosphorylation, then addition of phosphatase to the induced protein sample prior to electrophoresis would eliminate the appearance of protein #3. Peptide mapping of protein #3 and the protein to its right would definitively determine whether the proteins were the same regardless of the type of modification. As with consideration of proteins #2 and #3 as degradation products, this explanation is not inconsistent with the data showing activity of the MB-1 antiserum against protein #3 and the protein immediately to its right. However, even if protein #3 represents a modified protein, protein #2 might still be a protein induced by the light treatment.

The experiments shown in figure 5.10 provided a reasonable indication (at least with the presence of protein #2) that differential gene expression may have resulted from the light treatments employed in this work. This led to the consideration of several possible approaches to isolate the corresponding genes. It was thought that the considerable effort that seemed to be required to obtain reliable results in two-dimensional analysis of *in vitro* translated mRNA would be better spent on constructing and differentially screening a cDNA library: Isolation of the necessary mRNA and construction of a cDNA library can be done in less time than it takes to analyse *in vitro* translation products using two-dimensional SDS-PAGE. This would be a more sensitive technique for detecting differentially expressed genes and would have been attempted regardless of the results acquired from analysis of *in vitro* translation products.

At the same time, the production of a polyclonal antiserum against the proteins extracted from induced leaves seemed a reasonable investment of time and the antiserum would have had multiple uses. Immunoadsorbed antiserum could have been used to study expression at the protein level in a variety of induction experiments which would

have been incredibly laborious using two-dimensional SDS-PAGE. Other applications could have possibly included cytological localization of the two proteins of interest as well as use in the screening of an expression library which allows for more rapid analysis of a greater number of clones than does differential screening.

The failure of the immunoadsorption technique was surprising because of the great success achieved when this technique was used previously to clone genes that are developmentally regulated during embryogenesis in carrot (Choi *et al.*, 1987). It is possible that further efforts towards optimizing the immunoadsorption protocol might have eventually been successful. In the first instance, it would be interesting to see how the immunoadsorbed antiserum reacted on a two-dimensional protein blot. For example, if immuno-activity against protein #3 shown in figure 5.14 is removed by the immunoadsorption, it is possible that this protein may be serologically related to other proteins common to both induced and control leaf extracts, or it could be that trace amounts of protein #3 in control leaf extracts may enough to remove activity. Therefore, the technique would never be useful in this case. Alternatively, if the activity against these proteins remains but is merely hidden by the remaining activity against highly abundant proteins on one-dimensional analysis, further experiments to perfect or improve the immunoadsorption would be sensible.

The results obtained from differential screening of the cDNA library constructed from mRNA taken from induced leaves could be interpreted as meaning that at this level of sensitivity no differences were detectable. As indicated in section 5.2.11.2, difficulties with the hybridisations were encountered and the reason for these difficulties remains obscure. A total of three sets of primary hybridisations, one of which is

presented in figures 5.22 through 5.25, were conducted which all yielded filters where the radioactive signal was specific to plaques and the remainder of the filter was extremely clean with no background. At the time when secondary screens were attempted, the hybridisations produced horrible background that appeared to be completely unspecific, nor was it washed off when subsequent washes were incorporated into the protocol. Experiments designed to solve the problem by replacement of the various reagents never completely solved the problem in the time remaining as indicated by the filters shown in figures 5.26 and 5.27 which are clearly not of the same quality as those in figures 5.22 through 5.25.

It is possible that the false positives are an indication that the hybridisation conditions were unreliable and should have possibly been checked initially with an internal control such as adding a tiny portion of packaged globin cDNA to the plates and globin mRNA to one of the mRNA pools used in the screening. Another control might have been to prepare total mRNA from another tissue such as stem and use it to prepare probe that could be used in comparison with the induced probe. This would certainly have shown many differences and allowed for validation of the hybridisation protocol which could be questioned since differential signals (figures 5.22 through 5.25, positives 1 through 13) were only detected in one of the three separate primary hybridisation experiments.

It is possible that the differential screening technique did not provide the degree of sensitivity required. It would be useful, in the future, to make use of subtractive hybridisation procedures. Screening a subtracted library with a subtracted probe would clearly be the ultimate technique and would take into consideration that the genes of

interest may be expressed at very low levels. Differential screening of a subtracted library was recently used successfully to isolate the homeotic *deficiens* (*defA* $^{+}$) gene which is involved in the genetic control of flower development in *Antirrhinum majus* (Sommer *et al.*, 1990). After the clone for the *defA* $^{+}$ gene was isolated, the abundance of its corresponding transcript in the mRNA population was estimated to be ~10^{-5} which indicates the sensitivity of this technique.

Alternatively, a project might be done to look more specifically at the expression of the proteins seen in figure 5.10 and carefully determine the rate of their accumulation (as in proteins #2 and #3, figure 5.10) or decrease (as in protein #1, figure 5.10). Such a study might provide a basis for choosing an alternative time point to extract mRNA for library construction. For example, it is possible that the expression of the genes which code for proteins #2 and #3 (figure 5.10) is only transient, and therefore, it would be better to extract the mRNA at the time point when the proteins are actively accumulating or when they first begin to appear. One other possible approach would involve the purification and sequencing of the proteins of interest which would allow for the construction of olignucleotide probes which could be used for cloning the respective genes or for initial studies on mRNA expression.

Chapter 6 - General Discussion

The amount of time and effort that has been invested over the past fifty years into studies attempting to elucidate the mechanism of floral induction is vast and yet there is still no solid evidence to support any proposed model. Although, as discussed in the introduction, there is evidence for the involvement of differential gene expression in the induction of flowering, no specific genes have yet been identified and isolated. Furthermore, the importance of post-translational modification(s) has not been fully assessed and such molecular mechanisms may be involved. Thus, the difficulty of this developmental problem is easily appreciated.

Although it would be a tremendous achievement to be able to describe the exact mechanism of floral induction, the data that have accumulated from studies on numerous plants have, nevertheless, been of great benefit to the agricultural industry. From an agricultural view point, the ability to control floral induction is *currently* very useful. The control of flowering is an adaptive strategy in the plant kingdom that allows for the maximisation of seed production, dispersal , and survival in a given environment, and it is this evolutionary flexibility that has been exploited by man.

In the first instance, the need to control flowering for agricultural benefit has been accomplished by selection for desired characteristics. For example, fibre production in jute is benefitted if flowering is prevented, and therefore, cultivars have been produced which do not flower in the latitudes with inductive day-lengths (Evans, 1987). Indeed, many crop plants which produce a vegetative commercial end-product, such as lettuce, endive, celery and sugar beet have been bred and selected for in this way (Evans,

1987). Therefore, manipulation by selection and breeding has already played a significant role in permitting the spread of crops to new regions and environments and in the improvements of yield potential.

Flowering in certain crops has also been beneficially controlled by environmental and chemical manipulation. Sugar cane is an example of a field crop where light breaks have been used effectively to prevent flowering during the brief time during the year when exposure to intermediate day-lengths would ordinarily promote flowering (Evans, 1987). This treatment is naturally very costly and in the case of sugar cane alternative environmental treatments, such as water stress, which inhibit flowering have been developed (Evans, 1987). Environmental manipulation is much more routine in modern horticultural practices in the production of high value flower crops. For example, a drop in temperature triggers flower bud development in certain orchid species (Evans, 1987).

The wide variety of endogenous plant growth regulators and the vast amount of data that have accumulated from empirical testing of the effects of these and other chemicals on flowering has made chemical manipulation of flowering another important and effective horticultural and agricultural practice (Evans, 1987). For example, ethylene generating chemicals can be used for year-round production of pineapples and mangoes (Evans, 1987).

Manipulation of flowering for agricultural benefit has profitted from the great amount of existing physiological data and is certainly successful; however, it is possible that agriculture might gain a further advantage if crop plants could be genetically engineered such that reproduction could be controlled without the implementation of

chemicals or costly equipment (such as search lights for night breaks). Based on the implications that flowering is controlled by the action of specific genes (as described in section 1.5) and the hope that identification of these genes would be of academic as well as agricultural importance, the goal of this project was to develop an experimental plant system that would allow for the examination of gene expression that might be associated with floral induction in the leaves.

The difficulties that can be encountered in such an undertaking are numerous and have been dealt with throughout this project. The experiments with *Nicotiana plumbaginifolia* (chapter 3) demonstrated the importance of choosing a species in which flowering could be induced by a defined brief treatment; changes in expression could be monitored over a specific period during which an inductive signal was likely to be generated. However, not all species which show an absolute requirement for an inductive treatment lend themselves to molecular experimental studies (as was found with *Silene coeli-rosa*, chapter 4). Also, it cannot be presumed that changes in gene expression following the inductive treatment will readily be observed (as was the case with *Amaranthus caudatus* , chapter 4).

Nevertheless, it is possible that in one of the chosen subject species examined here, *Anagallis arvensis*, there was a reasonable indication that changes in gene expression did occur during floral induction, although it was not possible during the time available to isolate the corresponding genes. Future efforts could be made to more specifically characterise the nature of the expression either at the mRNA or protein level and to refine the techniques for cloning the genes as discussed below.

The genes could be studied in experiments which compare expression under different light treatments including the other controls such as expression in lower leaves which are not photoperiodically sensitive as discussed in section 1.9.4, but these experiments would be difficult to interpret. For example, if the genes are expressed in both the photoperiodically sensitive leaves as well as the lower leaves, it could not be concluded that the genes are not involved with floral induction because it might be that all of the leaves express the gene but action of the gene product is dependent upon the presence of other constituents in the leaves that are not available in older leaves. Therefore, the expression of the genes would have to be specifically inhibited using, for example, anti-sense mRNA techniques as described in section 1.8.1.4. It is clearly possible that quite a lot of effort could be spent attempting to characterise the function of isolated genes that do not have any detectable role in the developmental process under study. However, from an academic veiwpoint, the isolated genes might have an interesting function in an unrelated biochemical or developmental process, and therefore, the effort would not have been a waste of time.

The approach taken in this work is indirect and relies heavily on the assumption that genes involved with floral induction will be differentially (and detectably) expressed either at the mRNA level, which would allow for cloning using standard differential screening techniques, or at the protein level, which would allow for protein purification and sequencing. During the time period in which this research was carried out, these were the only realistic approaches that could be utilised. However, speculation on future experiments on floral induction must include discussion of recent developments (some published immediately prior to submission of this thesis).

Advances in the use of T-DNA gene tagging have recently been made (Feldman *et al.*, 1989; Marks and Feldman, 1989; Herman and Marks, 1989; Yanofsky *et al.*, 1990) and this may allow for a direct approach to the study of floral induction as opposed to the indirect approach that was taken in the work presented here. Feldman *et al* . (1989) have used a non-tissue culture method for the transformation of germinating *Arabidopsis thaliana* seeds (Feldman and Marks, 1987) to create a series of mutants displaying phenotypes including dwarf and other height variants, floral structure variants, stems lacking trichomes, and embryo lethals. Marks and Feldman (1989) and Herman and Marks (1989) have studied the T-DNA tagged *Glabrous 1* gene, which when mutated results in an altered trichome phenotype. Sequences flanking the T-DNA insert in the trichome mutants were identified by screening a genomic library made from DNA extracted from the mutant plants, and eventually, a 2.8 kb clone was isolated which was used to identify an uninterrupted *Glabrous 1* sequence from a wild-type genomic library. The wild type clone was subcloned and used in complementation experiments to test for its ability to revert the mutant phenotype. These experiments have allowed for the localization of the wild-type sequence to an 8.3 kb subclone which is currently being further characterized in smaller fragments in order to determine the precise location of the gene by further complementation experiments and by sequencing.

Another of the T-DNA tagged mutants (Feldman *et al.*, 1989), which causes an alteration in floral structure has also recently been cloned (Yanofsky *et al* ., 1990). This mutants displays a phenotype resembling *agamous* mutant *Arabidopsis* plants which lack stamens and carpels and appear as a flower within a flower. The T-DNA insert was found to co-segregate with the mutant flower phenotype and this together with the allelism of the tagged mutant to *agamous* allowed for the conclusion that the T-DNA

could be used to isolate the *agamous* gene. A cosmid genomic library was constructed from the DNA of transformed mutant plants and allowed for plasmid rescue of a clone containing the T-DNA and flanking sequences. The flanking sequences were used in further screening experiments of wild-type genomic libraries until clones were identified that would complement mutant plants. cDNA clones were identified which displayed hybridisation spanning the T-DNA insertion site and these clones were subsequently used for sequencing analysis. The deduced *agamous* protein sequence was found to share significant sequence similarity to transcription factors from humans, yeast, and the product of the homeotic *deficiens* gene from *Antirrhinum majus* . Similar work is currently being conducted to determine the gene structure of another homeotic regulatory gene, *apetala-2* , which when mutated results in the loss of petals, partial loss of stamens, and the transformation of one pair of sepals to carpels (Jofuku *et al.*, 1990).

T-DNA gene tagging in *Arabidopsis thaliana* could possibly be the best approach to the identification of genes involved in floral induction. *Arabidopsis* is a quantitative LDP but the timing of bolting or floral initiation can vary and specific loci have been identified which determine this phenotype (Redei, 1962). Recent reports in abstracts from the Fourth International Conference on *Arabidopsis* Research indicate that, indeed, efforts are currently being directed towards the isolation of genes that affect the timing and initiation of flowering in *Arabidopsis* using the T-DNA gene tagging approach. Two T-DNA tagged late flowering mutants have already been made by Feldman *et al.* (1990) and presumably, the tagged genes will eventually be characterized in the same manner as described for the *glabrous* and *agamous* genes described above.

An alternative strategy was also reported to be in progress in the effort to

characterise a mutation that maps to the *fg* locus on chromosome 5 (Koornneef *et al.*, 1990). When mutated, *fg* results in plants that flower later than wild-type plants in long days of 18 hours but at the same time as wild-type in short days of 10 hours (Koornneef *et al.*, 1990). It is believed that characterization of this gene will provide information on the biochemistry and timing of floral initiation and one group (Coupland *et al* ., 1990) is currently attempting to isolate the *fg* gene by chromosome walking starting from the chalcone synthase gene located 2 cM (280kb) from *fg* .

It seems that *Arabidopsis* will provide much information concerning various aspects of the flowering process given the ease of genetic manipulation and the availability of mutation and cloning strategies. It is not known whether the response to day length is controlled by genes acting in the leaves or at the apex, but certainly, if the various flowering genes are cloned, studies on their mode of action will follow and will be interesting and important. It is likely that work on *Arabidopsis* flowering genes will be more profitable than the indirect approaches that have been pursued in the past. For example, cloning of the genes shown to be differentially expressed during floral induction in *Pharbitis* and *Hyoscyamus* , using the *in vitro* translation technique, as discussed in section 1.6.2 has not yet been reported even though the observations were published several years ago.

Clearly, it is important to undertake molecular studies with species other than *Arabidopsis* . Given the diversity of flowering responses, it is definitely not certain that similar mechanisms will be found in all species. Whether T-DNA taggingwill be as useful in identifying genes in plants other than *Arabidopsis* is unknown. It would be desirable to be able to use T-DNA tagging in a species such as *Anagallis arvensis* to

identify a gene(s) involved with floral induction since this species is so strictly controlled by photoperiod.

It is important when using T-DNA tagging that a reasonable frequency of insertion into transcribed regions is achieved, and in *Arabidopsis*, this frequency was shown to be one to three out of ten insertions and this might have been because of the small genome size (Hanson, 1989). The chromosome number of *Anagallis arvensis* (n) is 20, and it has been reported to be polyploid and self-pollinating (Heiser and Whitaker, 1948). The larger genome size could present problems if a technique such as T-DNA tagging was to be employed, although comparable frequencies of insertion into transcribed regions have been noted in *Nicotiana* which has a much larger genome (Hanson, 1989). With regard to ploidy, *Anagallis arvensis* has been used since the 1800s for studies on the inheritance of flower color (Ballard, 1969) so it is possible that it would be appropriate for use in genetic studies (it is also possible that the report of polyploidy was incorrect or only applicable to the stock that was investigated). Also, transformation of *Anagallis* with T-DNA contructs has not yet been attempted, although it is a dicot and this may be possible.

The consideration of using a plant such as *Anagallis arvensis* for molecular genetic studies of floral induction may seem ridiculously ambitious, but at the same time, it is unwise to rely upon *Arabidopsis thaliana* for all of the answers to all of the developmental questions. It seems that in plant molecular biology the need for specific tools to study developmental processes is rapidly becoming satisfied. It will hopefully be only a matter of time before a wide variety of plants, other than *Arabidopsis*, will be useful in the study of flowering using genetics and molecular biology.

Chapter 7 - References

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