A Study of Vernalization and Cold-Induced Changes in Gene Expression in Sugar Beet (*Beta vulgaris*)

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List of Abbreviations

cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DTT	dithiothreitol
FBS	foetal bovine serum
MOPS	3-[N-Morpholino]propane-sulfonic acid
Na_2EDTA	ethylenediaminetetra-acetic acid (disodium salt)
PBS	phosphate buffered saline
PVP	polyvinylpyrrolidone
PMSF	phenylmethylsulphonyl flouride
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	tris[hydroxymethyl] aminomethane
TCA	trichloroacetic acid
ABA	abscisic acid
COR	cold-regulated
DNP	day neutral plant
E_A	activation energy
GA	gibberellin
IEF	iso-electric focussing
$L T_{50}$	temperature which kills 50% of the plants
LDP	long day plant
LHCP	light-harvesting chlorophyll a/b binding protein
PGR	plant growth regulator

pI	isoelectric point
RUBISCO	ribulose 1,5-bisphosphate carboxylase/oxygenase
SDP	short day plant
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSU	small subunit
A_{260}	absorbance at wavelength 260 nm
A_{280}	absorbance at wavelength 280 nm
cM	centi Morgan
kDa	kilo Daltons
nm	nanometer
rpm	revolutions per minute
v/v	volume to volume
w/v	weight to volume

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SUMMARY

Annual and biennial genotypes of sugar beet (*Beta vulgaris*) require long day lengths to induce flowering. In addition to long days, biennials must also be exposed to low temperatures (vernalization) before flowering will occur.

In this study physiological experiments were carried out with sugar beet to investigate 1) if only those leaves initiated from a vernalized meristem are able to respond to long daylengths to produce a floral stimulus or, if leaves which directly experience low temperatures become competent; and 2) whether an inhibitor of flowering is present in the old or unvernalized leaves. The results of defoliation experiments showed that leaves present, but not yet expanded, at the start of the cold treatment could be vernalized. Therefore a change in competence due to low temperature is not limited to leaves produced by a vernalized meristem. No data were collected which suggested that fully expanded leaves of biennial plants could be vernalized. Fully expanded leaves of annual plants were unable to respond to long daylengths and this suggests that as leaves age their ability to produce a floral stimulus in response to inductive daylengths is lost. The experiments provided no evidence of inhibitors in either the old leaves of annuals or biennials or in the unvernalized leaves of biennial plants.

To investigate vernalization at the molecular level, polypeptides present in the young unexpanded leaves of control and vernalized annual and biennial sugar beet were compared using two-dimensional electrophoresis. The resulting gels were stained with silver. No major consistent differences were observed between the polypeptide profiles of annuals and biennials. However, two acidic polypeptides of 22 and 23 kDa and acidic pI accumulated in the leaves of both annual and biennial plants exposed to low temperatures. These two polypeptides were not detected at the same levels in control leaves of different ages and are therefore not related to development. The polypeptides also accumulated in mature leaves of biennial plants exposed to low temperatures and could be detected on silver stained gels two days after transfer of plants to low temperatures. The polypeptides persisted in the leaves as long as plants were kept in the

cold. Because of the prevalence of the two polypeptides they were probed with antisera specific to two chloroplast proteins, the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase and the major light-harvesting chlorophyll protein. The antibodies did not bind to the polypeptides and it is therefore unlikely that the polypeptides are precursors or degradation products of these proteins. The cold-induced polypeptides were detected in leaf proteins extracted with a non-denaturing Tris buffer and are therefore unlikely to be integral membrane proteins. Two polypeptides of similar size and pI accumulated when biennial plants were wounded.

The function of the 22 and 23 kDa polypeptides is unknown. Their presence in the leaves of both annual and biennial plants does not rule out the possibility that they are involved in vernalization. The annual, though not requiring vernalization, can be vernalized. However the accumulation of the polypeptides in response to wounding, their presence in leaves of different ages, and their persistence, make it probable that they are involved in cold acclimation or cold tolerance.

Further experiments were carried out to investigate whether changes in gene expression could be detected at the mRNA level. The products of *in vitro* translation of mRNAs from the young leaves of control and cold-treated biennial plants were separated on one- and two-dimensional gels. Several differences between the profiles were seen, with some products increasing and others decreasing on exposure to low temperatures. It is possible that some of these translation products are encoded by cold-regulated mRNAs. However, translation products found in different samples from plants given the same treatment and in samples from plants given different treatments were quite variable. Interestingly, two-dimensional gels of *in vitro* translation products from mature biennial leaves included polypeptides of similar size and pI to the cold-induced polypeptides seen on silver stained gels of accumulated protein. One of the polypeptides was present in both control and cold-treated tissue. The other polypeptide was detected in samples exposed to low temperatures for 3 hours or more. A similar polypeptide was seen to increase in the leaf tissue of plants sprayed with 3 mM ABA. If these polypeptides are products of the mRNAs which code for the cold-induced

polypeptides the level of at least one of the polypeptides must be post-transcriptionally controlled.

Chapter One: Introduction

1.1 Introduction

Classical breeding techniques and manipulation of the physical environment were, until recently, the only means at the disposal of agronomists to extend the range and yield of crop species. To this end plant breeding programmes have been successful in producing varieties with temperature and daylength requirements tailored to suit those experienced in different regions, and in selecting crops with enhanced resistance to pests and disease. The use of irrigation and the application of plant growth regulators (PGR's), fertilizers, pesticides and herbicides have also had a beneficial effect on crop survival and productivity. However, these strategies do have practical and financial drawbacks. It may take 10 years or more for plant breeders to produce a new improved variety (Tanskley, 1989), and altering environmental conditions is costly and the timing of intervention crucial to.success. For example an estimated 4 billion dollars was spent on insecticides to protect cotton, rice and maize worldwide in 1987 (Hilder *et al.,* 1990). For these insecticides to be effective the emergence of specific pests must be predicted correctly since protection is transitory and may decline with time as the chemicals degrade or are washed away.

Since the early seventies when the first transgenic bacteria were engineered (Cohen *et al.,* 1973), new molecular genetic techniques have made feasible the rapid and stable transfer of genes conferring desirable characteristics to plants. The advantage of direct gene transfer over traditional plant breeding is that genes isolated from any species which confer desirable characteristics can be incorporated into an already highly desirable genotype in one step. So far agriculturally important genes have been transferred which confer resistance to various herbicides (Comai *et al.,* 1985; De Block *et al.,* 1987; Fillatti *et al.,* 1987; Llewellyn *et al.,* 1990), insect pests (Fischoff *et al.,* 1990; Hilder *et al.,* 1990) and viruses (Nelson 1990). If the distribution and productivity of crop species is to be improved through manipulation of developmental characters, clearly a prerequisite is knowledge of which genes are responsible for desirable phenotypes. Knowledge of the molecular events of all aspects of plant growth

and development is required. For example, knowledge of genes conferring tolerance to extremes of temperature and to drought would be necessary for constructing crops with resistance to these stresses. In addition the ability to alter metabolic pathways either to alter the levels of useful products or to produce new products holds great potential (Flavell *et al.*, 1990; Somerville *et al.*, 1990; Bennett *et al.*, 1990). Other important genes are those controlling major steps in development. For example, if the molecular events leading to flowering were known, genetic techniques could be used to manipulate vegetative and reproductive growth. The vegetative phase of plants grown for their vegetative organs could be lengthened, whilst in those plants grown for their fruit or seed the reproductive phase could be promoted. Reducing the life cycle would also be useful to hasten improvement through classical plant breeding programmes and for seed production.

1.2 Flowering: introduction

The events of flowering can be divided into three classes: floral induction, evocation and morphogenesis. Inductive events produce a change in the plant which by itself or in association with other changes results in flowering. Inductive conditions may cause the production of a floral stimulus or change the sensitivity of cells to such a signal. When the floral stimulus is received at the apex evocation results in the morphogenesis of flower parts (Vince-Prue, 1975).

Regulation may be autonomous, determined solely by the genotype of the plant. In this case flowering occurs when the plant reaches a certain developmental stage and the only necessary environmental conditions required are those which allow the plant to reach this stage. In other plants flowering may be dependent on the interaction of the genotype with specific environmental conditions. The two main environmental signals are light (quality and photoperiod) and low temperature. Responses to these environmental conditions often dictate the distribution of species, ensure flowering in the most favourable conditions and, in outbreeding plants, assures synchrony of flowering within a localised population.

1.3 Photoperiodism

1.3.1 Photoperiodic requirements

The control of flowering by daylength has been called photoperiodism (Garner and Allard, 1920). There are several photoperiod response types: short day plants (SDPs), long day plants (LDPs), day neutral plants (DNPs), long short day plants (LSDPs), and short long day plants (SLDPs). SDPs flower only after receiving less than a certain number of hours of light in the diurnal cycle; LDPs flower only when they experience more than a minimum number of hours of light, and DNPs flower irrespective of daylength. SLDPs first require short days (SDs) followed by long days (LDs), LSDPs require LDs followed by SDs. There may be quite some overlap in the daylengths which LDPs and SDPs respond to. Some LDPs, for example *Hyoscyamus niger,* will flower in a 12 hour photoperiod which would also induce many SDPs to flower, and some SDPs may be induced to flower in 14.5 hours of light which is sufficient to induce certain LDPs (Lang, 1965). The defining factor is that LDPs will stop flowering or flowering will be delayed if the daylength is reduced below a threshold and flowering will be promoted as the daylength is increased, whilst the opposite is true for SDPs.

A photoperiodic requirement may be facultative or obligate. Plants which absolutely require a particular photoperiodic treatment to induce flowering are often referred to as qualitative LDPs or SDPs, while species whose flowering is accelerated in certain photoperiodic conditions are said to have a quantitative response. The number of cycles of an inductive photoperiod required for the induction of flowering varies and may be altered depending on temperature, light intensity and the age of the plant. For example, *Chicorium intybus* may flower in LDs at high temperatures but in any daylength at low temperatures (Vince-Prue, 1975). Thus it may be that the difference between facultative and obligate requirements for daylength are not due to fundamental differences in the processes which lead to flowering, but rather the conditions of light and temperature at which the processes can proceed (Lang, 1965).

In *Pharbitis nil* (Vince-Prue and Gressel, 1985) and *Chenopodium rubrum* (Cumming, 1959) flowering can be induced when only the cotyledons are present.

However some plants have a juvenile phase when they do not respond to otherwise inductive conditions (Klebs, 1913 cited by Lang, 1965). Even when this juvenile phase is completed the minimum number of inductive daylengths required may decrease with age, for example, in *Perilla*, *Kalenchoë blossfeldiana* (Vince-Prue, 1975) and *Lolium temulentum* (Evans, 1960). However, sensitivity does not always increase with age. Ballard and Lipp (1964) have shown that in *Anagalis arvensis* sensitivity decreases with age.

1.3.2 Perception of daylength

Both LDPs and SDPs are induced to flower if only the leaves are exposed to inductive daylengths. Knott (1934 cited by Vince-Prue, 1975) was the first to discover the importance of the leaves: when he exposed only the leaves of *Spinacia oleracia* to LDs the plants flowered. A few years later, Hamner and Bonner (1938) showed with *Xanthium strumarium* that if only a single leaf was exposed to inductive daylengths and the remainder to non-inductive daylengths flowering would occur. Further evidence that the leaves are important sites of daylength perception and the consequent production of a flower promoting substance came from experiments in which excised leaves of *Perilla* placed in inductive conditions caused flowering when grafted to receptor plants kept in non-inductive daylengths (Zeevaart, 1986). Although leaves are the main site of photoinduction in a few cases defoliated plants have responded to daylength (Lang, 1965).

Species differ in the number of inductive daylengths they require for flowering. In some plants one cycle is sufficient whereas others may require exposure to inductive daylengths for several weeks. In some plants, such as soybean, *Anagallis arvensis* (Lang, 1965) and *Chrysanthemum morifolium* (Vince-Prue, 1975), exposure must continue until some morphological differentiation has occurred otherwise they revert to the vegetative state. Many other species will flower in any daylength once they have experienced inductive conditions. These responses may be connected with the type of changes exposure to inductive daylengths elicits. For instance, in *Perilla frutescens* leaves exposed to inductive daylengths remain permanently induced (Zeevaart, 1986)

even when they are returned to non-inductive daylengths, and the induced state persists until the leaves senesce. However, although induced leaves grafted to receptor plants in non-inductive conditions cause flowering, leaves taken from the flowering receptor plants are not capable of inducing flowering themselves. In contrast, in *Xanthium*, leaves remain induced for only a short while (4-5days) (Hamner and Bonner, 1938). The induced state is not localised but is transmitted to young leaves which have not been exposed to inductive daylengths and in this way the induced state is maintained (Lam and Leopold, 1960; Salisbury, 1963). Very few species have been studied, but it may be that in those plants which require continuous exposure to inductive daylengths until morphological changes have occurred, the induced state is neither long lived nor self-perpetuating.

In all species studied to date, once a certain number of inductive cycles has been given the appearance of the first flower primordia is not advanced by exposure to more inductive daylengths. Moreover, although exposure of one leaf or part of a leaf may be sufficient to cause flowering, the threshold number of cycles is not decreased if more leaves are exposed. However, increasing the number of inductive cycles increases the magnitude of the response, that is, how quickly the floral primordia develop and the number of flowers that are produced.

1.3.3 Models of the flowering process

Julian Sachs in the early 1880's (Lang, 1965) was the first to propose that flowering is mediated by a hormone induced by environmental conditions. In photoperiodic plants grafting experiments provide evidence that a stimulus produced in the leaves (Knott, 1934) during inductive daylengths is transported to the shoot meristem via the phloem, where it initiates the development of flower primordia. Chailakhyan (1936 cited by Vince-Prue, 1975) gave this transmissible substance the name "florigen", which seems to be similar if not identical in different species and families (Vince-Prue, 1975). Grafting experiments of Lang and Melchers (1948) suggest that an inhibitor of flowering may also be present in the leaves of non-induced plants and he called this "anti-florigen". The florigen/antiflorigen model of flowering

proposes that flowering occurs when these two substances reach certain threshold levels at the meristem. However, neither florigen nor anti-florigen have been isolated, and in most plants other factors can influence and totally override the photoperiodic control of flowering. These factors may work by altering levels of florigen and anti-florigen. On the other hand, the interaction of environmental stimuli, and the ability of chemicals and plant growth regulators to induce responses similar to stages seen during floral induction and evocation, led Bernier *et al.* (1981) to suggest a multifactorial model for the control of flowering. In this model various plant parts may respond to external and internal conditions, their reaction depending on the plant's genetic makeup and stage of development defined by previous growth conditions. Promotors and inhibitors may affect one common pathway to flower initiation or independent pathways, with flowering occurring only when the correct balance of inhibitory and promoting substances is achieved at an appropriate time. The primary effects of the various pathways may be exerted in different tissues or organ systems.

Which ever model is favoured the isolation of substances which have a crucial role in the induction of flowering has not yet been successful. This may be because 1) the substances are biologically active at undetectable levels, 2) they are unstable and therefore difficult to isolate, 3) the substances are common but the response depends on the sensitivity of a small localized group of cells, 4) that several substances are involved and it is their relative rather than absolute concentrations which are important, or 5) that other changes make it difficult to distinguish truly inductive phenomena. For example, Sachs and Hackett (1983) argued that nutrient levels elicit a flowering response from competent tissue either directly or through the physical stresses the competent cells experience as a result of changed growth rates elsewhere in the plant. Many factors controlling flowering also influence photosynthesis and/or assimilate availability. For example LDs, high irradiance light, CO_2 concentration and vernalization (response to low temperatures; see below) which can promote flowering, alter assimilate availability and distribution. However, it is very difficult to decide whether assimilate concentration *per se* induces flowering or whether it is only

important as an energy source, because if specific inhibitors and promoters of flowering travel in the phloem, altering the assimilate flow will also result in changed levels of these substances and *vice versa.*

1.4 Vernalization

1.4.1 Temperature requirements

Some plants require exposure to low temperatures before they are able to flower. Klippart (1857) showed that after exposure to low temperatures winter cereals became similar to spring cultivars in their response to long daylengths. The process which occurs during growth at low temperatures and results in the promotion of flowering is called vernalization. In many cold-requiring species low temperatures do not induce a change from the vegetative to the reproductive state, but confer the ability to enter into reproductive growth when other factors controlling flowering are no longer limiting. Exceptions do exist; for example, in *Brassica gemmifera* (brussel sprouts) and *Brassica rapa* (turnip) (Bernier *et al.,* 1981), flower initiation must occur at low temperatures. Whether this is due to vernalization has been questioned by Vince-Prue (1975), who restricts the term "vernalization" to the indirect inductive effects of temperature. However, in *Lunaria biennis* (Wellensiek, 1985), *Beta vulgaris* (Fife and Price, 1953) and *Campanula medium* (Bernier *et al.,* 1981) flower initiation may occur after prolonged exposure to low temperatures, though in the field these plants would normally flower after a much shorter exposure to low temperatures followed by the longer photoperiods of spring. In these species the use of the term is not debated and until the process occurring during vernalization is understood it is pointless to make such a division. Differences in threshold temperatures required for vernalization and floral evocation may be the cause of these apparently direct and indirect effects of low temperature (Lang, 1965).

Generally, temperatures between 0°C and 15°C cause vernalization, but the range of effective temperatures varies between and within species and the optimum temperature may vary with the age of the plant, the length of exposure to low temperatures and the daylength. Although vernalization will occur in Petkus rye

between -5°C and 15°C the optimum temperature is somewhere between 1 and 7°C (Purvis, 1948). In sugar beet the optimum temperature varies with the length of exposure and the photoperiod. The optimum temperature in SDs is about 5°C, whereas in LDs a higher temperature, between 8°C and 10°C is optimal (Curth, 1960). It is difficult to be precise, as between cultivars there is considerable variation and the optimum temperature for vernalization is influenced by other environmental conditions and the developmental state of the plant (Vince-Prue, 1975).

In *Silene armeria* it has been shown that vernalization occurs only during the dark phase of a SD (Lyndon, 1985), but in other species eg. *Beta vulgaris* vernalization procedes in any daylength including continuous illumination (Heide, 1973).

Vernalization is a quantitative process in that the longer a plant is exposed to low temperatures up to a maximum period, the greater the effect. SDs, high temperatures and high light intensity can in some species substitute for a low temperature requirement. In *Campanula medium* SDs can substitute for low temperature (Wellensiek, 1985), *Calceolaria* requires vernalization at low light intensity but is a LDP at high light intensities (Runger, 1975) and high temperatures substitute for low temperatures in *Schrophularia*, which flowers after six weeks at 3°C or three weeks at 32-37°C (Larrieu and Bismuth, 1985). In some LDPs, eg. *Poa pratensis*, SDs are ineffective and even inhibitory in the absence of low temperatures but promote floral initiation when given together with or before exposure to low temperature (Gardner and Loomis, 1953). In most plants vernalization in inductive daylengths accelerates flowering. This is probably due to the concurrent production of the floral stimulus as vernalization procedes.

1.4.2 Devernalization

Devernalization can be caused by SDs, high temperatures, anaerobiosis and, in chicory, by exposure to ethylene (Bernier, 1989). The stability of the vernalized state depends on the species. In some cases such as *Lunaria annua* (Wellensiek, 1985), and *Hyoscyamus niger* (Lang, 1985), after vernalization the plant can be grown under any photoperiod or temperature and the vernalized state persists. Furthermore, vernalized

seeds of *Sinapis* and *Cheiranthus* (Bernier, 1981) can be dried and stored at room temperature for up to six years without devernalization occurring. In other species the vernalized state may be "fixed" only if the conditions after cold treatment are favourable. Favourable conditions vary with species or variety, but in general low temperatures and inductive daylengths stabilize the vernalized state whilst high temperatures and photoperiods other than those which induce flowering, cause devernalization. Generally growth in the temperature range 12-15°C does not cause either appreciable vernalization or devernalization and growth at these intermediate temperatures in many cases is enough to fix the vernalized state (Bernier, 1981). The length of exposure to low temperatures and the light intensity can also influence the stability of the vernalized state. In rye, complete devernalization occurs after a short period of vernalization, but as the duration of low temperature treatment lengthens so the stability of the vernalized state increases (Chouard, 1960). In *Chrysanthemum* devernalization only occurs when the light intensity is low (Schwabe, 1985).

1.4.3 Interaction of environmental factors

From the previous two sections it is clear that, in certain species, factors which are able to substitute for vernalization can also cause devernalization. For instance in certain species, particular daylength regimes can accelerate vernalization, while in others they may cause devernalization. Moreover, the interaction between environmental factors is dependent on endogenous factors relating to the stage of development. To illustrate the complexity of these interactions, in *Silene armeria,* a facultative LDP, flowering is induced by high temperatures of around 35°C applied to the root in SDs, by LDs at 20°C, and is accelerated in LDs and SDs at 20°C after growth at 5°C in SDs. These three sets of conditions all seem to act on the same process. Genotypes of *Silene armeria* with a low or high requirement for one set of inductive conditions exhibit an equal requirement for the other two. The sets of inductive conditions interact in an additive fashion no matter in which order the plant experiences them, and suboptimal exposure to each results in reversal of the induced state in SDs (Wellensiek, 1985). It has been proposed that all of these conditions may

inhibit the synthesis of an inhibitor which is produced in SDs at intermediate temperatures. In *Campanula*, on the other hand, the evidence suggests that induction by low temperatures and SDs acts on separate processes. Sensitivity to SDs occurs one month after sensitivity to cold is acquired, and whereas the SD requirement remains constant the cold requirement decreases with age. Moreover, the number of LDs required to induce flowering after SD induction is fewer than after exposure to cold (Wellensiek, 1985).

1.4.4 Responses to gibberellins

Application of gibberellin (GA) can substitute for the environmental conditions required for flowering in many plants. Treatment with GA elicits flowering in a selection of DNPs and LDPs; and can substitute for either the cold or LD requirement of plants which require vernalization. *Impatiens balsamina* is the only SDP which has so far flowered in response to GA (Nanda *et al.*, 1967), though other SDPs grown under non-inductive conditions may show stem elongation and, in sub-optimal conditions, application of GA may reduce the number of short days required for flower induction (Krishnamoorthy, 1975).

It has been suggested that florigen is a GA, however GAs have so far failed to cause flowering in any caulescent LDPs under SDs. Moreover, in *Chrysanthemum morifolium*, Harada and Nitsch (1959) showed that whilst GA could induce flowering in cold-requiring cultivars, SD cultivars did not respond. However stalks of GA-induced cold-requiring varieties when grafted to a SD cultivar in LDs did cause flowering (Krishnamoorthy, 1975). In addition GA_3 can cause the LSDP *Bryophyllum diagremontianum* to flower in SDs only, but if GA_3-induced plants are grafted to vegetative receptors they flower regardless of daylength (Zeevaart and Lang, 1962).

Although GA can substitute for vernalization in cold-requiring plants, GA is not thought to act directly on the process of vernalization. Application of GA to the seeds of seed-vernalizable plants has no effect on flowering and in, for example, *Arabidopsis*, plants are most sensitive to GA application at a time when they are least responsive to low temperature (Krishnamoorthy, 1975).

If GA does not act directly on vernalization how does it influence flowering? A likely answer is that GA may be a product of the vernalized state necessary for flowering to occur. If a multifactorial model of flowering is accepted, the range of effects that GA application elicits may be due to the component stimuli required to initiate flowering being different in the different plant species. That is, in some plants perhaps GA is the only factor lacking whereas additional signals may be blocked in others.

1.4.5 Genetic basis of vernalization

A requirement for vernalization can be caused by one or more genes. One dominant gene confers the biennial habit in *Lunaria annua* (Wellensiek, 1973), *Digitalis purpurea* (Yaniv, 1985) and *Hyoscyamus niger* (Correns, 1904 cited by Lang, 1985). In *Hyoscyamus*, ann^{+}/ann^{-} plants have an intermediate response. They require less cold to complete vernalization than ann^{+}/ann^{+} plants and show a greater floral response than the homozygote to the same cold treatment. In *Beta vulgaris* (Abegg, 1936) and *Secale cereale* (Purvis, 1939) control is also monogenic but in these plants the presence of a dominant gene confers annuality .

Two dominant genes *Sh2* and *Sh3* and one recessive gene *sh* confer annuality in *Hordeum vulgare* (Deitzer, 1985). The presence of either one of the *Sh* genes or homozygous *sh* confers annuality, whereas the winter habit is caused by the presence of a dominant *Sh* or homozygous recessive *sh2* or *sh3*.

In *Triticum* (Pinthus, 1985) five major genes *Vrn 1*, *Vrn 2*, *Vrn 3*, *Vrn 4* and *Vrn 5* control vernalization. Plants are annual if *Vrn 1-4* and perhaps *Vrn 5* is present. When *Vrn 1* is present plants do not respond to vernalization at all. When the other *Vrn* genes are present plants exhibit varying degrees of response to vernalization. The winter habit is conferred by genes *vrn 1- 4* and perhaps *vrn 5*.

In *Arabidopsis* both dominant and recessive genes are involved. Koorneef *et al.*, (1990) found 4 loci *fca*, *fc*, *fy* and *ft* in late-flowering mutants which showed a more pronounced response to vernalization than the wild-type under both long and short days. Up to 40 mutants in which flowering is promoted by exposure to low

temperatures have been reported (Napp-Zinn, 1985) but it is not clear how many of these represent different mutations.

In *Pisum sativum,* a LD plant, several phenotypic flowering classes have been identified, using response to photoperiod and variables such as NFI, FT, TFI, RLA, FLR and AS (see Table 1.1). These phenotypes can be explained by the action of genes at six major loci : *veg*, *Sn*, *Dne*, *Hr*, *Lf* and *E* (see Table 1.2). Briefly, plants with a *veg* phenotype are unable to respond to a floral stimulus. The *Lf* locus has four alleles: lf^a, *lf*, *Lf* and Lf^d which determine minimum flowering nodes of 5, 8, 11, and 15 respectively. Evidence from grafting studies has led to the proposal that *Lf* acts at the apex to determine the level of flowering signal necessary to trigger flowering. *Sn* and *Dne* are concerned with induction events in the leaves and their product seems to be a graft transmissible inhibitor of flowering. Production of the inhibitor is prevented in continuous light and is diminished by low temperature. The activity of the *Sn Dne* system decreases with age. *Hr* magnifies the effect of *Sn Dne* and acts in the leaves and the stem but not in the apex. *E* operates in the cotyledons and probably decreases the amount of inhibitor produced by the *Sn Dne* system (Murfet, 1985).

In plants with genotype *lf e Sn Dne hr* exposure to continuous light eliminates the vernalization response. Since continuous light has been shown to switch off inhibitor production by the *Sn Dne* system it has been proposed that one process which vernalization affects is the production of this inhibitor (Murfet, 1985). Another effect of vernalization is evident in plants with an *Lf e Sn Dne hr* genotype. In these plants vernalization still occurs in continuous light and in this case it is thought to act at the apex altering its sensitivity to levels of promotor and inhibitor substances overriding control by the *Lf* gene (Amos and Crowden, 1969).

1.4.6 Perception

Localized cooling experiments on celery (Curtis and Chang, 1936), sugar beet (Chrobozek, 1934) and *Chrysanthemum* (Schwabe, 1954) indicate that low temperature is perceived by cells at the shoot apex. Grafting experiments in which vernalized and unvernalized shoot tips of *Hyoscyamus niger* (Melchers, 1936) and

Table 1.1 **Criteria used to assess the flowering response of different genotypes of *Pisum sativum*.**

(adapted from Murfet 1985)

1.	NFI	node of flower initiation
2.	FT	number of days from sowing to first open flower
3.	TFI	time of flower initiation obtained by dissection
4.	RLA	rate of leaf appearance
5.	FLR	flower development relative to the development of surrounding leaves
6.	AS	apical senescence

Table 1.2 Six loci and their action in *Pisum sativum* with regard to flowering.

(adapted from Murfet 1985)

E	causes early flower initiation in certain backgrounds
Dne	together with Sn confers photoperiod response and ability to produce an inhibitor of flower initiation
Hr	interacts with Sn and Dne to delay the activity of Sn and Dne with age
Lf	confers minimum node of flower initiation
Sn	see Dne
veg	mutant which completely prevents initiation of flowering regardless of other genes

radish (Tashima, 1957 cited by Vince-Prue, 1975) were exchanged and flowering occurred only from the vernalized meristems confirm this view. In *Lunaria biennis*, Wellensiek (1964) found that meristematic cells at the base of leaf petioles could be vernalized, and that the vernalized state was transferred to plants regenerated from these cells. Thus all dividing shoot cells, including those in the leaves, may be potential sites of vernalization. Plants regenerated from the axillary buds of vernalized *Lolium temulentum* (Arumugunathan *et al.*, 1991) formed during and after vernalization appeared to be vernalized. However, it is possible to vernalize winter rye (Lang, 1965) and *Cheiranthus* seeds (Vince-Prue, 1975) under conditions where cell division does not occur. In addition, recent experiments have shown that shoots regenerated from leaf cuttings of vernalized *Thalspi arvense* L. (Field pennycress), which were fully expanded prior to low temperature treatment, developed reproductively (Metzger, 1988). In this work 1-2 cm of the petiole was cut off to ensure that no meristematic tissue from axillary buds remained on the cutting. Root fragments and leaves taken from vernalized *Chicorum intybus* (Chicory) will also form flowers *in vitro* and in this species meristematic tissue is not necessary for vernalization (Paulet, 1985). Moreover, the chilling treatment substituting for the daylength requirement in *Pharbitis, Perilla* and *Blitum* has recently been reported to be perceived by the leaves and not the apex (Bernier, 1989).

Studies on the perception of low temperature are scant but the information cited above does seem to suggest the situation is more complex than has previously been admitted. It appears that low temperature may be perceived by more than one part of the plant at sites removed from the site of daylength perception and the eventual site of floral evocation. Furthermore, vernalization may not be dependent on the presence of meristematic cells.

The site at which devernalizing conditions are perceived is also unclear. In *Brassica pekensis* (Chinese cabbage) high devernalizing temperatures are perceived by the root. Bolting and flowering were delayed at least 55 days by exposing the root to 30°C, whilst this localized heating raised the temperature at the shoot to only 12°C, too low a temperature to cause devernalization (Pressman and Negbi, 1981). Conditions

causing devernalization are therefore perceived by the roots but, since they affect the leaves and apex, transmission of the stimulus must be occurring.

1.4.7 Transmission of the vernalized state

Whether or not vernalization occurs in meristematic tissue exclusively or also occurs in non-meristematic tissue, the general consensus is that the vernalized state is localized and can only be transmitted through cell division from vernalized cells to their daughter cells. This view arose from the results of early experiments which suggested that dividing cells were a prerequisite for vernalization (Purvis, 1961) and from the results of studies which showed that flowering only occurred when vernalized cells or cells derived from them were present. For example, when the main shoot of vernalized Petkus rye is removed, the tillers which are stimulated to grow act as though they have been vernalized even though they were not present when the plant experienced low temperatures (Purvis, 1961). This view of a localized change of state is further supported by results of localized cooling experiments with *Medicago arborea* L. (Chouard, 1960) and with olive (Hackett and Hartmann, 1964), where flowering occurred only in parts of the plant which had received the cold treatment. In *Cheiranthus* (Barendse, 1985) and in *Chrysanthemum* (Schwabe, 1954) removal of the plant part that is derived from the originally vernalized apex totally prevents flowering of the laterals that develop afterwards.

1.4.8 Vernalin?

Localization of the vernalized state and a change in the competence of these vernalized cells does not preclude the existence of a translocated and perhaps transient effect of vernalization. The hypothesis that vernalization resulted in the production of a mobile substance "vernalin" was put forward by Melchers in 1939 (Vince-Prue, 1975). Although numerous grafting experiments between vernalized donors and unvernalized recipients have been reported in the literature in support of the vernalin hypothesis (Lang, 1965; Vince-Prue, 1975) none distinguish unequivocally between flowering caused by the floral stimulus transported directly from an induced or flowering donor to

the recipient, and what might be the transmission of vernalin. One grafting experiment of interest is that carried out by Melchers (Lang, 1965) between SD Maryland Mammoth (MM) tobacco and LD unvernalized biennial *Hyoscyamus niger*. Both grafting partners were kept in LDs and the Maryland Mammoth remained vegetative whilst the *Hyoscyamus* recipient flowered. This outcome is attributed to a substance (vernalin) present in the vegetative MM being translocated to *Hyoscyamus* allowing it to flower in LDs. However, it should also be borne in mind that the thermal requirement of biennial varieties of *Hyoscyamus* can be fullfilled by small amounts of gibberellin. Ideally this experiment should be carried out with donor and recipient plants of the same species. If vernalin existed one would expect flower formation in a non-vernalized recipient in inductive daylengths if the donor was vernalized and in non-inductive daylengths. In this respect *Hyoscyamus* is a poor choice of plant as in SDs an inhibitor of flowering is produced.

1.4.9 Models

Salisbury (1963) and others (Lang, 1965) proposed that low temperatures could act by suppressing the action of a flower inhibitor relatively more than the activity of a promotor. This could occur via a differential effect on synthesis of the promotor/inhibitor substances due to different temperature coefficients of enzymes involved in their synthesis, such that production of the inhibitor is suppressed at low temperatures. Over the years various models have been constructed to try to account for the observed effects on vernalization of different environmental stimuli. Lang and Melchers (1947 cited by Chouard, 1960) formulated a model (see Figure 1.1) to account for observations they made on *Hyoscyamus*. The model incorporates a precursor (A) used in a reaction at low temperatures which gives rise to a thermolabile substance (B) which is lost at high temperatures but which at intermediate temperatures can be used to form a floral stimulus (C). Later models constructed to account for the responses of other species to low temperature are fashioned along the same lines with a few more intermediates invoked and, in the case of Napp-Zinn (1957) two alternative routes to the production of floral stimulus.

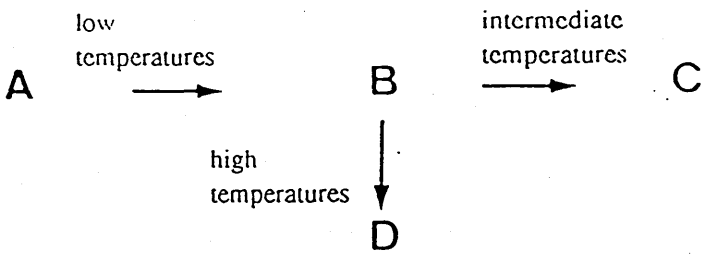


Figure 1.1
(taken from Chouard 1960)

A is used in a reaction at low temperatures which gives rise to B. B is thermolabile. At high temperatures it is used in a reaction to produce D. At intermediate temperatures it is used to produce a floral stimulus (C).

Alternatively the physiological effects of growth at low temperatures for example, the depression of leaf growth and altered assimilate levels at the shoot apex may result in vernalization. Depression of leaf growth by low temperatures may alter morphogenic gradients within the plant (Warren-Wilson and Warren-Wilson, 1984). Accumulation of carbohydrates at the shoot apex occurs at low temperatures and parallels the induction of flowering. If this is a requirement for floral meristem formation it could also account for the effect of other factors which alter assimilate availability and can substitute in certain species for low temperatures such as exposure to SDs, high light intensity and high nutritional levels (Evans, 1971).

McDaniel (1983) discussed the effects of vernalization in terms of the competence of tissues to respond to inductive conditions. In photoperiodic plants with a vernalization requirement, where daylength is perceived by the leaves and a floral stimulus of some sort is produced in response to inductive daylengths, the lack of response of unvernalized plants must mean that 1) the leaves are not competent to respond to daylength or 2) the apex is not competent to respond to the floral stimulus. Thus McDaniel proposed that vernalization may affect the meristem in either of two ways: a) the meristem starts producing leaves which are competent to respond to inductive daylengths with the production of floral stimulus; or b) an incompetent meristem becomes competent to respond to the floral stimulus. The response type a plant belongs to can be distinguished by grafting experiments (see Figure 1.2). The model is useful in that attention is focussed on the site of perception and action of the low temperature effect. As seen in pea, low temperatures affect both the leaves and the apex and there is good reason to believe that vernalization may act differently in different tissues and species.

Vernalization causes a localised change of state in groups of cells enabling them to respond to inductive conditions. The vernalized state is transmitted through cell division and may be extremely stable. It has been proposed that such a stable epigenetic state might be achieved via the phenomenon of habituation (Jackson and Lyndon, 1990). Habituation describes the situation where cells in culture requiring an exogenous supply of growth hormone, lose this requirement. From investigations of habituation in

Figure 1.2 a and b **Vernalization and two hypothetical response types.** (adapted from McDaniel 1983)

In a photoperiodic plant which requires vernalization flowering will only occur when the cold and daylength requirements have been met.

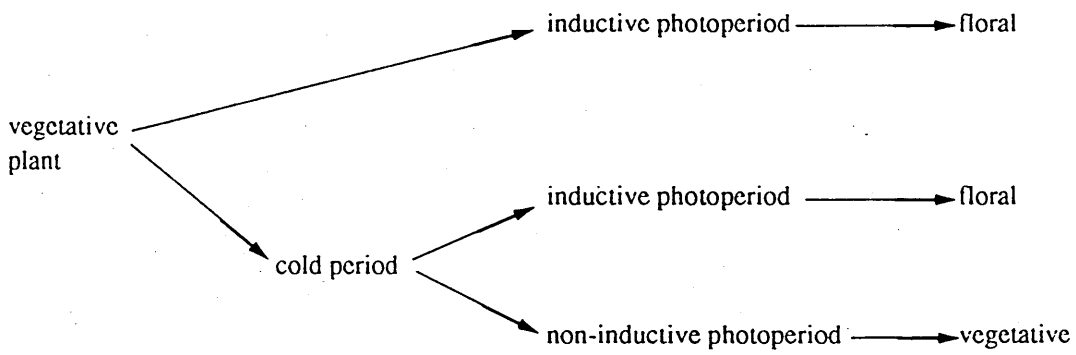
a Distinction between cold-requiring and non-cold-requiring genotypes

Plants not requiring vernalization flower in an inductive photoperiod without cold-treatment (upper line). Plants which require vernalization (lower lines) will only flower after cold-treatment and then only in the appropriate daylength.

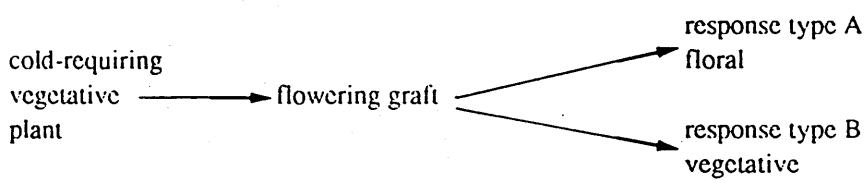
b Two hypothetical response types

In plants with response type A, the cold requirement is eliminated by grafting on a flowering shoot. In this type of plant the unvernalized meristem is evidently able to respond to floral stimulus translocated from the graft, but the leaves are unable to produce it. In plants with response type B, grafting on a flowering shoot does not induce flowering. In this type of plant the unvernalized meristem is unable to respond to floral stimulus.

a


inductive photoperiod
floral
vegetative plant
inductive photoperiod
floral
cold period
non-inductive photoperiod
vegetative

b


response type A
floral
cold-requiring vegetative plant
flowering graft
response type B
vegetative

plant callus culture it has been found that low concentrations of growth hormone can cause habituation for the same growth hormone or for a different hormone, and levels of only ten fold higher concentrations can cause reversion to dependence on an exogenous supply. One model proposes that the habituated and non-habituated states are autocatalytic (Meins, 1989).

Another mechanism, demethylation of DNA could be involved in maintenance of the vernalized state. Recently Dennis *et al.* (McDaniel, 1991) have reported that treating germinating seeds of vernalizable wheat and *Arabidopsis* with 5-azacytidine (which causes de-methylation) reduces the number of days to flowering.

1.5 Cold Acclimation

1.5.1 Physiology and biochemistry

Low temperatures not only cause vernalization but have a pronounced effect on vegetative growth (Pollock and Eagles, 1988; Guy, 1990; Thomashow, 1990). Plants tend to assume the temperature of their environment; thus to survive low temperatures they must be able to cope with the biochemical consequences. Plants are categorised on their ability to cope with low temperatures as either: chilling sensitive, chilling resistant or freezing resistant. Chilling temperatures are those usually below 10°C, which cause injury in chilling sensitive plants; freezing temperatures are those below 0°C. Many plants tolerate freezing temperatures better if they are first exposed to low non-freezing temperatures. This phenomenon is known as cold acclimation or cold hardening. In woody perennials cold acclimation and deacclimation are also triggered by seasonal changes in daylength (Thomashow, 1990).

Genetic factors involved in the response to cold are not well studied. However in wheat (Gullord *et al.*, 1975), oat (Jenkins, 1969) and barley (Enus *et al.*, 1962), when plants with different degrees of cold hardiness are crossed the offspring express a range of hardiness between and occasionally transgressing that of their parents. Thus in these species cold hardiness seems to be a quantitative trait.

Low temperatures affect membrane permeability and cell metabolism. Changes in membrane permeability occur over a range of temperatures and depend on membrane

composition. As the temperature is lowered, the phase transition of lipids changes the membrane from a fluid to a more solid state. This causes contraction of the membrane altering its permeability to small molecules and altering the conformational state of membrane-associated proteins affecting their ability to function. When chilling is sudden, contraction of the membrane may not be uniform and fractures may occur increasing permeability. At freezing temperatures ice crystal formation within the cell membrane or fracture of the cell membrane is lethal and must be avoided (Levitt, 1980). In addition the formation of extracellular ice crystals results in an increase in solute concentration outside the cell. In response to this, water moves out of the cell causing severe dehydration. Metabolic disruption at low temperatures occurs due to these changes and is also affected both by the inherent differences in the ability of enzymes to catalyse reactions at low temperatures and by protein denaturation due to low temperature weakening of hydrophobic bonds (Thomashow, 1990; Levitt, 1980).

Chilling resistant and freezing resistant plants are able to adjust their metabolism and structure to grow and survive at low temperatures. Studies of membrane composition indicate that the membranes of chilling resistant plants contain higher proportions of unsaturated fatty acids. Steponkus (1990) showed that rye protoplasts from non-acclimated plants exposed to low temperatures formed endocytotic vesicles upon dehydration which were not reincorporated into the membrane when the stress was removed, causing the protoplasts to burst as water entered the cell. In contrast, protoplasts from cold-acclimated plants produced exocytotic extrusions which remained in association with the membrane. Selective enrichment of non-acclimated protoplasts with an unsaturated species of dilinoleoylphosphatidyl-choline by liposome-protoplast fusion transformed the behaviour of the plasma membrane so that it produced exocytotic extrusions during osmotic contraction. Although the relative abundance of various lipid species changes on exposure of rye protoplasts to low temperature, Steponkus (1990) found no species unique to either acclimated or non-acclimated plasma membrane. The phospholipid-to-protein ratio in the plasma membrane of rye (Uemura and Yoshida, 1984) and orchard grass (Yoshida and Uemura, 1984) was found

to increase on exposure to low temperatures and changes in membrane proteins separated by 2-D PAGE have been identified.

Changes other than alterations in membrane structure and composition occur at low temperatures. Labile forms of enzymes may be replaced. To counteract decreased activities the concentration of enzymes may be increased or isozymes with lowered E_As produced. Cryoprotectants and repair proteins are induced and the subcellular location of proteins may be altered (Guy, 1990).

In chilling resistant plants morphological changes occur due to reduced cell extension, changes in the pattern of cell division and assimilate partitioning (Pollock and Eagles, 1988). Cell extension is affected as soon as plants are transferred to low temperatures. Cell extension relies on the presence of unextended cells and turgor pressure. Measurement of cell turgor in the extension zone of *Lolium temulentum* showed that turgor was independent of temperature between 2-20°C, thus the low temperature must be affecting cell walls making them more rigid (Pollock and Eagles, 1988). An increase in the glycoprotein extensin is known to be stress induced (Wilson and Fry, 1986). Extensin is thought to contribute to the strength and rigidity of cell walls. Weiser *et al.* (1990) found that the weight of cell walls increased as freezing resistance increased in pea seedlings as did the levels of extensin mRNAs.

Differences between mutants which show altered responses to temperature may help to elucidate the ways in which plants respond positively to low temperatures. For instance, the *slender* mutant of barley, which is caused by mutation of a single recessive gene, continues cell extension to -7°C compared to 2.5°C in the wild-type (Stoddart and Lloyd, 1986). The authors suggest that the mutation causes a change in the dynamics of wall turnover during extension. Hugly *et al.* (1990) reported the isolation of a chilling sensitive mutant of *Arabidopsis thaliana* (*chs1*). This is a recessive nuclear mutation which causes leaf chlorosis and electrolyte leakage on exposure to temperatures of 18°C or less. These are symptoms associated with chilling sensitivity. The biochemical difference observed between the mutant and the wild-type is the accumulation of steryl esters in the mutant following low temperature treatment. However it is not clear if the mutation affects sterol metabolism directly or whether

some other process has been affected and the effects on sterol metabolism are secondary.

1.5.2 Gene expression

To try to distinguish between proteins specifically involved in cold acclimation and general changes in gene expression due to growth at low temperatures numerous studies have compared proteins and mRNAs found in control and low temperature treated plants. The species studied include: alfalfa (Mohapatra *et al.*, 1987), barley (Cattivelli and Bartels, 1989; Hughes and Pearce, 1988), *Arabidopsis* (Gilmour *et al.*, 1988), mung bean (Kawata and Yoshida, 1988), spinach (Guy and Haskell, 1988), rapeseed (Meza-Basso *et al.*, 1986), wheat (Sahran and Perras, 1987; Uemura and Yoshida, 1984), tomato (Schaffer and Fischer, 1988) and potato (Tseng and Li, 1990). Both increases and decreases in the abundance of proteins and mRNAs have been observed, although in several species *in vivo* labelling of proteins and *in vitro* translation of mRNAs indicates that synthesis of most proteins continues at low temperatures and their relative abundance is unchanged.

In *Arabidopsis* (Gilmour *et al.*, 1988), alfalfa (Mohapatra *et al.*, 1987) and in wheat (Perras and Sahran, 1989; Danyluk and Sahran, 1990) increased LT_{50} values have been correlated with qualitative and quantitative changes in mRNA and protein found in freezing sensitive and freezing resistant cultivars at low and normal temperatures. Some of the proteins are induced in all cultivars at low temperatures and are probably involved in a general response to growth at low temperatures, whilst the others are only seen in freezing resistant cultivars and are therefore more likely to play a role in cold acclimation. In wheat (Danyluk and Sahran, 1990) and in *Arabidopsis* (Thomashow *et al.*, 1990) the fate of these mRNAs and proteins has been monitored after 1 days deacclimation and they have been found to return to control levels. Time course studies showed that new proteins were present within 1 day of exposure to low temperatures in potato (Tseng and Li, 1990) and that new mRNAs were present within 2 days in barley (Hughes and Pearce, 1988).

Some of these mRNAs from cold grown plants have been cloned by

differential hybridization from alfalfa (Mohapatra et al., 1989), *Arabidopsis* (Kurkela and Franck, 1990; Thomashow et al., 1990), barley (Cattivelli and Bartels, 1989; Dunn et al., 1990), tomato (Schaffer and Fischer, 1988) and *Brassica napus* (Pallas and Jenkins, unpublished). Only Mohapatra *et al.* (1989) have attempted to correlate the expression of these cloned transcripts in different cultivars with varying degrees of cold tolerance. Cold regulated (COR) is an apt term for these transcripts (Hajela *et al.*, 1990). Using labelled cDNA probes the COR transcripts have been shown to be present as early as one hour after transfer of plants to low temperatures (Hajela *et al.*, 1990). Characterization of these cDNAs and the genes they represent should provide information about the biochemical changes directly responsible for the changes associated with cold acclimation.

1.5.3 ABA

Freezing tolerance can be induced in certain species at non-acclimating temperatures by the application of ABA. This has been achieved in *Brassica napus* (Orr *et al.*, 1986), potato (Chen *et al.*, 1983) and in cell suspension cultures of *Bromus mermis* (Reaney and Gusta, 1987). In *Arabidopsis thaliana* (Lång *et al.*, 1989), and in *Brassica napus* cell suspension cultures (Johnson-Flanagen and Singh, 1986) it has been found that ABA induces some of the proteins which accumulate at low temperatures.

One of the *Arabidopsis* cold acclimation-specific (CAS) gene products identified by Kurkela and Frank (1990) accumulates in response to ABA. This gene codes for a small hydrophobic protein which has an amino acid sequence similar to that of the fish antifreeze proteins which are thought to exert their effect by binding to ice crystals (Yang *et al.*, 1988). Lee *et al.* (1990) transferred a gene coding for an "anti-freeze" protein to tobacco which resulted in depression of the freezing point of the transgenic plant by 3-5°C.

1.6 Sugar Beet (*Beta vulgaris*)

Clearly several different species have been used to study vernalization and cold acclimation and each of these has its merits. Sugar Beet (*Beta vulgaris*) was selected for the research presented in this thesis. The features of this plant demonstrating its suitability as a subject for this research are outlined below.

1.6.1 Introduction

Sugar beet (*Beta vulgaris*) belongs to the genus *Beta*. All the cultivated beets are included in the section *vulgaris*. The subspecies *vulgaris* contains several cultivars including: sugar beet, beetroot, mangolds, chard and spinach beet. Sugar beet is an important crop plant. Cultivated sugar beets are biennial; they are sown in early spring and harvested in the autumn of the same year. During vegetative growth sugar beet grows as a rosette and excess sugar is stored in an enlarged tap root, the "beet". Sugar is extracted from the root, and what remains of the root and the top foliage is used for animal fodder.

Annual and biennial varieties of sugar beet exist, and all are obligate LD plants (Munerati, 1924; Lexander, 1985; Smit, 1983). However, certain biennial varieties absolutely require vernalization in addition to LDs for the initiation of flowering. This makes them very suitable from the physiological standpoint, for studies of vernalization. The reproductive phase involves the rapid production of an elongated flowering stalk (termed bolting) which utilizes energy stored in the beet (Lexander, 1985).

The annual habit is determined by a single dominant gene, the *B* gene (Abegg, 1936), but is influenced by many recessive genes (Lexander, 1985). Although plant breeders continually select for an increased vernalization requirement the presence of these numerous recessive genes means that there is always a small percentage of seed in a batch tending towards annuallity; that is, plants which require a shorter exposure to low temperatures. If sufficiently low temperatures are experienced for a period of time in the spring some plants will bolt before harvest. This premature bolting decreases the yield of sugar (Longden, 1980), and if left to complete fruiting and shed seed these

early bolters give rise to "weed beet" in the following years. These weed beet are plants which have inherited a low vernalization requirement either because they have selfed or crossed with wild annual beets (Hornsey and Arnold, 1979).

The yield of sugar is directly related to the amount of sunlight intercepted (Scott and Jaggard, 1978), therefore higher yields are obtained if plants are well developed early in the year and are able to intercept a lot of the available sunlight during the long days of spring and summer. In northerly latitudes this means sowing in early April after which there is still a very real possibility of cold weather which may cause vernalization of the crop. For this reason the development of a triennial variety would be welcomed by farmers. Unfortunately this would be contrary to the needs of breeders and seed producers. Plants requiring long periods of vernalization have a longer life cycle which means that seed production and selection to enhance or reduce characters are slowed considerably.

Due to the agricultural importance of sugar beet its physiological requirements have been well studied. Although the requirements for both vegetative and reproductive growth are well defined the processes occurring during vernalization remain a mystery. If vernalization could be understood at the molecular level then perhaps ways could be found to engineer the ideal crop plant. For instance, if vernalization depends on the production of a protein at low temperatures, then translation of the mRNA encoding it could be inhibited by antisense RNA (Schuch, 1991). More recent approaches for blocking gene expression include the blocking of transcription with triple helix forming sequence-specific oligonucleotide analogues (Riorden and Martin, 1991) and the expression *in vivo* of antibodies to specific proteins (Hiatt *et al.*, 1989; Hiatt, 1990). These methods have so far been used to inhibit gene expression in animal cells but in the future they may be developed for the control of plant gene expression. On the other hand, if vernalization inhibits the production of a protein the mRNA coding for it could be cloned behind a cold specific promoter and transformed into plants. To enable seed production a mechanism for turning on or off the construct would need to be incorporated.

1.6.2 Temperature requirement for vernalization

Low temperatures between 4-10°C (Curth, 1960) are responsible for vernalization. The necessary length of exposure to these temperatures varies and depends on variety and age (Chrobozek, 1934; Wellensiek and Hakkaart, 1955; Curth, 1960). Some varieties known as resistant bolters require up to ten weeks vernalization, whilst three weeks may suffice for easy bolters. These are minimum times and if such plants are left longer at low temperatures flowering times can be reduced and seed yields increased (Curth, 1960). Moreover, annual forms will flower faster if they experience vernalizing temperatures (Chrobozek, 1934), thus annual and biennial varieties differ only in their degree of dependence on vernalization.

There is no juvenile stage with respect to vernalization in sugar beet. Sugar beet of all ages can be vernalized, but sensitivity to low temperature increases with age (Wellensiek and Hakkaart, 1954). Seed can be vernalized even while still attached to the mother plant (Wood *et al.*, 1980), but seed and young seedlings require longer exposure to cold to complete the vernalization process.

The temperature and daylength experienced after vernalization are critical since there is no stabilization of the vernalized state. Stout (1946) used the percentage of plants bolting and the bolting rate as a measure of the rate of vernalization occurring in biennial plants which he stored at a range of different temperatures. He found that the rate of vernalization was slower the lower the temperature within the range known to cause vernalization, and that the rate of devernalization increased as the temperature outwith this range increased. He hypothesized that temperature operated upon biochemical rather than physiological processes, such that at low temperatures the biochemical equilibrium favours the production of a substance required for flowering. At high temperatures this equilibrium alters so that its production is inhibited.

1.6.3 Photoperiod and vernalization

Vernalization can occur in any daylength and even in total darkness (Fife and Price, 1953). There is an interaction between temperature and daylength such that the longer the photoperiod during vernalization the shorter the subsequent time to

flowering. Furthermore, the longer plants are left at low temperatures, the fewer the number of long days required after vernalization to induce flowering and *vice versa.* (Chrobozek, 1934; Wellensiek and Hakkaart, 1955; Heide, 1973; Junges, 1959; Margara, 1968 and Curth, 1960).

Just as the optimum temperature for vernalization differs between cultivars, so does the number of LDs required to induce flowering and also the number of hours of light which constitutes a LD (Heide, 1973). If short days and/or high temperatures >15°C are experienced after vernalization devernalization occurs and the plants remain in the vegetative state (Chrobozek, 1934; Curth, 1960). Even when bolting has started unfavourable conditions will cause reversion to the vegetative state and the plants develop a "perched" rosette. These devernalized plants can be revernalized (Chrobozek, 1934; Curth, 1960).

SDs extended with low intensity light can cause flowering. As light intensity is increased so the time to flowering is reduced (Curth, 1955, 1960). When the ratio of red (600-700) to far red (700-770) light increases flowering is later in the annual (Lane *et al.*, 1965).

1.6.4 The effect of gibberellin

The effects of growth promoting and retarding hormones and chemicals have been studied in an attempt to find substances which could be used to control development in sugar beet.

The impetus for many experiments using GA was the hope that it would shorten the life cycle and allow plant breeders to reduce the generation time when selecting for plants requiring long periods of vernalization. GA can substitute at least partially for the cold requirement of biennial beet but not for the photoperiodic requirement (Wheatley and Johnson, 1959). In SDs only seed stalk formation and elongation occurs (Margara, 1968) whereas in LDs elongation and flowering result (Gaskill, 1957). As the concentration of GA applied increases, so the response rate increases, but if plants which have had sufficient vernalization are sprayed with GA there is no advantage. Experiments with different varieties and clones of a single variety have shown that GA

does not cause any synchronization of flowering, rather that as much variation occurs in timing and stage of development reached as with the response to vernalization (Snyder and Whittwer, 1959). Lexander (1975,1981) has found a correlation between bolting susceptibility and the state of membrane proteins before vernalization. Bolting susceptible varieties have greater amounts of available reactive -SH groups. She suggests that changes in the state of the membrane proteins may determine the sensitivity of plants to GA.

1.6.5 Perception of Light and Temperature

1.6.5.1 Grafting

When bolting resistant unvernalized scions of *Beta vulgaris* are grafted onto flowering stocks the scions flower only after complete defoliation (Curtis, 1964) or when they are kept in the dark (Margara, 1960). This suggests that a food deficit is required for transport of the graft transmissible flowering signal. In the one reported experiment in which flowering of unvernalized scions took place in the light the scions were grafted on at an early cotyledon stage and presumably were still importing carbohydrates (Curtis and Hornsey, 1964). Stout (1945) grafted annual scions to unvernalized biennial stocks and *vice versa*, and the stock or scion was kept in continuous illumination or in complete darkness (see Table 1.3). Both stock and scion flowered only when the annual part was kept in the light. Stout concluded that the floral stimulus was the same in both annual and biennial forms because floral stimulus translocated from annual plants in LDs resulted in flowering of unvernalized biennial recipients. In addition, flowering of the unvernalized biennial stock suggests importantly, that the unvernalized meristem is competent to respond to the floral stimulus, whereas the unvernalized leaves are unable to produce or transport it.

1.6.5.2 Localized cooling

Localized cooling experiments have shown that if the top of the beet just below the base of the leaf petioles is kept cool (~12°C), vernalization occurs (Chrobozek, 1934), and devernalization is prevented (Curth, 1960). Sample sizes in both

Table 1.3 **Flowering response of grafts of unvernalized annual and biennial sugar beet in different light regimes.**

Unvernalized biennial scions were grafted to annual stocks and *vice versa*, and the stock or scion kept in continuous illumination or total darkness. Both stock and scion flowered only when the annual part was kept in continuous illumination.

		Flowering	
Darkness	Continuous illumination	stock	scion
annual stock	biennial scion	-	-
biennial stock	annual scion	+	+
biennial scion	annual stock	+	+
annual scion	biennial stock	-	-

experiments were extremely small and they do not rule out the possibility that other parts of the plant may be vernalized.

1.6.5.3 Defoliation

Only one defoliation experiment has been reported in the literature. Sugar beet plants were vernalized for four weeks at 2.5°C and then placed in flower-inducing conditions. After 4, 6, 8, 10 and 12 weeks a number were completely defoliated then put back with the others in LDs (Curth, 1960). Up until 6-7 weeks after the end of the cold treatment this defoliation significantly delayed bolting and flowering and it was concluded that the green leaves were needed to receive and conduct the photoperiodic stimulus. The results also indicate that a substance produced by the leaves is required not only for initiation of flowering but also for the completion of the later stages of floral morphogenesis.

1.6.6 Tissue culture and transformation

If any sort of genetic manipulation is eventually to be carried out in sugar beet, transformation and regeneration will have to be perfected. Regeneration is not yet a routine technique in sugar beet, but various workers have had some success in regenerating whole plants from anther callus (Rogozinska and Goska, 1982), flower buds (Margara, 1970), petiole callus (Tetu *et al.*, 1987), petiole explants (Ritchie *et al.*, 1989) shoot-base tissue (Lindsey *et al.*, 1990) and mesophyll protoplasts (Krens *et al.*, 1990). Tetu *et al.* (1987) regenerated whole plants via somatic embryogenesis. Attempts to induce haploids from anther culture have so far been unsuccessful (Rogozinska and Goska, 1982), but Lux *et al.* (1990) produced haploid sugar beet plants from unpollinated ovules.

Transformation of sugar beet with *Agrobacterium tumefaciens* has been successful (Krens *et al.*, 1988, Lindsey *et al.*, 1990). Methods of direct DNA uptake by electrical or chemical stimulation or particle bombardment have not been reported for sugar beet to date.

1.7 Approaches to understanding the molecular basis of flowering.

Since many aspects of the flowering process are ultimately controlled by a plant's genotype, molecular genetic techniques can be used to identify and isolate the genes involved in controlling flowering. Three different strategies may be followed. The first depends on the detection of differential expression of genes between induced and non-induced plants or between mutant and wild-type plants. The second depends on the recovery of mutants caused by the insertion of a mobile genetic element (transposon) or T-DNA, and the third strategy relies on knowledge of the position of the genes of interest in the genome.

RNA/DNA hybridization experiments have shown that different tissues in a plant contain both common and specific RNA populations (Goldberg, 1986). Comparisons of mRNA and protein extracted from different tissues may identify differences due to differential gene expression. Transcripts present at different levels can be isolated from a cDNA library. Clones of transcripts present at 0.1-0.05% abundance containing sequences of interest can be isolated by differential hybridization with probes derived from RNA from induced and non-induced plants. Differentially expressed rare transcripts (<0.01-0.0001% of total mRNA) (Rhyner *et al.*, 1986) may be isolated by selectively enriching mRNAs present at low copy number by removing sequences common to both pools of mRNA before screening the library. Recently the polymerase chain reaction (PCR) has been used to amplify cDNA derived from a small amount of tissue and used to construct libraries (Jepson *et al.*, 1991) or to screen them (Kelly *et al.*, 1990). Potentially this technique could be used to amplify RNA from a single cell (Kaiser, 1990. unpublished review).

Qualitative and quantitative changes in total accumulated protein and protein synthesized *in vivo* and *in vitro* can be detected by 1 and 2-dimensional gel electrophoresis. If changes in protein are detected it is possible to purify proteins for which there is no known assay by separating them in a 2-D gel. The proteins can be blotted directly onto nitrocellulose, and from this ng quantities can be microsequenced (Bauw *et al.*, 1989). The protein sequence can then be used to generate oligonucleotides to screen a cDNA or genomic library for the corresponding gene.

Alternatively, the protein can be eluted and antibodies raised against it (Meyer et al., 1988). These antibodies can be used to screen a cDNA expression library for a clone containing the corresponding coding sequence.

These specific clones can be used to investigate the function of the proteins they code for. Sequence comparison with that of known proteins may indicate a possible function. For example sequence comparison of the *Arabidopsis* homeotic *agamous* gene, concerned with controlling morphogenesis, showed that it was highly homologous with the DNA binding region of transcription factors of both yeast and humans (Yanofsky *et al.,* 1990). Alternatively, transformation of the species in question with the gene of unknown function, such that expression is not specific to a developmental stage, may provoke a change from the expected phenotype and the new phenotype may indicate the function of the protein. A changed phenotype may also be brought about by introducing antisense RNA (Smith *et al.,* 1988; Grierson et al., 1990) or engineered ribozymes to prevent expression *in situ* (Ecker and Davis, 1986; Walbot and Brueing, 1988).

Alternatively the generation of tagged mutants by insertional mutagenesis can be used to identify genes of interest. In this approach mobile genetic elements (transposons) or T-DNA are used to create insertion mutants. When the element inserts into a gene, that gene function is lost and the plant acts as a mutant. The transposon or T-DNA is then used to probe a genomic library made from the mutated plant DNA for a clone containing the gene of interest. Sub-clones of the gene sequences flanking the inserted DNA can then be used to recover the wild-type gene. Transformation of a mutant plant with this gene should restore the wild-type phenotype.

This approach is feasible only in those species harbouring endogenous, cloned transposable elements or which can be transformed with known transposable elements or T-DNA. Recently the transposon *Ac* has been shown to transpose in transgenic potato, tomato, carrot, *Arabidopsis* and petunia. However, so far only a handful of plant genes have been cloned using this method from maize, *Antirrhinum* and *Arabidopsis* (Balcells *et al.,* 1991). The need to screen large numbers of tagged individuals means this approach is most relevent in identifying genes active early in development and

causing easily spotted mutant phenotypes. With regard to flowering, isolation of *deficiens* (Sommer *et al.*, 1990), a gene required for the normal development of petals and stamens in Snapdragon, and of *floricaula* (Carpenter and Coen, 1990), which is thought to control the developmental switch from vegetative to floral meristem, has been achieved. Coupland *et al.* (1990) are attempting to tag the late flowering locus *fg* in *Arabidopsis* using derivatives of the *Ac* system. Investigation of this gene should provide information on the biochemistry of floral induction and initiation. T-DNA has been used successfully by Yanofsky *et al.* (1990) who isolated *agamous* from *Arabidopsis*.

In the third approach positional information is used to clone genes. The position of the gene of interest, identified as a mutation, is located on the genetic map by following its segregation. The location of the gene on the genetic and physical maps is aligned using restriction fragment length polymorphisms (RFLPs). Physical maps of the species may be long range restriction maps or overlapping fragments (contigs) of the genome cloned in vectors such as cosmids, plasmids or yeast artificial chromosomes (YACs). The cloned genomic sequences are arranged through computer matching of fingerprints (Coulson *et al.*, 1986) and/or by multiplex analysis (Evans and Evans, 1990).

An RFLP showing linkage to a locus identified on a genetic map may still be several cM distant from the locus. In *Arabidopsis*, which has a small genome, one cM is estimated to equal several thousand bp. Further fine structure mapping (Hauge *et al.*, 1991) in the region of the locus can be carried out to orientate the position of the RFLP in relation to the locus and enhance the resolution of the RFLP map.

From the linked RFLP a chromosome walk can be initiated using overlapping cosmid/YAC clones to cover the predicted distance to the locus. Once a set of clones covering the estimated region of the gene has been identified transformation of mutants with a cosmid containing the wild-type gene may cause functional complementation. If no mutant is available, or if complementation doesn't work, the only way to find the gene may be to sequence and analyse large tracts of DNA for candidate genes.

Bleecker (1991) has reported the isolation of a gene involved in the response to ethylene in *Arabidopsis* utilizing the above approach. Hauge *et al.* (1991) are attempting to clone genes for ABA and GA responses in *Arabidopsis*.

1.8 Aims of the research

The aim of this project was to investigate the molecular basis of vernalization in sugar beet. If vernalization of sugar beet could be prevented crops of beet could be sown earlier in the year or overwintered and the yield of sugar increased due to earlier establishment coupled with prevention of bolting. Moreover, if the requirement for vernalization could be controlled the life cycle could be shortened and seed production increased.

From the previous sections it is clear that the promoting effect of low temperatures on the flowering process may affect the synthesis of promoter and inhibitor substances. Species evidently differ in this respect. There may be fundamental differences in the nature of the processes occurring or, for a given process, different chemical substances may function as the active molecules. Because of these differences it is important to study individual species rather than generalise too widely from model systems.

The three main approaches available for the investigation of vernalization at the molecular level were outlined in the last section. As yet gene tagging is not feasible in sugar beet since there are no known endogenous transposons and the transformation system has not been fully optimised. In addition the number of plants which would have to be screened and the nature of the screen would require large amounts of space and would be very time consuming. A chromosome walk to the *B* gene could be initiated, however the amount of data on genetic, physical and RFLP maps required before this could be done is immense. Thus an approach based on the detection of differential gene expression was followed. It was therefore important to determine which tissues are affected by vernalization in sugar beet and are therefore most likely to show differential gene expression.

Vernalization influences the developmental fate of certain plants by affecting their ability to respond to inductive daylengths. Grafting experiments of Stout (1945) indicate that in sugar beet the shoot apex is competent to respond to inductive signals in the absence of vernalization. Hence it is most likely that vernalization changes the competence of the leaves to produce the floral stimulus which is subsequently transported to the apex in response to inductive daylengths. However it is not clear which leaves are changed, only those initiated from a vernalized meristem, or all those exposed to low temperatures. In addition, there is no mention in the literature of experiments which investigate the possibility that inhibitors of flowering might be present in the leaves. The defoliation experiment carried out by Curth (1960) to determine the role of the leaves in the perception of daylength indicates that flowering in sugar beet is not simply controlled by an inhibitor, but this does not rule out the possibility that an inhibitor might interact with other factors and affect the degree of the flowering response. The first part of this thesis is concerned with the site(s) of vernalization and investigating the possible presence of an inhibitor substance.

Localised cooling experiments showed that cooling the growing tip of sugar beet was sufficient to cause vernalization. However, this need not mean that perception is restricted to this region. From the work cited above, it is reasonable to assume that differences in gene expression might be detected in both the leaves and the meristem. The annual phenotype in sugar beet is conferred by the presence of the *B* gene. It is not known if this gene codes for a protein which is always present or one which is induced or modified in LDs. Moreover, it is not known if the same or an alternative protein is required for flowering in the biennial. If the same protein is necessary, again it is not known whether during the period of low temperature, production of this protein is induced or its concentration increased to a threshold level, or if its activity is altered by modification. Such changes may be brought about by changes in transcription, translation or processing of mRNA or protein, mRNA or protein turnover, or the post translational modification of proteins. The assumption was made that it might be possible to detect any such changes by comparing proteins separated by 2-D electrophoresis. If large quantitative or qualitative changes of either accumulated

protein or translation products of mRNA were detected the techniques of protein purification or differential screening of cDNA libraries would be used to investigate their significance.

Chapter Two: Materials and Methods

2.1 Chemicals

All chemicals were obtained from BDH (Poole, Dorset.), Fisons (Loughborough.) and Sigma (Poole, Dorset.) unless stated otherwise in the text, and were of the highest grade obtainable.

2.2 General Methods

2.2.1 Sterilization of equipment and solutions

Equipment and solutions were sterilized in a Prestige Hi-Dome pressure cooker or an Astell Heavson Portaclave at 15 lb/in^2 for 20 minutes. Solutions used in RNA work were treated with diethyl pyrocarbonate (DEPC). DEPC was added to a concentration of 0.1% (v/v) and left overnight at room temperature or for 2 hours at 25°C. After this time the solutions were autoclaved for 20 minutes at 15 lb/in^2. Glassware was baked for 8 hours at 180° C.

2.2.2 pH measurement

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

2.2.3 Preparation of phenol

Phenol was redistilled, 8-hydroxyquinoline added to a final concentration of 0.1% (w/v), and the solution saturated with 10 mM Tris-HCl pH 7.6 (Boehringer-Mannheim UK (Diagnostics and Biochemicals) Ltd).

2.2.4 Quantification of RNA

Aliquots of each RNA sample were scanned in a Pye Unicam SP8-500 spectrophotometer over the range 220 - 300 nm. A reading of 1.0 at 260 nm is equal to approximately 40 µg/ml RNA. The ratio of A260 to A280 readings gave an indication of the purity of each sample. A ratio of 2.0 should be obtained for pure RNA.

2.2.5 Quantification of protein

Protein concentration was determined by the method of Bradford (1976), using a protein dye concentrate (BioRad, Watford, Hertfordshire.). A calibration curve was plotted using triplicate samples of known concentrations of BSA versus their average absorbance at 595 nm. This was used to determine the concentration of protein in samples.

2.2.6 Estimation of protein molecular mass

To estimate the molecular mass of polypeptides separated by polyacrylamide gel electrophoresis marker proteins of known molecular mass were run on the same gel. Proteins migrate through SDS-polyacrylamide gels at rates which are inversely proportional to their molecular mass. Thus the distance the marker proteins migrated through the gel was plotted against the logarithim of their molecular mass. From the plot the size of other proteins on the gel could be calculated by measuring the distance they had migrated and using the standard curve to read off their molecular mass.

2.3 Illumination and Growth of Plants

2.3.1 Measurement of spectral quality of the light sources

Spectral photon distributions of the light sources (see Figure 2.1) were determined using a Macam spectroradiometer.

2.3.2 Growth rooms - light and temperature regimes

Warm short day (SD) growth room:

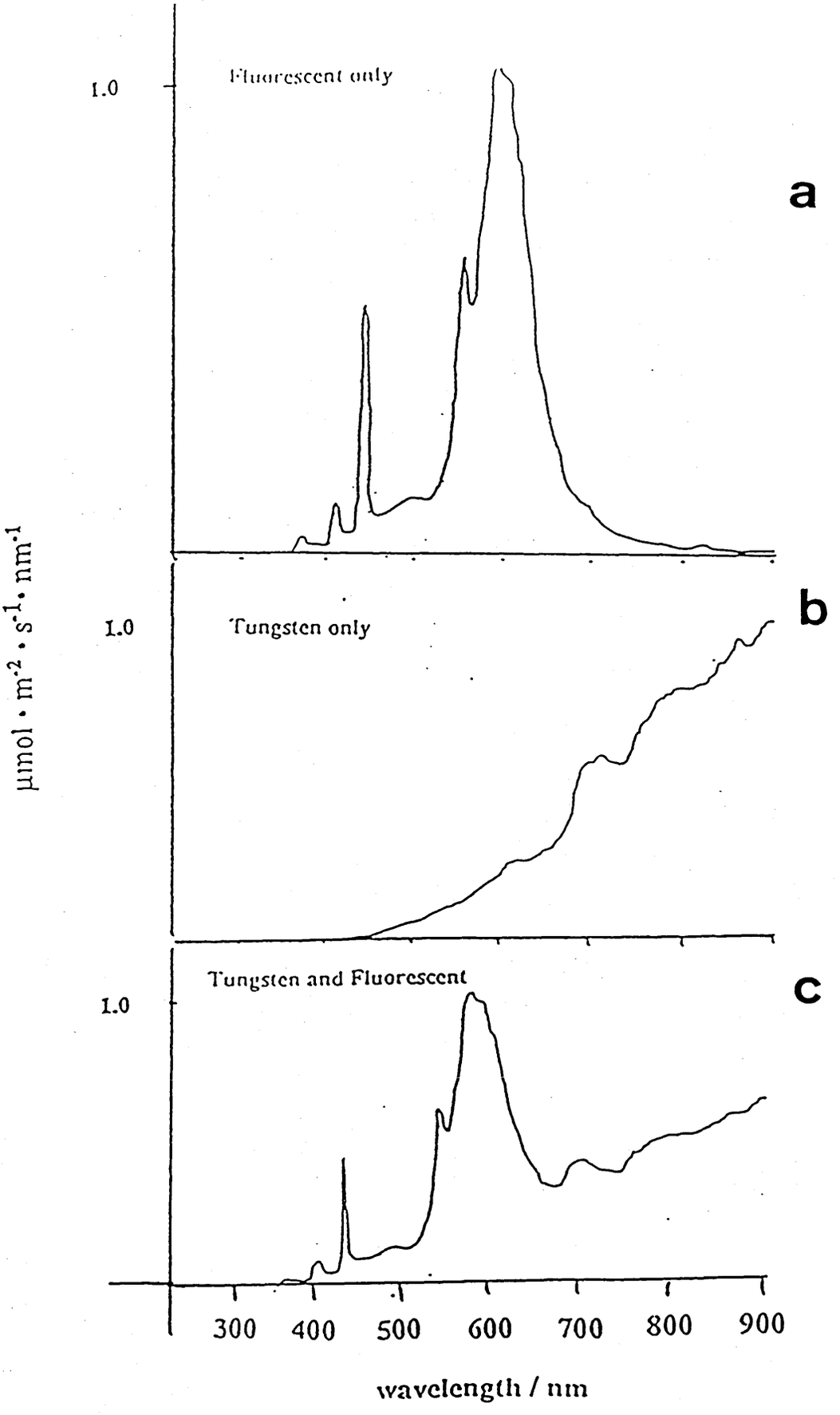
Temperature at plant height 20 - 23°C ; 8 hours fluorescent (Osram 45 W warm white) light (100-120 µmol m^{-2} s^{-1}; 400-700 nm) as measured with a Li-COR LI-185B quantum sensor.

Cold SD growth room:

Temperature at plant height 6-11°C , 8 hours fluorescent (Osram 45 W warm white) light (100-120 µmol m^{-2} s^{-1} ; 400 - 700 nm).

Figure 2.1 Spectral photon distributions of light sources

Spectral photon distributions were measued at wavelengths from 300nm-900nm using a spectroradiometer for **a** : fluorescent light, **b** : incandescent light, and **c** : both fluorescent and incandescent light.


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

Cool long day (LD) growth room:

Temperature at plant height 13-15°C, 22 hours fluorescent (Osram 45 W warm white) light supplemented with 8 150 W tungsten lights (150-200 µmol m^{-2} s^{-1} ; 400 - 700 nm).

Warm LD growth room:

Temperature at plant height 22 - 24°C, 22 hours fluorescent (Osram 45 W warm white) light supplemented with 8 150 W tungsten lights (150-200 µmol m^{-2} s^{-1} ; 400 - 700 nm).

2.3.3 Growth of plants

Seeds of diploid annual (7S 31660) and tetraploid biennial (5P 606) sugar beet were obtained from Dr W. Schuch, ICI Seeds, Bracknell, UK. Seeds were soaked overnight to leach out inhibitors of germination, then sown in autoclaved compost in flat trays and left to germinate in the warm SD room. When the first pair of true leaves emerged (after approximately 3 weeks) the seedlings were potted out into 4" pots and grown on to at least the 6 - 8 leaf stage.

2.4.1 Sampling

Samples of leaf tissue for the RNA and protein work were taken between 3pm and 4pm. The leaves were removed, their outline traced, and directly after this they were frozen in liquid nitrogen (BOC). Samples were stored at -80° C.

For the dissection of apical tissue, large leaves were removed from the region of the shoot apex which was then embedded in 1.8% agarose in a petri dish. The remaining leaves were removed with fine micro-dissection scissors under a dissecting microscope. When the apical meristem was visible it was dissected out, wrapped in a small amount of aluminium foil and placed in liquid nitrogen. For long term storage the tissue was kept at -80° C.

2.4.2 Harvesting and microscopy of apical tissue

Apices were fixed in 3% (v/v) glutaraldehyde (Aldrich Chemical Company Ltd., Dorset.) for a minimum of 48 hours. Mr Eoin Robertson, Department of Chemistry, Glasgow University embedded, sectioned and stained the tissue. Briefly, after fixing in 3% glutaraldehyde the tissue was rinsed in distilled water and dehydrated through emersion in solutions of increasing concentration of ethanol. The tissue was then cleared in xylene and after decanting most of the xylene, covered in wax pellets and placed in a 60°C oven overnight. This wax was replaced with fresh melted wax and put back in the oven. This was repeated twice more, and the tissue was then embedded in wax in preshaped plastic containers.

Sections of 10 μm thickness were cut on a rotary microtome, separated on water and collected on standard glass slides. The sections were dewaxed in xylene hydrated with water and mordanted in 1% (w/v) ferric ammonium sulphate for 40 minutes, rinsed in water, then transferred to 0.5% (w/v) haematoxylin (Heidenheins) and left overnight. Slides were regressively destained in 1% (w/v) ferric ammonium sulphate and the destaining process stopped by a 1 hour wash in tap water. Finally the sections were dehydrated through an ethanol series and counterstained in 0.5% (w/v) Fast Green in xylene and mounted in D.P.X. mounting medium.

2.4.3 Determination of the leaf area and dry weight of plant tissue

Leaf area was measured using a Delta T area meter (Delta Devices, Cambridge, England). The leaves were removed and numbered from the base. After leaf area measurement, the leaves and roots were dried in an 80°C oven in paper bags and the dry weights taken after 2 and 3 days by which time the weight remained constant. All leaf measurements included the lamina and the petiole.

2.4.4 Wounding

Plants were wounded by scoring the leaves several times with a razor blade.

2.4.5 Absisic acid (ABA) treatment

A measured quantity of ABA was first dissolved in a small amount of acetone and then distilled water was added to give a final concentration of 3 mM. This solution was sprayed on the plants to run-off.

2.5 RNA Methods

2.5.1 Extraction of total RNA

RNA was extracted as described by Wallace (1987). Plant tissue was ground under liquid nitrogen using a mortar and pestle. The resulting powder was transferred to a 200 ml conical flask containing extraction buffer [0.25 M NaCl, 0.025 M NaAc, 0.0025 M Na_2EDTA, 5% (w/v) SDS pH 5.0], SDW (sterile distilled water), and phenol /chloroform (1:1) in the ratio 1 : 4 : 5. The flask was shaken for five minutes in a 65°C-70°C water-bath. The mixture was transferred to a 30 ml Corex tube and centrifuged at 5000 rpm in a Beckmann (J12-21 Model) centrifuge in a JS-13.1 rotor for 20 minutes at 4°C. The upper aqueous phase was extracted first with an equal volume of phenol/chloroform followed by an equal volume of chloroform. The upper phase was saved and nucleic acids precipitated overnight at -20°C by the addition of 1/10 volume 3 M NaAc pH 5.4 and either 2½ volumes ethanol (James Burrough Ltd., Witham, Essex) or 1.1 volumes of iso-propyl alcohol. The precipitate was pelleted at 9000 rpm in a Beckman J12-21 centrifuge (JA-20 rotor) for 45 minutes at 4°C and resuspended in 2 M LiCl. Finally the RNA was pelleted by centrfugation at 9000 rpm in a Beckman J12-21 centrifuge (JA-20 rotor) for 30 minutes at 4°C, washed with 95% (v/v) ethanol and dried under vacuum. Pellets were resuspended in DEPC-treated water and 1 µl (25 units) of human placental ribonuclease inhibitor (Amersham International plc.) added. The samples were stored at -80° C.

2.5.2 Extraction of total RNA from small quantities of tissue

The above procedure was followed except that the lower phase from the first extraction was re-extracted by the addition of more extraction buffer and water and after spinning the upper phases were combined. After phenol/chloroform extraction the

lower phase was re-extracted with water and the upper phases combined before extraction with chloroform.

2.5.3 *In vitro* translation of mRNA

mRNA was translated *in vitro* using nuclease-treated wheat germ extract (Amersham International plc, Aylesbury). Potassium ion and mRNA concentrations were optimized and translation in the presence of 25 µCi L-(^{35}S)methionine, specific activity 1138 Ci/mmol (ICN Biochemicals Ltd., High Wycombe), carried out according to the manufacturers' protocol. Samples containing 1 x 10^5 cpm or more for one-dimensional electrophoresis were added to 2 volumes of SDS loading buffer (see section 2.7.4) and boiled for 3 minutes before loading. Samples for two-dimensional electrophoresis containing 1 x 10^6 cpm were first incubated with ribonuclease A at 5 µg/ml for 10 minutes at room temperature, then mixed with an equal volume of 2 times concentration IEF sample buffer (see section 2.7.3).

2.5.4 Estimation of incorporated radioactivity in *in vitro* translation products

The amount of incorporation in 2 µl aliquots of the reaction mixture was measured firstly by stopping the reaction with 1 ml of stop solution (1 ml 10 M NaOH, 10 mg methionine in 30 ml water) and incubating at 37°C for 10 minutes. 1 ml 25% (w/v) TCA and 10 µl 2% (w/v) BSA were added and the protein left to precipitate for 15 minutes in an ice-bath. The precipitate was collected on Whatman GF/A glass fibre filter discs, washed twice with 8% (w/v) TCA and once with 95% (v/v) ethanol. The discs were air dried, placed in scintillation vials along with 6 ml Ecoscint A (National Diagnostics, Manville, NJ, USA) scintillation fluid and the incorporated radioactivity measured in a scintillation counter.

2.6 Protein Extraction

2.6.1 Extraction of proteins in the presence of SDS

Protein extraction was carried out according to Colas des Francs *et al.* (1985). Plant tissue was ground under liquid nitrogen using a mortar and pestle. An

equal volume of extraction buffer [50 mM Tris-HCL (pH 8.5 at room temperature), 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 25 μg ml^{-1} leupeptin and 40% (w/v) insoluble polyvinylpyrrolidone (PVP, average molecular weight 40,000)] was added to the powder and the mixture vortexed for 40 seconds. The extract was then boiled for 3 minutes and centrifuged in either a MSE Microcentaur microcentrifuge at 13 000 rpm or in a Beckman centrifuge (J12-21) at 9 000 rpm (JA-20 rotor) at 4°C for 20 minutes. The supernatant was decanted into a fresh tube and the protein precipitated with 10 volumes of acetone overnight at -20°C. The precipitate was collected by centrifugation as above, the pellet washed in 95% (v/v) ethanol, then dried down under vacuum. The dried pellet was resuspended in either SDS (see section 2.7.4) or IEF (see section 2.7.3) loading buffer.

2.6.2 Extraction of proteins in a non-denaturing Tris buffer

Protein was extracted according to Granier (1988). Plant tissue was ground as above. An equal volume of extraction buffer [30 mM Tris-HCl (pH 8.7), 1 mM DTT (Boehringer-Mannheim UK), 1 mM Na_2EDTA, 5 mM $MgCl_2$ and 40% (w/v) insoluble PVP (average molecular weight 40 000)] was added to the powder and the mixture was vortexed for 40 seconds and then centrifuged at 13 000 rpm in an MSE Microcentaur microcentrifuge for 20 minutes at 4°C. Proteins were precipitated from the supernatant at -20°C for 1 hour or more by the addition of 10 volumes of a solution of acetone and 10 mM 2-mercaptoethanol. The precipitate was collected and treated as in section 2.6.1.

2.6.3 Extraction of proteins with phenol

Protein was extracted according to Guy and Haskell (1988). An equal volume of extraction buffer [80% (v/v) distilled phenol buffered wiith 120 mM Tris-HCl pH 6.8, 50mM Na_2EDTA, 100 mM KCL, 1 mM PMSF, 2% (v/v) 2-mercaptoethanol] was added to the powdered tissue and vortexed for 40 seconds. The mixture was centrifuged for 10 minutes at 4°C in an MSE Microcentaur microcentrifuge at 13 000 rpm. The upper phase was discarded and the lower phase

extracted 3 times with 1 ml of extraction buffer. After the final spin the proteins were precipitated from the phenol for 2 hours at -80°C following the addition of 5 volumes of 100 mM ammonium acetate, 1% (v/v) 2-mercaptoethanol in absolute methanol. The precipitated protein was pelleted by centrifugation as in section 2.6.2. The pellets were washed twice with 1ml of the ammonium acetate solution, then twice with 1 ml of acetone containing 1% (v/v) 2-mercaptoethanol, and finally air dried.

2.7 Gel Electrophoresis

2.7.1 Formaldehyde gel electrophoresis of RNA

1% agarose gels were prepared by dissolving by heating 0.5 g of agarose in 5 ml of 10 times MOPS buffer (0.2 M MOPS, 0.05 M NaAc, 0.01 M Na_2EDTA pH 6.5) and 42.5 ml SDW. The melted agarose solution was cooled to 50°C and then 2.7 ml 37% formaldehyde mixed in. The solution was poured into a gel former and left to set. RNA samples were mixed with 2 volumes of loading buffer (0.72 ml formamide, 0.16 ml 10 x MOPS buffer, 0.26 ml 37% (v/v) formaldehyde, 0.18 ml SDW; 0.1 ml glycerol and 0.08 ml saturated Bromophenol blue). The samples were heated at 60°C for 10 minutes then chilled on ice before loading. The gel was run at 100 V until the dye front had migrated 2/3 the length of the gel.

2.7.2 Phast System protein electrophoresis

Apical proteins were separated and stained in the Phast system (Pharmacia) using precast 10-15% SDS gels and buffer strips (Pharmacia). The gels were stained in the Phast system development unit with silver following the manufacturers protocol.

2.7.3 Iso-electric focussing

Iso-electric focussing was carried out according to O'Farrell (1975). The resolving gel [5.5g urea (Gibco, Paisley) 1.3 ml 28.3% (w/v) acrylamide and 1.62% (w/v) methylenebisacrylamide, 2 ml 10% nonidet P-40, 1.95 ml water, 0.4 ml Ampholine carrier ampholytes pH 5-7 (Pharmacia/LKB), 0.1 ml Ampholine carrier ampholytes pH 3.5-10 (LKB), 10 µl 10% (w/v) ammonium persulphate and 5 µl

TEMED] was poured into warm tubes (12cm x 1.5 mm), overlayed with 25 µl SDW and left to polymerize for 30-40 minutes. After polymerization the water was replaced with 25µl of IEF sample buffer [9.5 M urea, 5% (v/v) 2-mercaptoethanol, 2% (v/v) nonidet P-40, 1.6% (v/v) Ampholine carrier ampholytes (pH 5-7), 0.4% (v/v) Ampholine carrier ampholytes (pH 3.5-10)] and left to equilibrate for 20 minutes. After this the tubes were placed in the electrophoresis apparatus (LKB) with 10 mM H_3PO_4 in the lower reservoir and 20 mM NaOH in the upper reservoir. 25 µl of fresh IEF sample buffer was layered onto the gels and they were pre-run at 200 V for 15 minutes, 300 V for 30 minutes and 400 V for 30 minutes. Prior to loading the samples, the liquid above the gels was aspirated and fresh 20mM NaOH placed in the upper reservoir. The samples were then loaded, overlayed with 25 µl of IEF sample buffer and run at 400 V for 13.5 hours or more (up to 15 hours). For the final hour of electrophoresis the voltage was increased to 500 V. After electrophoresis the gels were extracted from the tubes by applying pressure to one end, placed in equilibration buffer [2.3%(w/v) SDS, 5%(v/v) 2-mercaptoethanol, 10%(v/v) glycerol, 62.5M Tris-HCl pH 6.8, 0.1% (w/v) Bromophenol blue] and left at room temperature for 1 hour. Gels were then wrapped in foil and stored at -80°C or run in the second dimension by placing along the top of an SDS-PAGE slab gel (section 2.7.4), making sure no air bubbles were trapped between the IEF and SDS gels.

2.7.4 SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970) in 12% or 15% polyacrylamide gels with 3% stacking gels. Resolving gel [6.6 ml buffer 1 (1.5 M Tris-HCl, 0.4% (w/v) SDS, pH 8.8), 9.6 ml of a 30% (w/v) acrylamide and 0.8% (w/v) methylenebisacrylamide solution, 1.6 ml 50% (v/v) glycerol, 8 µl TEMED, 90µl 10% ammonium persulphate and SDW to 24 mls] was poured between glass plates (16 cm x 18 cm) separated by 1.5 mm spacers. After pouring the gel approximately 500 µl of 0.1% SDS was layered on top to ensure complete polymerization. Once the gel had set (approximately 60 minutes) a 3% stacking gel [3.75ml buffer 2 (0.5M Tris-HCl, 0.4% (w/v) SDS, pH 6.8), 1.5 ml of a 30%(w/v) acrylamide 0.8%(w/v)

methylenebisacrylamide solution, 8 µl TEMED and 150µl 10% ammonium persulphate and SDW to 15 ml] was poured on top with a sample well former in place and left to set for 1 hour. The gels were run in LKB electrophoresis tanks, with running buffer [0.025 M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS] in both the upper and lower reservoirs. Samples were added to 2 volumes of loading buffer [50 mM Tris-HCl pH 8.0, 2% (w/v) SDS, 10% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.1% (w/v) bromophenol blue] and boiled for 2 minutes. After loading the samples electrophoresis was conducted at 65 mA for approximately 2.5 hours (or until the dye front reached the bottom of the gel).

2.8 Staining and Autoradiography

2.8.1 Ethidium bromide staining of RNA gels

A stock solution of 10 mg/ml ethidium bromide in water was prepared and stored at room temperature in a vial covered with aluminium foil. After electrophoresis, gels were left shaking gently in a solution of 0.5 µg/ml ethidium bromide for 15-20 minutes, and then examined under ultraviolet illumination. If destaining was required the gels were shaken in SDW for 10-15 minutes.

2.8.2 Silver staining proteins in SDS-PAGE gels

Gels were silver stained according to Morrissey (1981) except that 12 ml of 2.3 M citric acid was added per 100 ml developer to stop the staining reaction. Briefly, gels were shaken gently for 30 minutes in 100 mls of each of the following solutions: (1) methanol : SDW : acetic acid (5:4:1) : (2) SDW : methanol : acetic acid (83:10:7): (3) 10% (v/v) glutaraldehyde (Aldrich Chemical Company Ltd.). Excess glutaraldehyde was removed and the gels soaked in several changes of SDW over a 2 hour period or in several volumes of SDW overnight. After a final water wash the gels were shaken gently in a solution of DTT (5 mg/l) for 30 minutes. The DTT solution was replaced by a 0.1% (w/v) solution of silver nitrate (John Matthey Materials Technology) and the gels shaken for a further 30 minutes. Following this the gels were rinsed quickly in a small volume of SDW, then shaken for 1 minute in a small amount

of developer [3% (w/v) sodium carbonate, 50µl 47% formaldehyde / 100 ml], then shaken in 100 ml of developer until the desired level of staining was achieved. To stop the staining reaction 12 ml of 2.3 M citric acid was added directly to the developer and the solution agitated for 10 minutes. After rinsing several times in SDW the stained gels could be stored in SDW for several months.

2.8.3 Coomassie Blue Staining

Gels were shaken gently in a solution of ethanol : SDW : acetic acid (50 : 45 : 5) containing 0.2% (w/v) Coomassie Brilliant Blue R250 for 1 hour at room temperature. They were then transferred to destaining solution of ethanol : SDW : acetic acid (20 : 75 : 5) and left shaking for several hours.

2.8.4 Autoradiography

Gels were fixed in 7% (v/v) acetic acid for 1 hour then placed on Whatman 3 MM paper and dried under vacuum using a gel dryer (Bio-Rad). The dried gels were placed in a film cassette with Fuji X-Ray film type RX and left at room temperature for an appropriate length of time. Intensifying screens were used if gels had been exposed to fluor, and the film cassettes left at -80° C.

2.8.5 Fluorography using EN^{3}HANCE

Gels were fixed in 7% (v/v) acetic acid for 1 hour then shaken gently in EN^{3}HANCE (NEN (Dupont), Biotechnology Systems Division, Stevenage.) for 1 hour at room temperature. The EN^{3}HANCE was removed and the gels rinsed for 30 minutes under running tap water before being dried down.

2.9 Western blotting and immunodetection of proteins

The Western blotting and immunodetection procedures which were followed are described by Beisiegel (1986). Following electrophoresis, the separated proteins were transferred for 2.5 hours at 350 mA to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using an electroblotting unit (LKB). The

transfer buffer consisted of 25 mM Tris-HCl pH 6.8, 0.16 M glycine, 20% (w/v) methanol and 0.02% (w/v) SDS.

The nitrocellulose membranes were shaken gently in 100 ml of phosphate buffered saline (PBS ; 0.17 M NaCl, 3.4 mM KCL, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and 5% (v/v) foetal bovine serum (FBS : Northumbria Biologicals Ltd., Northumberland) for 1 hour. The membranes were then washed 3 times over a 30 minute period with PBS and then incubated overnight at 4°C or for 2 hours at room temperature in a solution of PBS and 5% (v/v) FBS and a 1:200 dilution of the appropriate polyclonal antiserum. The membrane was then washed in PBS for 30 minutes followed by a 1:1000 dilution of the peroxidase-linked donkey-antirabbit antiserum conjugate (Scottish Antibody Production Unit, Carluke) for 1 hour at room temperature. This was followed by another 30 minute PBS wash. The membrane was then developed by incubating with 0.3% (w/v) 4-chloro-1-napthol in methanol: PBS: 0.01% H_2O_2 (3:14:1) until the protein(s) were visible. To stop the reaction the membranes were rinsed several times in SDW. The substrate (0.3% (w/v) 4-chloro-1-napthol in methanol) was stored in the dark at 4°C

RUBISCO antibody was obtained from Dr. A Keys (Rothampsted) and Dr. L. K. Barnett (Warick) provided the LHCP antibody.

2.10 Statistical Tests

Standard deviations were calculated using the formula :

$$s = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$$

where s = the standard deviation, x = the value of an observation, $\bar{x}$ = the mean of a group of values, and n = the number of observations.

Standard multivariate analysis of variance was carried out using the General Statistics Package, (GENSTAT), and missing values estimated by an iterative method whereby a suitable estimate is one that reduces the residual for the missing observation to zero (Lane *et al.*, 1987). The F ratio was used to test the departure of the variance ratio of samples.

Chapter Three: Results and interpretation of physiological experiments

3.1 Effective growth conditions for flower and seed-set

It was important at the start of this research to define conditions of temperature, light quality and daylength which would allow the plants to be properly vernalized, induced to flower and set seed. If sugar beet plants are vernalized and the subsequent light conditions are inadequate some stem elongation and flowering may occur but flowering does not reach anthesis (Chrobozek, 1934; Fife and Price, 1953; Margara, 1968). The responses of different genotypes to environmental conditions vary considerably (Margara, 1968). Different varieties of sugar beet are known to have different daylength and temperature requirements. For example, some easy bolting varieties require only two or three weeks exposure to vernalizing temperatures whilst resistant bolting varieties may need ten to twelve weeks at low temperatures. The minimum daylength required is also highly variable (Lexander, 1975).

A very large amount of space and a considerable period of time would have been required to make a detailed study of the conditions required for vernalization and flowering by the varieties used in this work. It was known that flowering in LDs occurs in the annual (7S31660) without prior exposure to low temperatures; the biennial (5P606) is an easy bolting variety which requires approximately 6 weeks vernalization followed by LDs before flowering occurs (W. Schuch pers. comm.). However, because the varieties are not isogenic a slightly longer period of exposure of two months was chosen to ensure that all the individuals in an experiment would receive sufficient vernalization and that bolting and flowering would occur after exposure to a minimal number of LD cycles. The time taken to bolting and flowering has been shown to decrease with increasing periods of vernalization (Lexander, 1985). Although a shorter period might have been sufficient, if too short a time was chosen a lot of time would have beeen lost repeating the experiment. After vernalization, plants were grown in continuous light to reduce the chance of devernalization occurring at the higher temperatures and to decrease the number of LDs required for bolting and flowering.

Seedlings of annual (7S31660) and biennial (5P606) sugar beet were grown

up to the 6 leaf stage in the warm SD growth room (22°C, 8 hours fluorescent light). Half were then transferred to the cold SD room (6-11°C, 8 hours fluorescent light), and both lots grown on for a further two months. All the plants were then transferred to the cool LD room (≤15°C, 22 hours fluorescent and tungsten light). Under these conditions devernalization is avoided whilst induction of flowering proceeds resulting in morphological change at the shoot apex (Chrobozek, 1934). After 3 weeks the temperature was raised to 23°C. Growth at this temperature hastens elongation of the inflorescence stalk, and seed set should occur.

Figure 3.1 shows photographs of longitudinal sections of apices of annual and biennial plants sampled at the end of the growth periods in the warm SD room (22°C, 8 hours fluorescent light), the cold SD growth room (6-11°C, 8 hours fluorescent light), and after growth in LDs at temperatures of ≤ 15°C. The apices of both the annual and biennial plants sampled show elongation after exposure to temperatures of ≤15°C for 3 weeks when the LD illumination was provided by both fluorescent and tungsten light.

Table 3.1 shows the number of control and vernalized plants bolting after various periods of time in the warm LD growth room (23°C, 22 hours fluorescent and tungsten light). The results of this experiment show that two months exposure to low temperatures is sufficient to cause vernalization of the biennial seedlings. The earlier bolting of annual plants exposed to low temperature compared to control plants is evidence that the annuals respond to vernalization even though they do not absolutely require it.

3.2 Leaf production and expansion in plants grown under different conditions

Growth of the control and vernalized annual and biennial plants differed considerably. The petioles of the leaves of plants grown at low temperatures were much shorter, and the leaves did not expand as much as those of control plants (Figure 3.2). In addition it was observed that on return to higher temperatures a greater number of leaves emerged from vernalized plants than from control plants. This is consistant with the work of Terry (1968) who studied the developmental physiology of sugar beet at

Figure 3.1. Photographs showing sections of apices of plants grown under different light and temperature regimes.

The figure shows photographs of longitudinal sections of the apices of annual (**a** - **c**) and biennial (**d** - **f**) plants grown in warm SDs (22°C, 8 hour fluorescent light) (**a** and **d**), warm SDs followed by growth in the cold SD room (6-11°C, 8 hours fluorescent light) for two months (**b** and **e**), and plants treated as in **b** and **e** which were subsequently grown on for three weeks in the cool LD growth room (≤15°C, 22 hours fluorescent and tungsten light) (**c** and **f**). The sections were prepared as described in section 2.4.2 and examined by standard light microscopy.

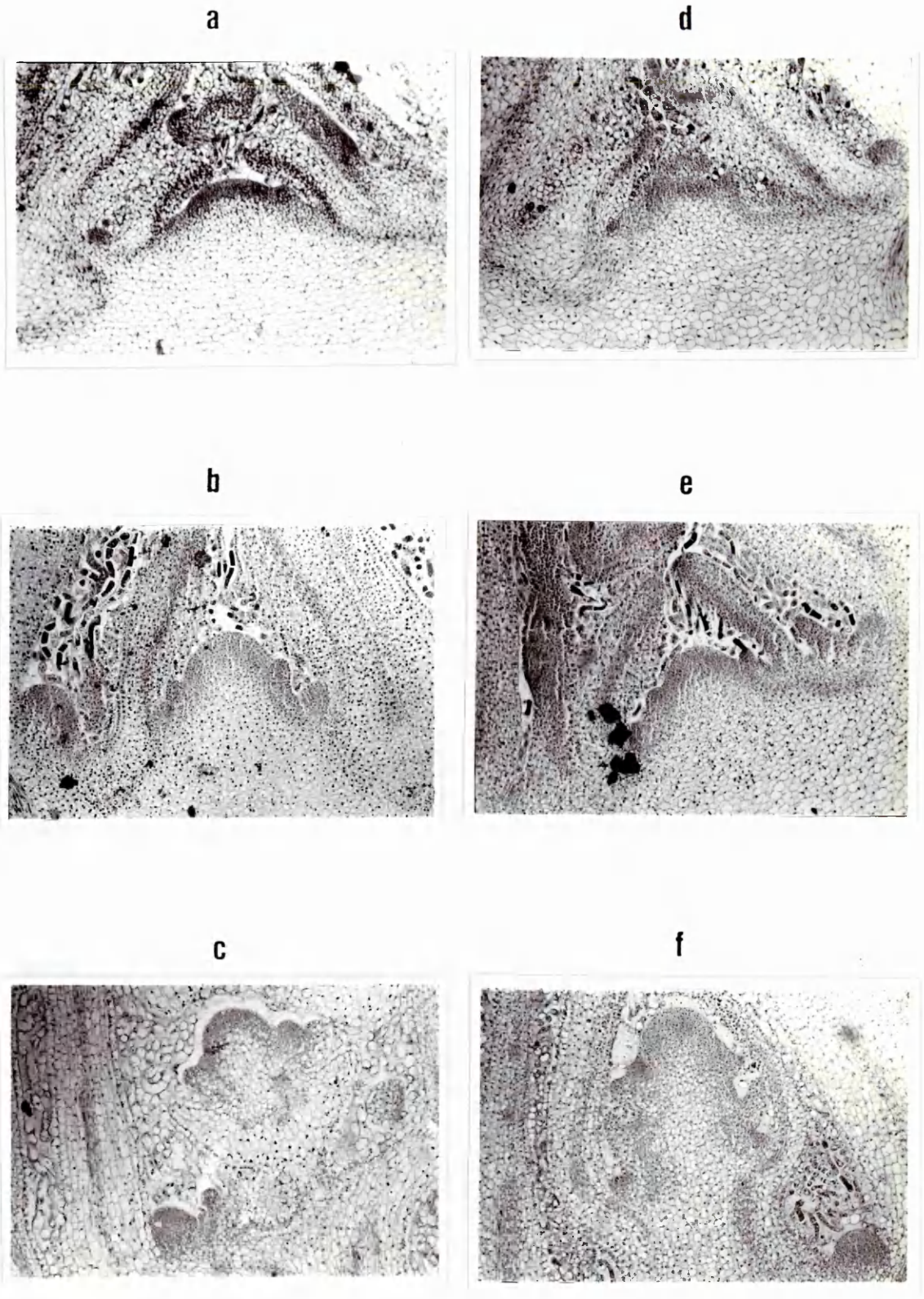

a
d
b
e
c
f
0·1mm

Table 3.1 The influence of vernalization on bolting in annual and biennial sugar beet.

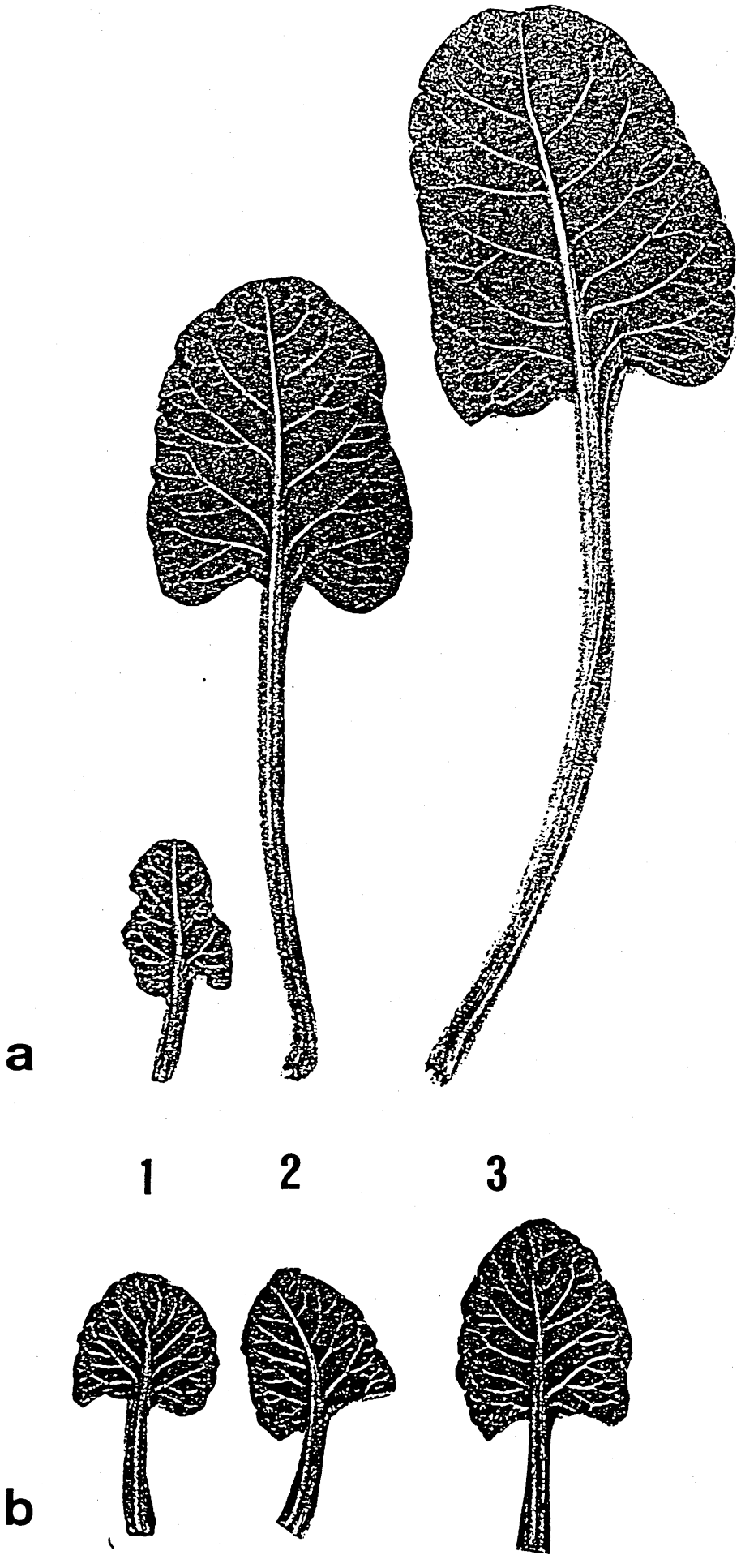
Seedlings of annual (7S31660) and biennial (5P606) sugar beet were grown to the 6 - 8 leaf stage in the warm SD growth room (22°C, 8 hours fluorescent light). Some were then transferred to the cold SD room (6-11°C, 8 hours fluorescent light). Annuals and biennials were vernalized for two months. At the end of the vernalization period all of the plants were transferred to the cool LD room (≤ 15°C, 22 hours light) for 3 weeks and then grown in the warm LD room (23°C, 22 hours fluorescent and tungsten light).

The table shows the number of plants bolting at various times following transfer to the warm LD room (23°C, 22 hours fluorescent and tungsten light). Plants were said to be bolting when the inflorescence stalk exceeded 1cm in length.

		Number of plants bolting following transfer to long days at 23°C							
		Number of long days at 23°C							Total number of plants/treatment
Plants	Treatment	2	7	11	15	17	19	21	
Annual	Vernalized	1	3	4	4	4	5	5	5
	Control	-	-	1	1	1	2	3	5
Biennial	Vernalized	-	-	2	4	4	4	5	5
	Control	-	-	-	-	-	-	-	5

Figure 3.2 Comparison of leaves of biennial sugar beet plants produced at high and low temperatures

Plants were grown up in the warm short day room (22°C, 8 hours fluorescent light) to the 6-8 leaf stage. One was then transferred to the cold SD room and both were grown on for 2 months. The figure shows the size of three leaves produced by **a** : the warm grown plant and **b** : the cold-treated plant. Leaf number **1** is the first leaf from the apex with an area greater than $1cm^2$, leaf number **2** the next oldest leaf and so on.


a
1
2
3
b

temperatures in the range 10-24°C. To define these differences when sugar beet are grown at 6-11°C an experiment was carried out in which the leaf area and dry weight of leaves of control and vernalized annual and biennial plants were measured. Only those leaves with a leaf area of 0.5cm^2 or greater were included. 20 annual and 20 biennial seedlings were grown up to the 6-8 leaf stage in the warm SD room (22°C, 8 hours fluorescent light). Half were left in the warm SD room and half were transferred to the cold SD room (6-11°C, 8 hours fluorescent light). Plants were grown in these conditions for two months after which time the dry weights and leaf areas from 5 plants in each group were measured (see section 2.4.3). Leaves were numbered from the oldest leaf to the apex ie. the oldest leaf was designated leaf number 1, the next oldest leaf, leaf number 2 and so on. The smallest leaf possible (with a leaf blade between 0.5 and 1cm in length) on each of the remaining plants was tagged and they were transferred to the cool LD room (≤ 15°C, 22 hours light) and grown on for 3 weeks. Before raising the temperature to 23°C the smallest leaf possible was again tagged. After one week's growth at this higher temperature leaf area and dry weight were measured and the number of leaves which emerged during growth at ≤15°C and at 23°C were counted.

Figure 3.3 shows graphs of leaf area and leaf dry weight versus leaf number for annual and biennial control and vernalized plants. Plants which were vernalized for two months did not produce as many leaves > 0.5cm^2 as the control plants. Control plants had on average produced 5 more leaves than vernalized plants and the leaf area and dry weight of leaves of the same age was generally much greater than that of the vernalized plants. After further growth in LDs for 4 weeks (Figure 3.4) annual control plants still had produced on average 4 more leaves than vernalized annuals but vernalized and control biennial plants by this time had produced the same number of leaves though the leaves produced from vernalized plants were still much smaller.

Table 3.2 shows the number of leaves which emerged from control and vernalized annual and biennial plants when they were subsequently grown in LDs at ≤15°C for three weeks and for 1 week at 23°C. Vernalized plants, particularly of the biennial, produced more leaves than controls, which is consistent with the data in

Figure 3.3 Leaf area and dry weight of leaves of control and vernalized annual and biennial plants

Plants were grown to the 6-8 leaf stage in the warm SD growth room (22°C, 8 hours fluorescent light). Half were then placed in the cold SD room (6-11°C, 8 hours fluorescent light) and all were grown on for a further two months. At the end of this time leaf area and leaf dry weight were measured (section 2.4.3).

The graphs show leaf area and leaf dry weight versus leaf number. Leaves were numbered from the oldest leaf to the apex. i.e. the oldest leaf was designated leaf number 1, the next oldest leaf number 2 and so on. The horizontal lines indicate means +1 standard deviation.

a Leaf area versus leaf number of control and vernalized annual plants

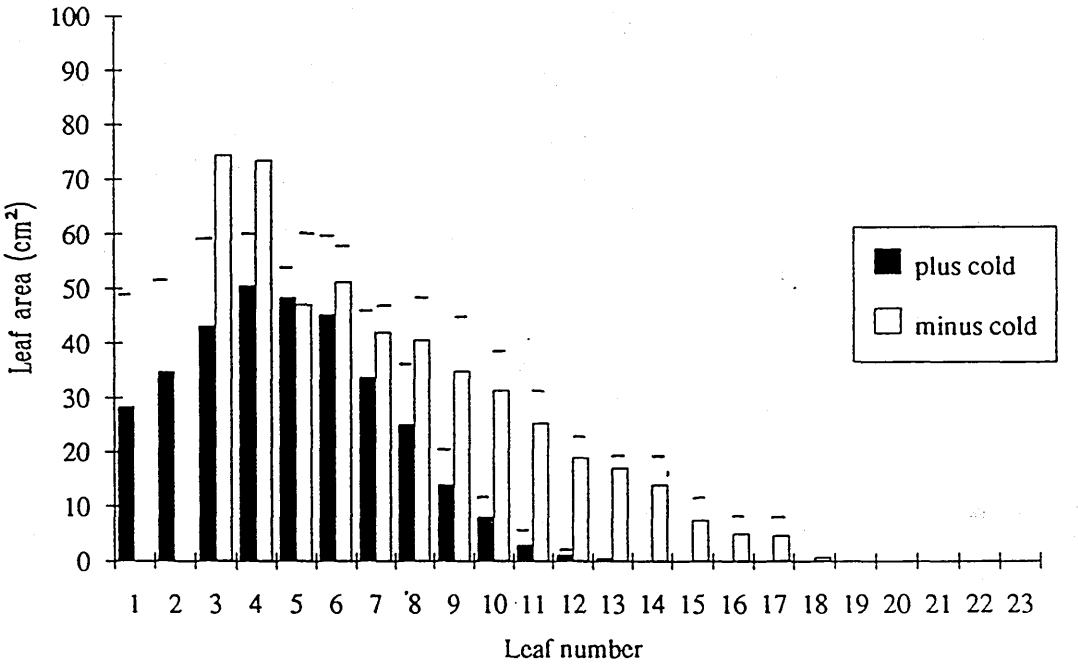
b Leaf area versus leaf number of control and vernalized biennial plants

c Leaf dry weight versus leaf number of control and vernalized annual plants

d Leaf dry weight versus leaf number of control and vernalized biennial plants

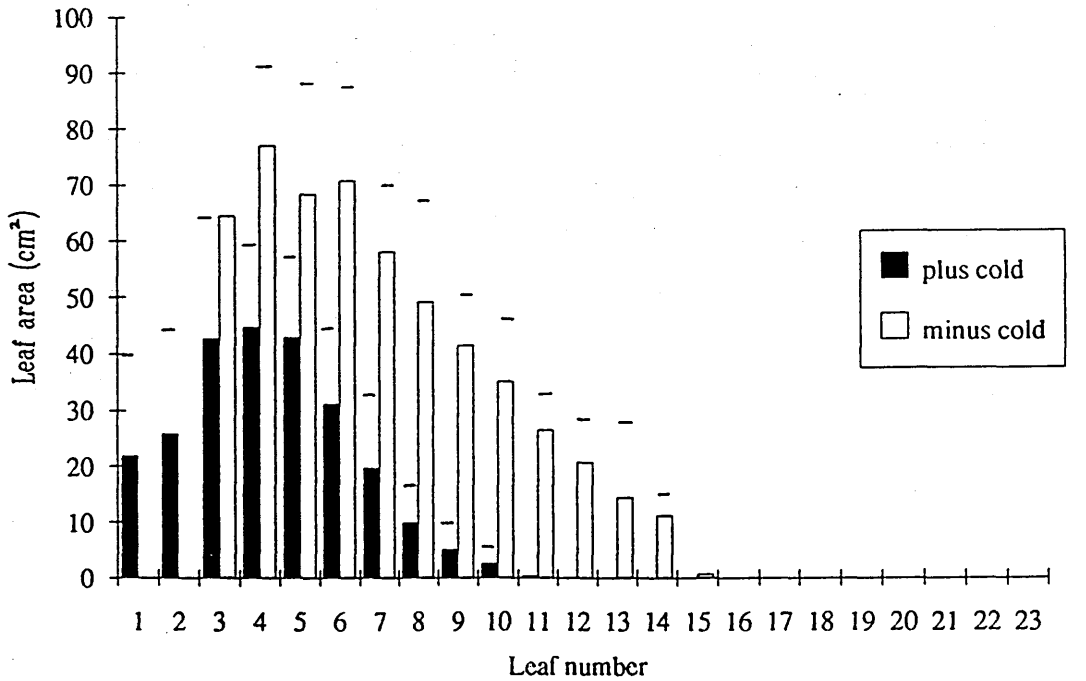
a

Leaf area versus leaf number of control and vernalized annual plants



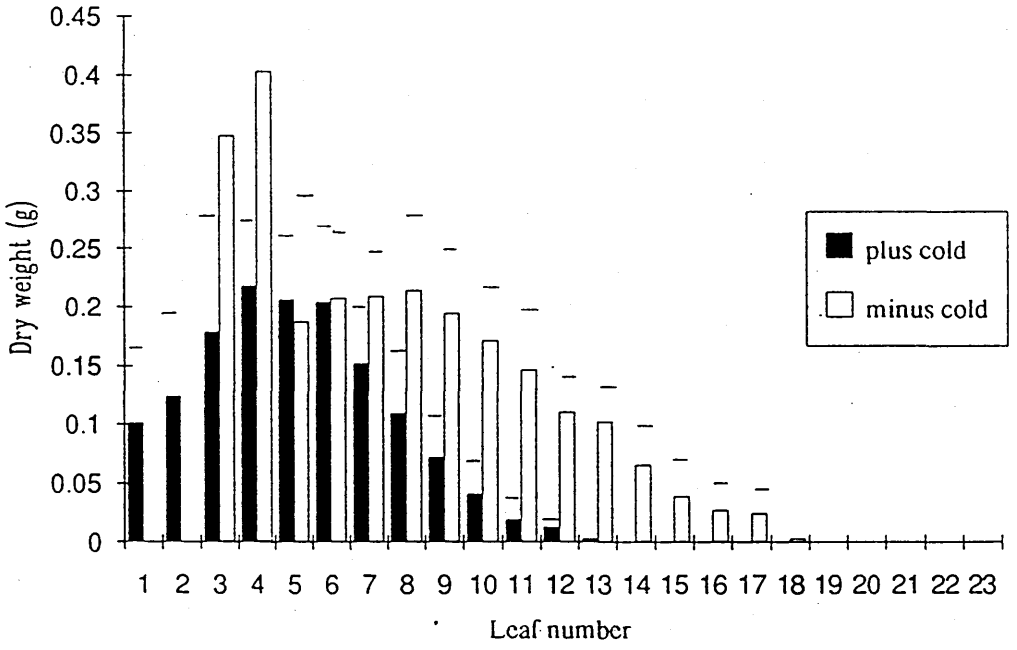
b

Leaf area versus leaf number of control and vernalized biennial plants



c

Leaf dry weight versus leaf number of control and vernalized annual plants



d

Leaf dry weight versus leaf number of control and vernalized biennial plants

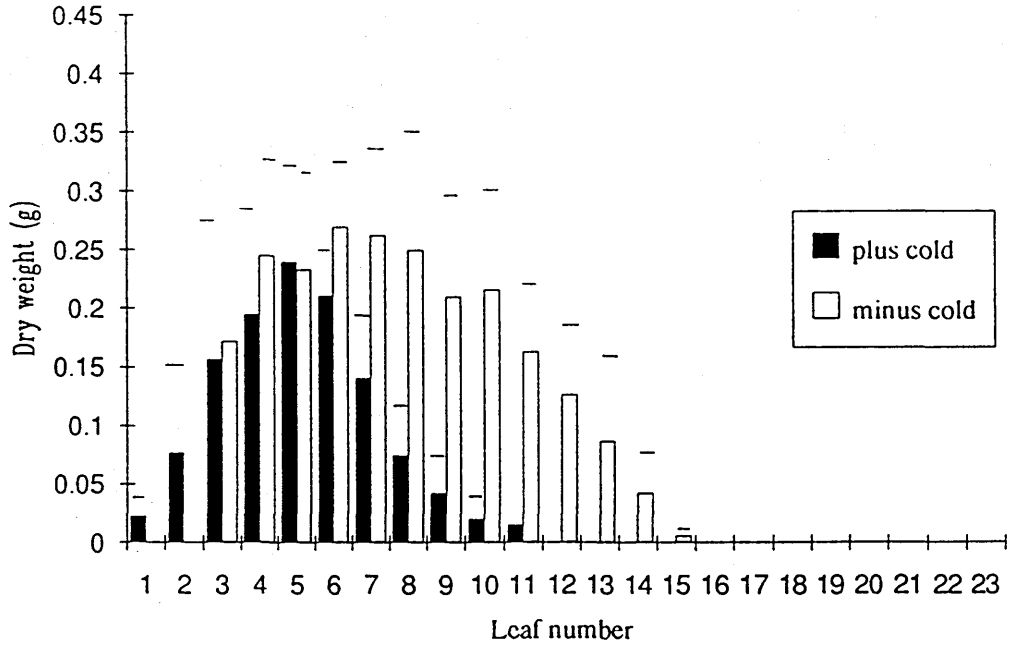


Figure 3.4 Leaf area and dry weight of leaves of control and vernalized annual and biennial plants after growth in inductive LDs for 4 weeks

Plants were grown to the 6-8 leaf stage in the warm SD growth room (22°C, 8 hours fluorescent light). Half were then placed in the cold SD room (6-11°C, 8 hours fluorescent light) and all were grown on for a further two months. At the end of this time all the plants were transferred to the cool LD room (≤ 15°C, 22 hours fluorescent and tungsten light) for three weeks before the temperature was raised to 23°C. After one week at this temperature leaf area and leaf dry weight were measured (section 2.4.3).

The graphs show leaf area and leaf dry weight versus leaf number. Leaves were numbered from the oldest leaf to the apex. i.e. the oldest leaf was designated leaf number 1, the next oldest leaf number 2 and so on. The horizontal lines indicate means+1 standard deviation.

a Leaf area versus leaf number of control and vernalized annual plants

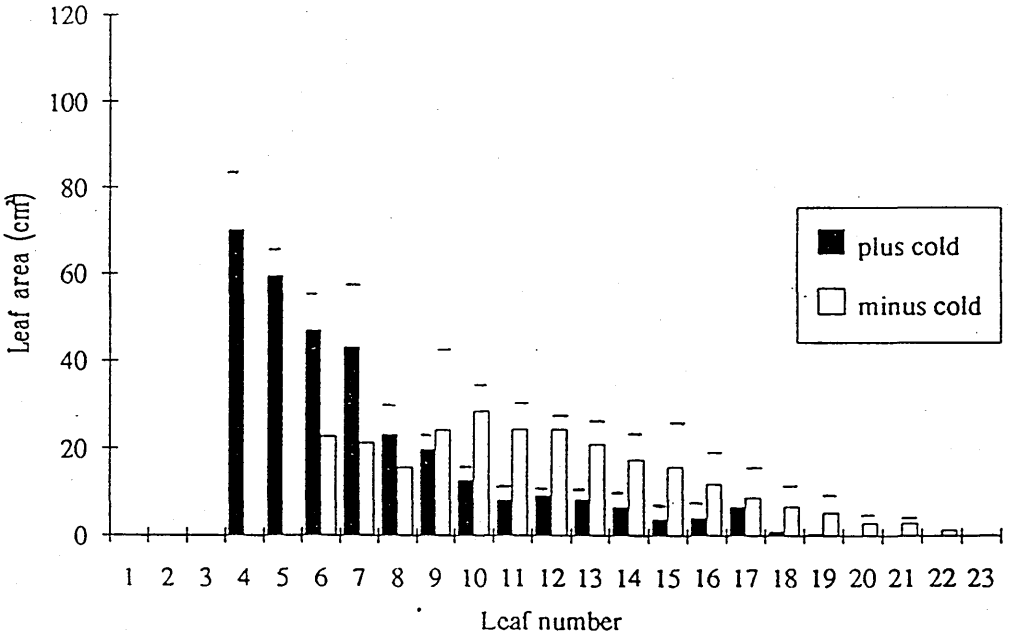
b Leaf area versus leaf number of control and vernalized biennial plants

c Leaf dry weight versus leaf number of control and vernalized annual plants

d Leaf dry weight versus leaf number of control and vernalized biennial plants

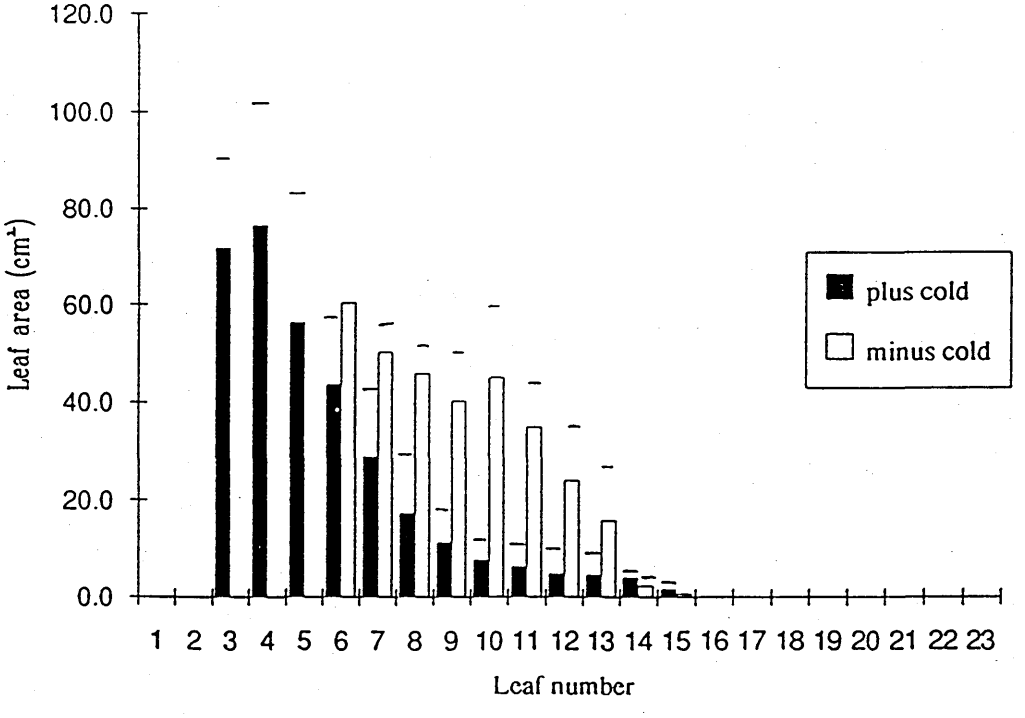
a

Leaf area versus leaf number of control and vernalized annual plants grown on for 4 weeks in LDs



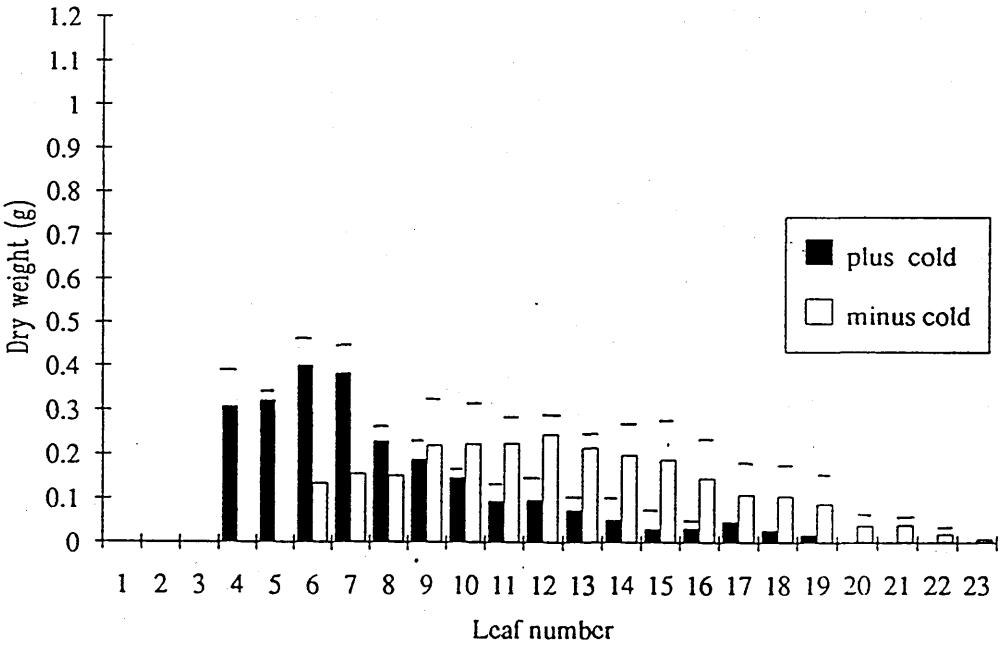
b

Leaf area versus leaf number of control and vernalized biennial plants grown on for 4 weeks in LDs



c

Leaf dry weight of control and vernalized annual plants grown on in LDs for 4 weeks



d

Leaf dry weight versus leaf number of control and vernalized biennial plants grown on in LDs for 4 weeks

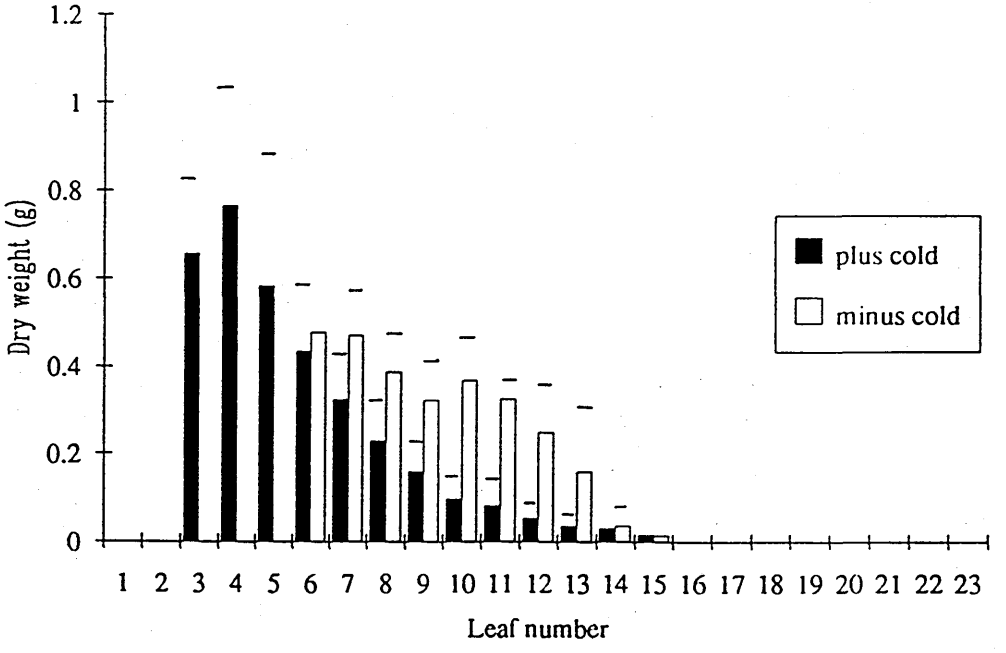


Table 3.2 Number of leaves produced by control and vernalized annual and biennial plants on transfer to inductive LDs

Twenty annual and twenty biennial plants were grown up in the warm SD room (22°C, 8 hours fluorescent light). When they reached the 6-8 leaf stage half were transferred to the cold SD growth room (6-11°C, 8 hours fluorescent light) and grown on for two months, the other half remained in the warm SD room. After two months the youngest leaf possible was tagged (usually leaves with leaf blades approximately 0.5 cm in length), and all the plants were transferred to the cool LD room (≤ 15°C, 22 hours fluorescent and tungsten light). After three weeks the smallest leaf which could be marked was again tagged and the temperature was raised to 23°C.

The table shows the average number of leaves produced per week at ≤15°C and 23°C by control and cold-treated annual and biennial plants. The number of leaves produced was calculated by counting the number of leaves between the two tagged leaves, and between the second tag and the next leaf with a leaf blade of approximately 0.5cm.

	Average number of leaves produced / week at 15°C	Average number of leaves produced / week at 23°C
Control annual	0.7	1.75
Vernalized annual	1.1	2
Control biennial	0.3	1
Vernalized biennial	1.2	2.6

Figure 3.4. This increase in the number of leaves which emerge could be the result of the increase in temperature, the increase in irradiation or the increased photoperiod (Milford and Lenton, 1976).

From these observations it is obvious that plants grown at low temperatures produce fewer expanded leaves than those grown at higher temperatures, therefore in subsequent experiments plants to be used as controls were sown 4 weeks after those which would be vernalized for two months and 6 weeks after those which were vernalized for 3 months. This meant that after the vernalization period control and vernalized plants had produced approximately the same number of leaves.

3.3 Defoliation Experiments

3.3.1 Experiment 1

3.3.1.1 Experimental treatments

Studies of vernalization in several species have shown that the process is dependent on the presence of meristematic tissue (Lang, 1965). McDaniel (1985) hypothesized that vernalization alters the competence of the meristem to 1) respond to floral stimulus and/or 2) to produce leaves able to produce a stimulus in response to LDs. However work reported by Metzger (1988) on field pennycress suggests that fully expanded leaves of this species can be vernalized, and in *Pisum sativum* physiological experiments suggest that production of an inhibitor of flowering made in the leaves is reduced at low temperatures, though whether this is only true for young leaves produced from a vernalized meristem is not clear (Murfet, 1985).

The aim of the experiment described in this section was to find out if 1) only those leaves initiated from a vernalized sugar beet meristem are able to respond to long days or if all leaves which experience low temperatures become competent, and 2) whether an inhibitor of flowering is present in the leaves.

Plants were grown up in the warm SD room (22°C, 8 hours light). After 2 months the first sown seeds had reached the 6 - 8 leaf stage. Before transferring them to the cold SD room (6-11°C, 8 hours fluorescent light) the smallest leaf possible was tagged. Forty annual and forty biennial plants were then vernalized for 3 months. After

vernalization both control and vernalized plants were treated as follows (10 plants/treatment). Treatments 1-3 are shown diagramatically in Figure 3.5.

Group	Treatment
1.	All leaves were left on
2.	All leaves formed before vernalization were removed i.e. all leaves before and including the tagged leaf. (and leaves of equivalent size removed from the controls)
3.	All leaves formed during and after vernalization removed i.e. tagged leaf and all subsequent leaves removed (and similar sized leaves left on controls).
4.	All leaves removed.

Figure 3.6 shows plants in groups 1-3. The apices of plants in group 3 were covered with aluminium foil and soil to prevent light reaching developing leaves. Every 1-2 days newly emerging leaves were removed and the cover replaced. Plants in group 4 were not covered, but the new leaves were removed every 1-2 days as they emerged.

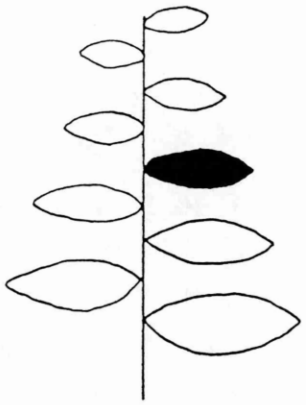
The annual and biennial control and vernalized plants in each group were given a number between 1 and 16 and placed randomly in 10 blocks in the cool LD room ($\leq 15^{\circ}C$, 22 hours fluorescent and tungsten light). Each block contained a representative of each treatment type. The plants were kept in this room for 3 weeks after which time the temperature was raised to $23^{\circ}C$. The presence or absence of an inflorescence stalk, the date the first flower opened and the length of infloresence stalk was noted.

When interpreting the results of such an experiment three factors which may affect bolting and flowering in each group must be borne in mind. Firstly, the inherent sensitivity of leaves to respond to LDs may vary with leaf age. In many species maximum sensitivity to respond to photoperiod is reached in the leaves approaching full expansion, thereafter declining until the oldest leaves show low sensitivity; however,

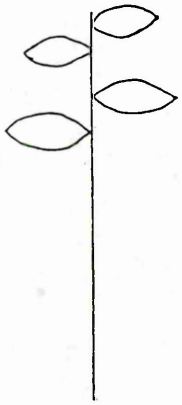
Figure 3.5 Diagramatic representation of plants in Experiment 1 treatment groups 1-3

1. All leaves left on.
2. All leaves formed before vernalization including tagged leaf removed.
3. All leaves formed during and after vernalization including tagged leaf removed.

1



2



3

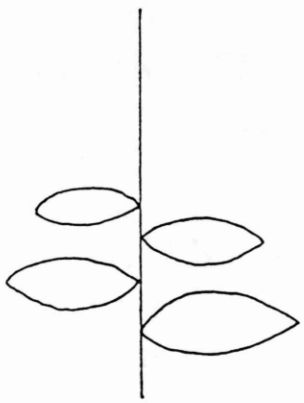


Figure 3.6 Photographs of plants in the different experimental groups in the defoliation experiments.

All seedlings were grown up in the warm SD room (22°C, 8 hour fluorescent light). Control plants were sown 6 weeks later than plants which were vernalized. When the first sown seeds reached the 6 - 8 leaf stage the smallest leaf possible was tagged before transferring the plants to the cold SD room (6-11°C, 8 hours fluorescent light). These plants were vernalized for three months, whilst control plants continued to grow in the warm SD growth room. At the end of the three month vernalization period all the plants were divided into four groups and treated appropriately (see text).

The photographs 1-3 show from left to right: **VB** - vernalized biennial, **CB** - control biennial, **VA** - vernalized annual, and **CA** - control annual plants in the different experimental groups **1** - control plants from group 1 with all leaves left on, **2** - group 2 plants with all leaves formed before exposure to low temperatures removed and **3** - group 3 plants with those leaves expanded during and after exposure to low temperatures removed.

VB CB VA CA

1

2

3

which leaves are sensitive has been found to vary between species (Vince-Prue, 1975). Secondly, the sensitivity of leaves at different stages of development to the process of vernalization may vary. ie. whether only those leaves initiated from a vernalized meristem are competent to respond to LDs or whether young or fully expanded leaves initiated before vernalization commenced can be vernalized. Finally, the action of inhibitor substances may prevent or retard bolting and flowering in certain groups. If for instance an inhibitor is produced in mature leaves then plants in group 2 may bolt and flower earlier than those in group 1. If all the expanded leaves of annual or vernalized biennial *Hyoscyamus* are removed and this state is maintained the plants flower in any photoperiod. In sugar beet it is possible that 1) an inhibitor is produced in the leaves in non-inductive daylengths, 2) in the old leaves or 3) in the unvernalized leaves of the biennial.

3.3.1.2 Affects of the defoliation treatments on bolting

Table 3.3 shows the number of plants with inflorescence stalks at various times following transfer to the warm LD room (23°C, 22 hour fluorescent and tungsten light). Bolting was recorded when the inflorescence stalk had reached a length of 1cm. The first conclusion which can be drawn from the control plants (group 1), is that the biennial plants without any vernalization do not produce an inflorescence stalk. This is as expected and is entirely consistent with previous studies of biennial sugar beet (Lexander, 1975) and the data shown in Table 3.1. It can also be concluded from the control plants that the annuals do respond to vernalization since both the number of inflorescence stalks and the time at which they were produced increased as a result of the vernalization treatment. This is also consistent with the data in Table 3.1.

The number of plants producing inflorescence stalks and the time taken to produce them is very similar in group 1 and group 2 plants. There is therefore no evidence that the old expanded leaves have either a beneficial or detrimental influence on the time taken to produce an inflorescence stalk. Furthermore the young leaves are clearly able to perceive LDs and are evidently competent in producing a flowering stimulus provided, in the case of the biennials, that they have received a cold treatment.

Table 3.3 The influence of low temperature and treatments 1-4 on inflorescence stalk formation in defoliation Experiment 1.

All seedlings were grown up in the warm SD room (22°C, 8 hours fluorescent light). Half were vernalized in the cold SD room (6 - 11°C, 8 hours fluorescent light) for three months while the control plants were left in the warm SD room. Control plants were sown six weeks later than plants which were vernalized. After the three month vernalization period the control and vernalized annual and biennial plants were separated into four groups each containing 10 plants, and various leaves were removed (see text and Figures 3.5 and 3.6). The plants were then placed in the cool LD room (≤15°C, 22 hours fluorescent and tungsten light) for three weeks and thereafter in the warm LD room (23°C, 22 hours fluorescent and tungsten light). The table shows the number of plants bolting in each group at various times following transfer to the warm LD growth room. Plants were recorded as bolting if the inflorescence stalk was 1cm or greater in length.

Number of plants induced to bolt after various times following transfer to long days at 23°C

Treatment	Plants	Number of days at 23°C																	
		4	8	11	14	17	20	23	26	29	32	35	38	41	44	47	50	59	65
Group 1	Vernalized annual	3	8	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Control	Control annual	-	-	-	-	-	-	-	2	2	2	2	2	2	3	3	3	3	3
All leaves left on	Vernalized biennial	3	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 2	Vernalized annual	5	7	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Leaves formed before	Control annual	-	-	-	-	-	-	-	1	1	1	1	1	3	3	3	4	4	4
vernalization removed	Vernalized biennial	6	9	9	9	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 3	Vernalized annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All leaves formed	Control annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
during and after	Vernalized biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
vernalization removed	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 4	Vernalized annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All leaves removed	Control annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vernalized biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

None of the plants in group 3 bolted. To explain this result for the biennial it could be argued that leaves formed from a vernalized meristem are essential for bolting and flowering to occur. However, even the annual plants in this group did not bolt. Hence the most likely explanation is that the ability of the leaves to respond to inductive photoperiods is lost or much reduced as they get older and insufficiently young leaves were present for either the annuals or the biennials to bolt.

Group 4 plants were included as controls. None of the plants bolted indicating that leaves are essential for the production of the floral stimulus, as expected (Curth, 1960). The apices of the plants in this group were not covered with soil or aluminium foil and although young leaves were removed every 1-2 days, very small leaves and leaf primordia would have been exposed to the LD photoperiod. Lack of bolting and flowering in this group shows that the presence of these very young leaves does not promote inflorescence stalk production. By the end of the experiment all the plants in this group had died. All the annual plants and most of the control biennials in this group were dead after 10 days. Vernalized biennials only started to die after 28 days.

3.3.1.3 The influence of the treatments on the date of flower opening

The initiation of an inflorescence stalk might not be affected by an inhibitor substance, but such a substance might affect productivity or flowering time. Therefore the final height of the inflorescence stalks and the date on which the first flower opened on plants in each group was noted.

Table 3.4 shows the number of plants in each group with open flowers after various periods of time in the warm LD room. There are no obvious differences between plants in group 1 and group 2. The numbers of control and vernalized annual and biennial plants with open flowers at various times in each group are very similar and there is therefore no evidence that the presence of mature leaves has any effect on the date the first flower opened. Opening of flowers in control annuals in group 2 seems to be delayed by comparison with the control annuals in group 1. However, a very small

Table 3.4 The influence of low temperature and treatments 1 and 2 on flowering in defoliation Experiment 1.

All seedlings were grown up in the warm SD room (22°C, 8 hours fluorescent light). Half were vernalized in the cold SD room (6 - 11°C, 8 hours fluorescent light) for three months while the control plants were left in the warm SD room. Control plants were sown six weeks later than plants which were vernalized. After the three month vernalization period the plants were separated into four groups of 10 plants and various leaves were removed (see text and Figures 3.6 and 3.7). The plants were then placed in the cool LD room (≤15°C, 22 hours fluorescent and tungsten light) for three weeks and thereafter in the warm LD room (23°C, 22 hours fluoresent and tungsten light). The table shows the number of plants with open flowers in groups 1 and 2 at various times following transfer to the warm LD growth room.

Number of plants with open flowers at various times after transfer to long days at 23°C

Treatment	Plants	Number of long days at 23°C																	
		4	8	11	14	17	20	23	26	29	32	35	38	41	44	47	50	59	65
Group 1	Vernalized annual	-	-	-	-	-	-	3	6	9	10	10	10	10	10	10	10	10	110
Control	Control annual	-	-	-	-	-	-	-	-	-	1	1	1	1	2	2	2	2	3
All leaves left on	Vernalized biennial	-	-	-	-	-	-	3	4	6	6	8	8	8	8	8	8	8	8
	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 2	Vernalized annual	-	-	-	-	-	-	5	8	8	10	10	10	10	10	10	10	10	10
Leaves formed before	Control annual	-	-	-	-	-	-	-	-	-	-	-	1	1	1	1	1	2	3
vernalization removed	Vernalized biennial	-	-	-	-	-	-	4	7	9	9	9	9	10	10	10	10	10	10
	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

number of plants were scored and it is likely that this delay is a result of natural variation.

3.3.1.4 The influence of the defoliation treatments on inflorescence stalk length

Two-way analysis of variance (ANOVA) was carried out on the final lengths of the inflorescence stalks produced by vernalized annuals and biennials in groups 1 and 2. Only eight out of ten of the vernalized biennials in group 1 bolted and flowered. The two plants which did not produce an inflorescence stalk grew vigorously and the only explanation for their failure to bolt is that they are seedlings which require a longer vernalization period. Therefore it was decided that they should be excluded from the analysis. Since ANOVA can only be carried out on groups containing the same number of sampling units GENSTAT was used to carry out the analysis since this package can estimate missing values (see section 2.10).

From the photographs of flowering annual and biennial plants in groups 1 and 2 (Figure 3.7.1 and 3.7.2) it can be seen that the plants produce a main infloresence stalk and a variable number of lateral stalks. Three separate analyses were therefore carried out on: 1) the final lengths of the main stalks, 2) the total length of all lateral stalks and 3) the combined length of main stalks and lateral stalks.

Tables 3.5 a, b and c show values for the degrees of freedom (d.f.), sums of squares, mean square, variance ratios and tables of means for each of the analyses. Since the test depends on the properties of normal distribution curves, where there was non-symmetrical distribution of data, logarithmic transformation was performed.

The length of the main inflorescence stalks produced by vernalized annuals and biennials and between plants in groups 1 and 2 were found to be significantly different, $p=0.05$. Vernalized biennials produced longer stalks than vernalized annuals and plants in group 1 produced longer stalks than those in group 2. Vernalized annuals produced a significantly greater total length of lateral stalks than vernalized biennials ($p=0.05$) and plants in group 1 produced a significantly greater total length of lateral stalks than those in group 2 ($p=0.05$). However, when the total length of all stalks was analysed, the results showed that plants in group 1 produced a greater total length of

Figure 3.7 **Photographs of plants in different groups in defoliation Experiment 1 after growth in inductive conditions warm LDs for two months.**

Photographs **1** - **5** show a representative selection of plants in defoliation groups **1** - **3** 11 weeks after transfer from vernalizing (6-11°C, 8 hours fluorescent light) or control temperatures (22°C, 8 hours fluorescent light), to flower inducing conditions (3 weeks at ≤15°C, 22 hour fluorescent and tungsten light; followed by 8 weeks at 23°C, 22 hours fluorescent and tungsten light).

VA = vernalized annual, **CA** = control annual, **VB** = vernalized biennial, **CB** = control biennial.

1 - vernalized annual plants from group 2 and group 1.

2 - vernalized biennial plants from group 2 and group 1.

3 - control biennial plants from group 1 and control annual plants from group 1.

4 - control biennial plants from group 2 and control annual plants from group 2.

5 - control annuals from group 3 and vernalized annuals from group 3.

1

2

CB Grp 1 CA Grp 1

3

CB Grp 2 CA Grp 2

4

CA Grp 3 VA Grp 3

5

Table 3.5 a, b and c Results of ANOVA carried out on the inflorescence stalk length of vernalized annual and biennial plants

The lengths of the inflorescence stalks of vernalized plants in groups 1 and 2 (see text) were measured after two months growth at 23°C in LDs. The tables show the results of ANOVA and tables of means for **a** - the length of the main inflorescence stalk, **b** - the total length of lateral inflorescence stalks, and **c** - the combined length of the main and the lateral inflorescence stalks. Data for annual and biennial sugar beet appears opposite the heading **variety**. Data for treatment types appears opposite the heading **foliage.**

a

Source of variation	d.f.	s.s.	m.s.	v.r.	significance
Variety	1	47165	47165	5.13	P<0.05
Foliage	1	45128	45128	4.91	P<0.05
Variety* Foliage	1	515	515	0.06	N.S.
Residual	34(2)	312339	9186		
Total	37(2)	394405			

Table of means

variety	annual	biennial
	49.6cm	56.5cm
foliage	on (group 1)	off (group 2)
	56.4cm	49.7cm

b

Source of variation	d.f.	s.s.	m.s.	v.r.	significance
Variety	1	1.3285	1.3285	4.86	P<0.05
Foliage	1	1.9223	1.9223	7.03	P<0.05
Variety*Foliage	1	0.3909	0.3909	1.43	N.S.
Residual	33(3)	9.0248	0.2735		
Total	36(3)	12.607			

Table of means

variety	annual	biennial
	70.9cm	49.1cm
foliage	on (group 1)	off (group 2)
	69.8cm	50.2cm

C

Source of variation	d.f.	s.s.	m.s.	v.r.	significance
Variety	1	0.13762	0.13762	1.39	N.S.
Foliage	1	0.6558	0.6558	6.64	P<0.05
Variety*Foliage	1	0.06644	0.06644	0.67	N.S.
Residual	33(3)	3.2607	0.09881		
Total	36(3)	4.08137			

Table of means

variety	annual	biennial
	120.5cm	105.6cm
foliage	on (group 1)	off (group 2)
	126.2cm	99.9cm

stalk than those in group 2 (p=0.05), but that there was no significant difference between the total length of stalk produced by annuals and biennials. Thus, to summarise, removing the old leaves reduced the total length of stalk produced by both annual and biennial plants. This could be because the older leaves contribute to the energy required for stalk formation through photosynthesis, but there is no direct evidence for this. The growth habit of annual plants differed from biennials in that they produced shorter main stalks but a greater total length of lateral stalks.

Figure 3.7 1-5 shows photographs of a selection of plants from several of the groups two months after transfer to LDs at 23°C. Vernalized annuals (Figure 3.7.1) and biennials (Figure 3.7.2) in groups 1 and 2 produced inflorescence stalks with lateral branches and set seed. Figure 3.7.3 and 3.7.4 show control annual and biennial plants in groups 1 and 2. None of the biennials produced inflorescence stalks. Control annuals in these groups had by this time started to produce inflorescence stalks. Figure 3.7.5 shows control and vernalized annuals from group 3 plants. Neither annual nor biennial plants belonging to this group produced flowering stalks. From comparison of the leaves of these plants at the start (Figure 3.6) and at the end of growth in long days it is obvious that the oldest leaves, some of which had started to senesce when the plants were defoliated, have regreened. Therefore lack of bolting and flowering in this group was not due to lack of vigour.

3.3.2 Experiment 2

3.3.2.1 Experimental treatments

The defoliation experiment described above was repeated and extended to investigate the effects of low temperature and inductive LDs on two further groups of plants. In the first experiment control plants were sown after vernalized plants to ensure that both lots of plants had reached approximately the same leaf number when they were transferred to LDs. Earlier bolting of vernalized plants compared to controls was attributed to an effect of the low temperatures, however it could be argued that part of the effect might be due to an increase in responsiveness to LDs in older plants. To test this hypothesis annual and biennial plants of the same absolute age as those which were

vernalized were used as one set of controls. The second new group contained plants that were defoliated but for a single leaf, which was initiated but unexpanded before the start of the low temperature treatment. On return to higher temperatures (13°C - 15°C) vernalized plants quickly produced leaves and this new group of plants was included to make sure that no leaves initiated from a vernalized meristem were exposed to the long days.

In this experiment plants were vernalized for two months. The control and vernalized plants were treated as follows (5 plants/treatment).

Group	Treatment
1.	All leaves left on ; plants to be vernalized sown 4 weeks earlier than controls.
2.	All leaves formed before vernalization were removed. ie. all leaves before and including the tagged leaf. (and leaves of equivalent size removed from controls)
3.	All leaves formed during and after growth at low temperatures removed (and similar sized leaves left on controls).
4.	All leaves left on; control annual and biennial plants sown at the same time as those vernalized plants in the other groups.
5.	One leaf initiated well before exposure to low temperatures left on (ie. the leaf 3 leaves younger than the tagged leaf left on). All others removed.

As in experiment 1, the apices of group 3 and 5 plants were covered with aluminium foil and soil and every 1-2 days new emerging leaves were removed. In group 5, once the production of an inflorescence stalk was noted no more leaves were removed.

3.3.2.2 The influence of the defoliation treatments on bolting

Table 3.6 shows the number of plants with inflorescence stalks at various times after transfer of the plants to the warm LD room. The number of vernalized annual and biennial plants bolting in groups 1-3 is consistent with the results of the previous experiment. However, bolting of the vernalized biennial plants does seem to have been delayed slightly, perhaps because of the reduction in the length of time plants were exposed to low temperatures. Control annual plants in group 1 produced inflorescence stalks earlier than those in the last experiment whereas control annuals in the other groups did not bolt within the time allowed for the experiment (which was less than in Experiment 1). The small number of plants in each group and the length of the experiment prohibits ruling out natural variation as the cause of this difference.

The plants in group 4 did not bolt. Rather than older plants becoming more sensitive to photoperiod the opposite seems to be true. Therefore earlier bolting and flowering of annuals which had been exposed to low temperatures is not due to increased sensitivity to daylength as the plants age but due to the process of vernalization. Since only 5 plants were included in each group it may be that variation between individuals accounts for the lack of bolting of the annuals in this group.

Vernalized plants in group 5 with only one leaf bolted just as quickly as those in groups 1 and 2. Therefore it can be concluded that leaves initiated before plants are exposed to low vernalizing temperatures can be vernalized. These leaves, which were very small and unexpanded at the start of vernalization, are made competent to respond to LDs and produce enough floral stimulus to make the plants bolt as quickly as plants which have not been defoliated. It is not known if these leaves are changed by the direct action of the low temperatures or whether a signal is sent from the vernalized meristematic tissue which changes their developmental state. Figure 3.8 shows vernalized biennial plants from this group. Control plants in this group died shortly after transfer to LDs.

Table 3.6 The influence of low temperature and treatments 1-5 on inflorescence stalk formation in defoliation Experiment 2.

All seedlings were grown up in the warm SD growth room (22°C, 8 hours fluorescent light) to the 6 - 8 leaf stage. Half were then vernalized in the cold SD room (6 - 11°C, 8 hours fluorescent light). Control plants were sown four weeks later than plants which were vernalized except for control plants in group 4 which were sown at the same time. After the two month vernalization period the plants were separated into 5 groups, each of 5 plants, and various leaves were removed (see section 3.3.2.1). The plants were then placed in the cool LD room (≤15°C, 22 hours fluorescent and tungsten light) for three weeks and thereafter in the warm LD room (23°C, 22 hours fluorescent and tungsten light). The table shows the number of plants bolting in each group at various times following transfer to the warm LD growth room. Plants were recorded as bolting if the inflorescence stalk was 1cm or greater in length.

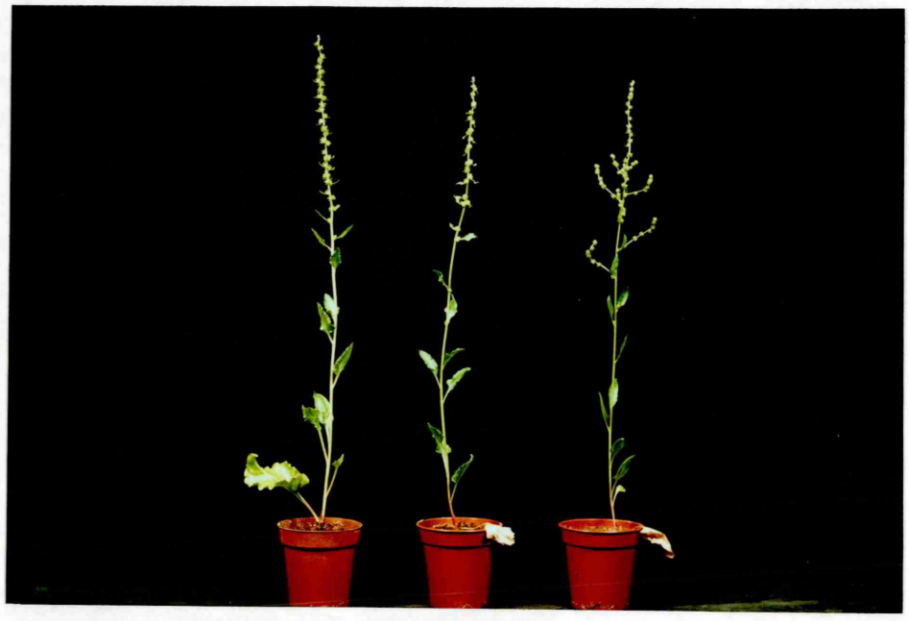
Number of plants induced to bolt at various times following transfer to long days at 23°C

Treatment	Plants	Number of long days at 23°C													
		2	4	5	7	8	12	15	17	19	21	23	26	29	31
Group 1	Vernalized annual	1	1	1	1	3	4	4	4	5	5	5	5	5	5
Control	Control annual	-	-	-	-	-	-	1	1	2	3	3	3	3	3
All leaves left on	Vernalized biennial	-	-	-	-	-	2	4	4	4	4	4	4	4	4
	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 2	Vernalized annual	-	-	1	1	2	4	5	5	5	5	5	5	5	5
Leaves formed before	Control annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-
vernalization removed	Vernalized biennial	-	-	-	-	-	-	2	2	3	4	5	5	5	5
	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 3	Vernalized annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All leaves formed during	Control annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-
and after growth at low	Vernalized biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-
temperatures removed	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 4															
All leaves left on control annual and biennial	Control annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-
plants sown at the same time as the vernalized plants	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 5															
One leaf initiated well	Vernalized annual	1	2	3	4	4	4	5	5	5	5	5	5	5	5
before exposure to low	Control annual	-	-	-	-	-	-	-	-	-	-	-	-	-	-
temperatures left on. All	Vernalized biennial	-	-	-	-	-	-	1	3	3	3	3	3	3	3
other leaves removed.	Control biennial	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 3.8 Photograph of three vernalized biennial sugar beet plants from group 5 in defoliation Experiment 2.

The photograph shows three vernalized biennial plants from group 5 in Experiment 2; ie. one leaf initiated well before exposure to low temperatures was left on, and all others were removed.

The plants were grown to the 6 - 8 leaf stage in the warm SD room (22°C, 8 hours fluorescent light), then transferred to the cold SD room (6-11°C, 8 hours fluorescent light) and grown on for two months. Before transferring to inductive LD conditions (3 weeks at ≤15°C, 22 hours fluorescent and tungsten light, then 23°C, 22 hours fluorescent and tungsten light), all leaves present bar one leaf 3 leaves younger than the tagged leaf were removed. All subsequent leaves were removed as they appeared until the inflorescence stalk emerged.



3.4 Summary

Exposure of sugar beet to low temperatures for two months was sufficient to cause vernalization of the easy bolting variety (5P606). Vernalization was essential for flowering of the biennial whereas it accelerated flowering of the annual. The emergence and expansion of leaves was greater in plants at normal temperatures than those grown at low vernalizing temperatures. Comparison of leaf emergence of control and vernalized plants on their return to higher temperatures and LDs showed that vernalized plants produced more leaves than the controls.

The results of the defoliation experiments showed that as leaves age their competence to respond to LDs to produce a floral stimulus is lost or greatly reduced. Leaves present but unexpanded at the onset of vernalization could be vernalized and therefore a change in competence due to low temperature is not limited to leaves produced from a vernalized meristem. The number of leaves present does not affect initiation of bolting nor the time at which the first flowers open, but it does influence the final length of the inflorescence stalk. Finally, no evidence was found to suggest the presence of a specific inhibitor of flowering in the old leaves of annual or biennial plants or in non-vernalized leaves of the biennial.

Chapter Four: Analysis of proteins in control and cold-treated leaf tissue

4.1 Introduction

Vernalization affects gene expression in that after vernalization genes involved in flowering are switched on. Whether switching on or off of genes occurs during vernalization or whether molecules already present are modified is not known. The initial aim of this work was to investigate whether the vernalized state is a product of changes in gene expression occurring at low temperatures. In principle, gene expression may be regulated at the level of transcription, translation, processing of mRNA, the post-translational modification of proteins or through differential rates of mRNA or protein turnover. To look for such changes it is necessary to compare the properties of vernalized and non-vernalized plants. Moreover, the action of low temperatures in relation to vernalization must be studied using tissue that is known to either perceive or be affected by vernalization.

The initial objective was to investigate the protein content of control and vernalized annual and biennial plants. If vernalization of sugar beet results in the synthesis, modification or degradation of proteins changes in the spectrum of proteins should be observed during exposure to low temperatures. It is possible that such changes would make the protein profile of the vernalized biennial similar to that of the annual. Thus, by comparing proteins extracted from control and vernalized annual and biennial plants significant changes could be identified. Of course, any changes occurring when plants are exposed to low temperatures could equally be due to the process of cold acclimation or could be general changes associated with growth at low temperatures. Proteins involved in these processes would be of great interest and further experiments could be devised to investigate exactly which processes the protein(s) played a part in.

4.2 Visualization of protein extracted from apical meristems

Evidence from the literature strongly suggests that in several species, including sugar beet, meristematic tissue is the site of the vernalization response (Lang, 1965).

Initially it was decided that proteins extracted from apical meristems of annual and biennial sugar beet plants should be compared. Plants were vernalized for two months. Although a shorter exposure to low temperature may have been sufficient, it was thought that leaving the plants at low temperatures for longer might decrease variation due to differing response times in different genotypes.

The small size of the apical meristems, which were 0.1 mm in diameter (see Figure 4.1), made dissection of the meristem from the surrounding young leaf tissue difficult, though by no means impossible with practice. Each apex yielded approximately 1µg of total protein. Pooling several apices made protein extraction from such small amounts of tissue easier and should help to overcome the problem of variation between individuals within a treatment group.

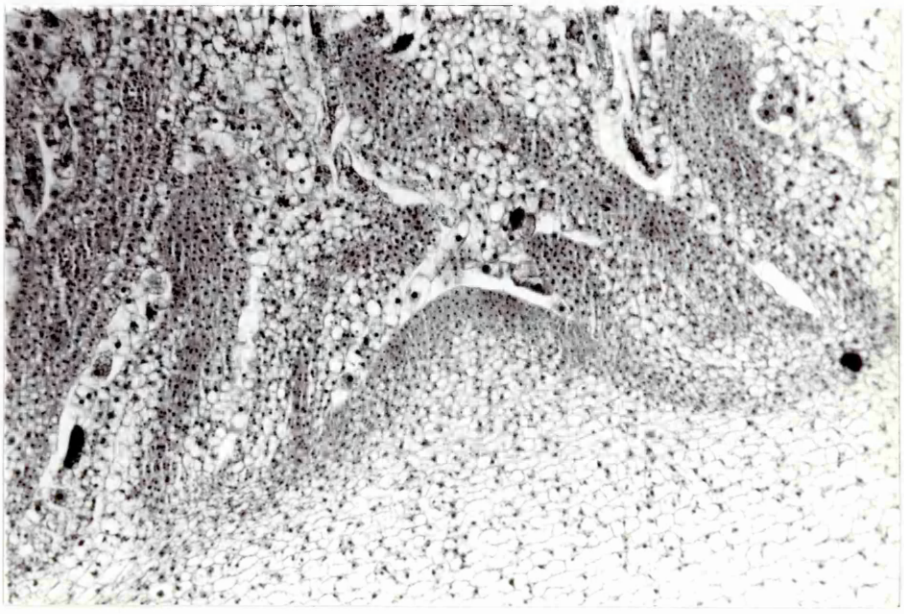
10µg of separated protein can be visualized on a silver stained mini- polyacrylamide gel (LKB) and the polypeptides in only 1µg of protein can be separated on a silver stained Phast gel (see Figure 4.2.). Although a lot of time was spent perfecting the dissection of apical tissue, when results of physiological experiments suggested that young leaves, present but not expanded, could be vernalized, it was decided that changes in gene expression in these leaves should be investigated. Using leaf tissue has several advantages. Firstly, sampling leaf tissue is a lot easier and less time consuming. Secondly, fewer plants have to be grown to obtain sufficient amounts of protein, and finally 2-D separation can be carried out on standard-sized gels since more protein can be obtained from the leaf samples.

4.3 Comparison of protein in annual and biennial leaf tissue

To minimize variation in the protein patterns between samples from one variety, leaf tissue from three individual plants was pooled and protein extracted from this tissue was treated as one sample. Since it was important at the outset to be sure that differences in protein patterns arose from real differences between samples the reliability of the 2-D separation was investigated. Preliminary experiments showed that protein patterns obtained in different 2-D separations of the same protein sample were highly reproducible. When aliquots from one sample were separated in the first and

Figure 4.1 **Longitudinal section of the apical meristem of a biennial sugar beet plant grown in warm short days.**

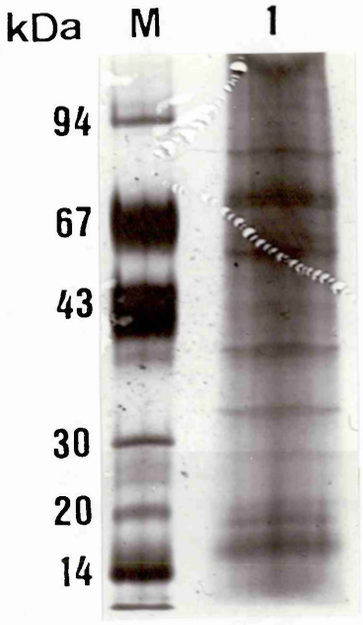
The figure shows a longitudinal section of an apex taken from a biennial sugar beet plant which was grown to the 6 - 8 leaf stage in the warm SD growth room (22°C, 8 hours light). The section was prepared as described in section 2.4.2 and examined by standard light microscopy.



0·1mm

Figure 4.2 1-dimensional SDS Phast gel of apical protein from annual plants.

Apices were dissected out from control annual plants and total protein extracted. 1µg of the sample was separated in a mini-SDS gel using the Phast system (Pharmacia). Lane **1** - total protein extracted from the apices of annual plants. Lane **M** contains molecular mass markers whose sizes are indicated to the left.


kDa
M
1
94
67
43
30
20
14

second dimensions at the same time not only were the densities of corresponding spots very similar but so were the position of the proteins relative to one another (results not shown).

Figures 4.3, 4.4 and 4.5 each show a set of six 2-D gels of proteins extracted from two biennial varieties (Figures 4.3 and 4.4) and an annual sugar beet variety (Figure 4.5). Gels **a**, **b** and **c** in each figure show 2-D separation of three separate samples of proteins extracted from control leaf tissue; **d**, **e** and **f** show 2-D separation of three separate samples of proteins extracted from vernalized leaf tissue. The patterns of protein found in all samples are overall very similar, though the presence and absence of some proteins and the quantity of others taken from plants grown under identical conditions varies considerably. For example protein 1 in Figure 4.3 is detectable in each sample except **e**. Protein 2 is present in all samples, but in quite different amounts. Thus comparison of replicate samples from plants grown under each set of light and temperature regimes is essential if any meaningful conclusions are to be drawn.

Differences in the quantity of many of the proteins detected are evident between the annual and biennial varieties. Some of the spots representing lower abundance proteins seem to be unique to either the annual or biennial protein profiles. However, because of the variability even within varieties in the concentration of individual polypeptides it is not known whether these differences are real or artifactual. Hence it was decided that only prevalent spots showing clear and reproducible differences could with certainty be said to change.

No major differences between control annual and control biennial proteins are evident, (compare Figure 4.4 **a** and **b** with Figure 4.5 **a** and **b**). However, growth at low temperature consistently induced the accumulation of two acidic polypeptides of approximately 22 and 23 kDa. These polypeptides accumulated to similar levels in both annual and biennial leaf tissue which had been exposed to low temperatures (see Figures 4.3, 4.4 and 4.5 gels **e**, **f** and **g**).

Figure 4.3 2-dimensional PAGE of total leaf protein from control and vernalized biennial (ASCEB) sugar beet.

Young unexpanded leaves with leaf blades ≤ 1 cm in length were harvested from three control and three vernalized plants as described in section 2.4.1. Vernalized plants had been exposed to low temperatures (6-11°C, 8 hours fluorescent light) for 2 months. Total protein was extracted as described in section 2.6.1 using a denaturing SDS-containing buffer. 100µg of protein from each sample was separated by IEF/SDS PAGE (section 2.7.3 and 2.7.4) and the gels stained with silver (section 2.8.2). **a**, **b** and **c** show patterns of total leaf protein from 3 separate samples extracted from control plants; **d**, **e** and **f** patterns of total leaf protein from 3 separate samples extracted from vernalized plants. Arrows with numbers indicate variable polypeptides referred to in the text. Other arrows point to polypeptides which were induced as a result of the cold treatment.

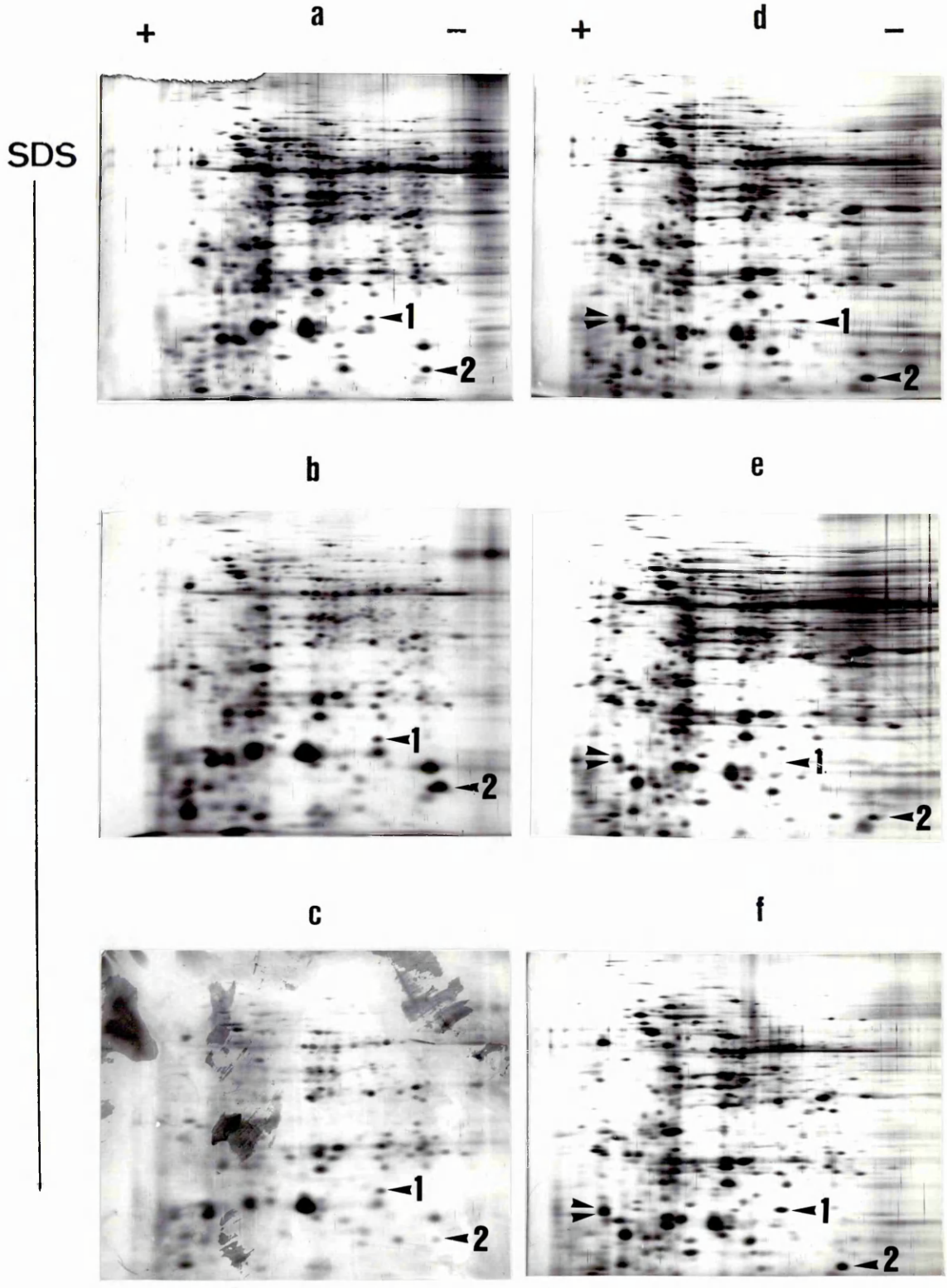

+
a
–
+
d
–
SDS
1
2
b
e
c
f

Figure 4.4 2-dimensional PAGE of total leaf protein from control and vernalized biennial (5P606) sugar beet.

Young unexpanded leaves with leaf blades ≤ 1 cm in length were harvested from three control and three vernalized plants as described in section 2.4.1. Total protein was extracted as described in section 2.6.1 using a denaturing SDS-containing buffer. 100µg of protein from each sample was separated by IEF/SDS PAGE (section 2.7.3 and 2.7.4.) and the gels stained with silver (section 2.8.2). **a**, **b** and **c** show patterns of total leaf protein from 3 separate samples extracted from control plants; **d**, **e** and **f** show the patterns of total leaf protein from 3 separate samples extracted from vernalized plants. Arrows point to polypeptides which were induced as a result of the cold treatment.

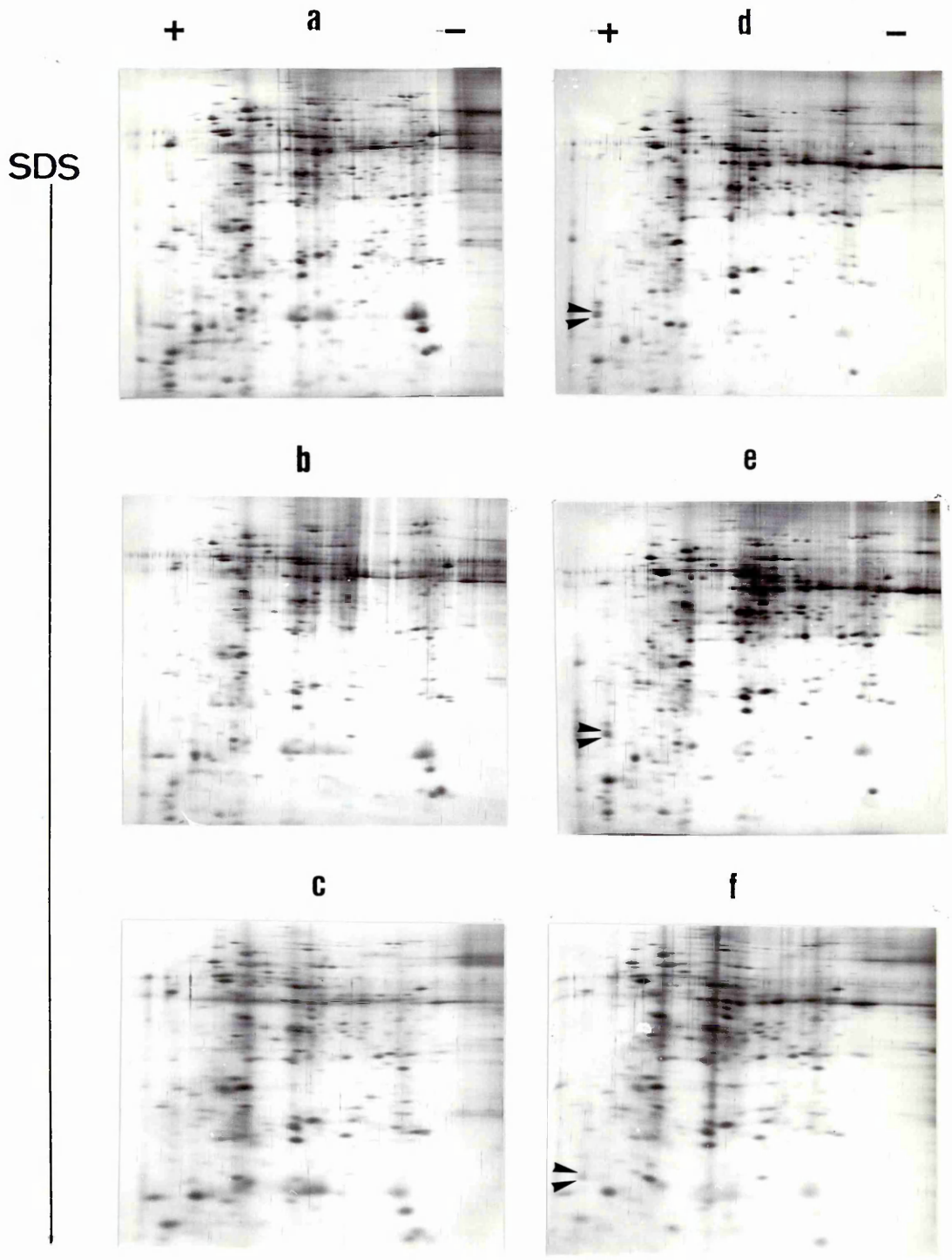
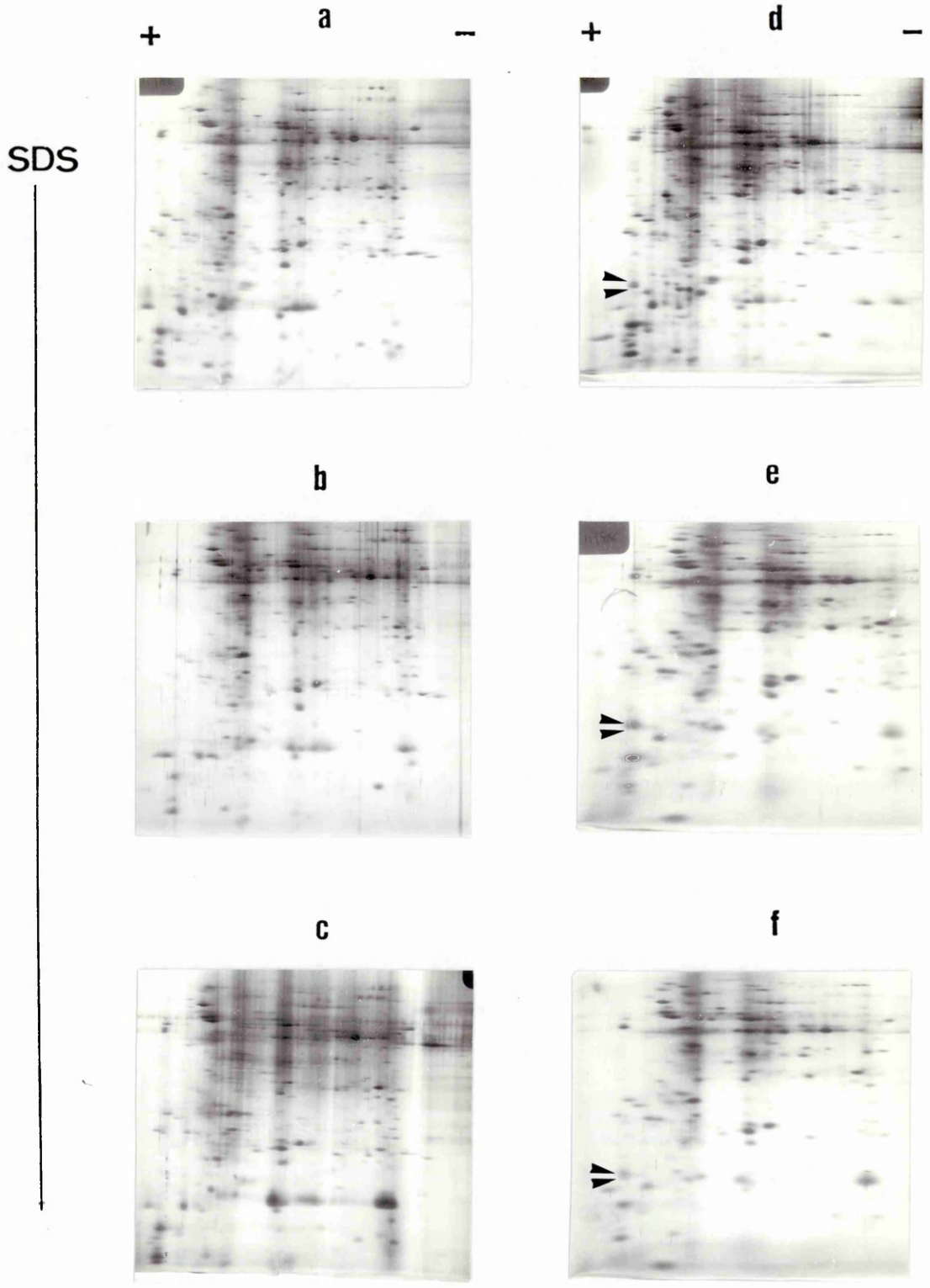

+
a
−
+
d
−
SDS
b
e
c
f

Figure 4.5 2-dimensional PAGE of total leaf protein from control and vernalized annual (7S31660) sugar beet.

Young unexpanded leaves with leaf blades ≤ 1 cm in length were harvested from three control and three vernalized plants as described in section 2.4.1. Vernalized plants had been exposed to low temperatures (6-11°C, 8 hours fluorescent light) for 2 months. Total protein was extracted as described in section 2.6.1 using a denaturing SDS-containing buffer. 100µg of protein from each sample was separated by IEF/SDS PAGE (section 2.7.3 and 2.7.4) and the gels stained with silver (section 2.8.2). **a**, **b** and **c** show patterns of total leaf protein from 3 separate samples extracted from control plants; **d**, **e** and **f** patterns of total leaf protein from 3 separate samples extracted from vernalized plants. Arrows point to polypeptides which were induced as a result of the cold treatment.


+
a
–
+
d
–
SDS
b
e
c
f

4.4 Molecular mass and pI of the cold-induced polypeptides

Figure 4.6a and 4.6b show the polypeptide profiles of mature leaves taken from control and cold-treated biennial sugar beet. The cold-induced polypeptides are not detected in the control. Using the molecular mass markers a plot of log. molecular mass versus distance run on the gel was used to calculate the approximate mass of the cold-induced polypeptides (see section 2.2.6 and Figure 4.7). The cold-induced polypeptides are approximately 22 and 23 kDa in molecular mass. The polypeptides are clearly acidic. Although measurements of pH across the first dimension gel were not made, the roughly even spread of proteins suggests that the pH gradient was not particularly uneven. It is therefore likely that the pI of the polypeptides would be between 4.0 and 5.5.

4.5 Pattern of total protein extracted from leaves of different ages taken from control biennial plants

The results presented in section 3.2 (see Figure 3.2) show that the leaves of sugar beet grown at low temperatures do not expand as much as those grown at higher temperatures. It was thought that the appearance of the "cold-induced" proteins might be related to development ie. that they accumulate as development progresses. Thus, even although the leaves sampled from control and vernalized plants were approximately the same size, the unexpanded leaves taken from cold-grown plants, although positionally equivalent, might be further advanced developmentally. Therefore protein was extracted from control leaves of different ages to see if the 22 and 23 kDa polypeptides were present (Figure 4.8A). In this experiment, proteins from individual leaves taken from two plants were extracted separately and run on 2-D IEF/SDS gels. Tracings of the leaves from which protein was extracted are shown in 4.8B. Protein profiles from the leaves of only one plant are shown, although the result was similar in both cases. No polypeptides with molecular mass and pI similar to the cold-induced polypeptides are detectable at the same levels as in the cold-treated plants, indicating that the 22 and 23 kDa polypeptides are unlikely to be the products of developmental changes.

Figure 4.6 2-dimensional PAGE of total proteins extracted from control and cold-treated leaf tissue.

Total protein was extracted (section 2.4.1) from a fully expanded leaf of **a** : a control and **b** : a biennial sugar beet plant grown at low temperatures for 1 month (6-11°C, 8 hours fluorescent light). 100µg of the sample was loaded onto each first dimension gel. Protein molecular mass markers were included on the second dimension SDS gels and the gels were stained with silver (section 2.8.2). The arrows point to the two polypeptides induced during the cold-treatment. Sizes of molecular mass markers are indicated to the right.

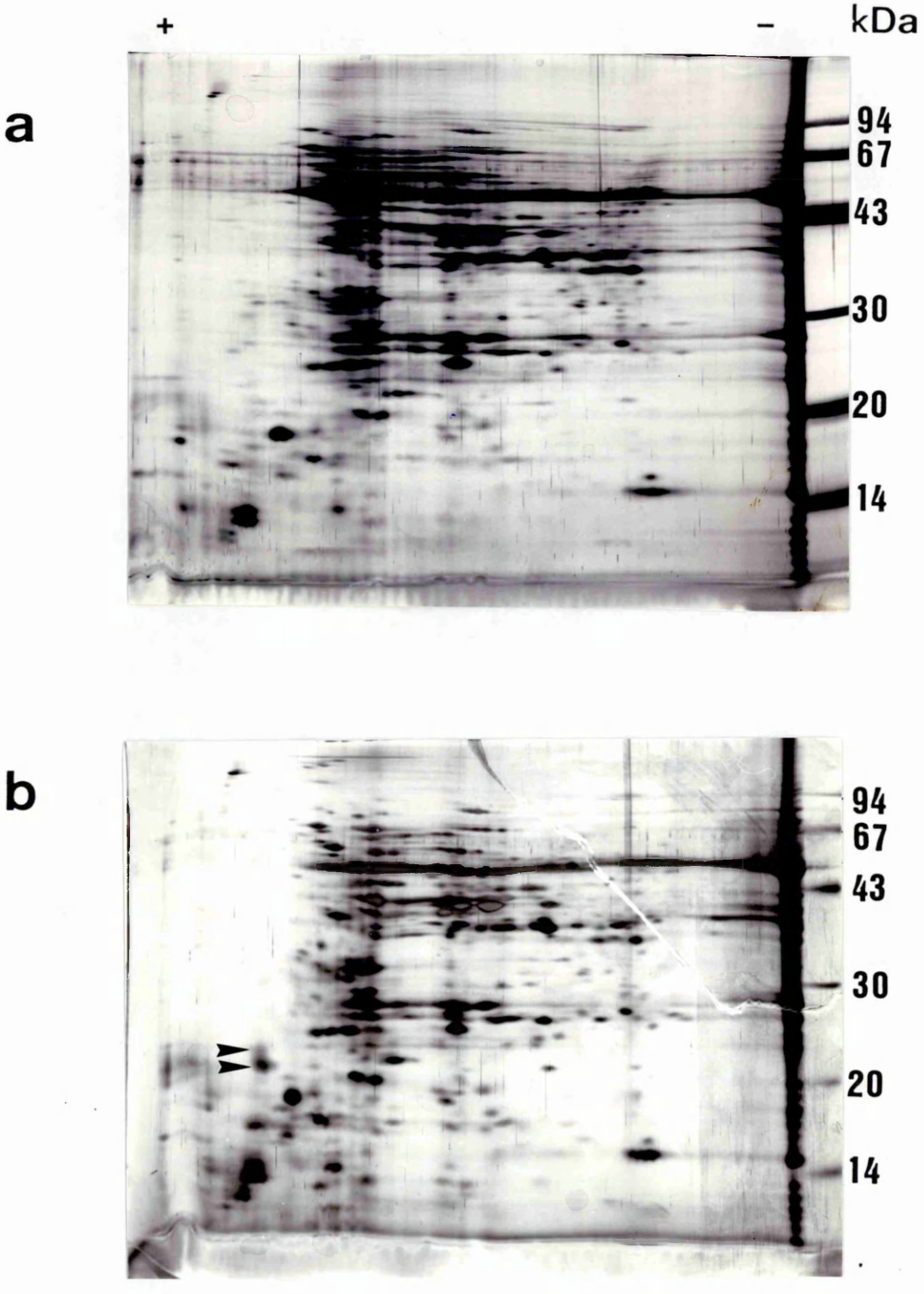

a
+
–
kDa
94
67
43
30
20
14
b
94
67
43
30
20
14

Figure 4.7 Plot of distance migrated against the log. molecular mass of protein markers

Proteins of known molecular mass were separated on an SDS polyacrylamide gel. The distance the proteins migated was plotted against the logarithm of their molecular mass.

Log. molecular weight of protein markers versus distance run on polyacrylamide SDS gel

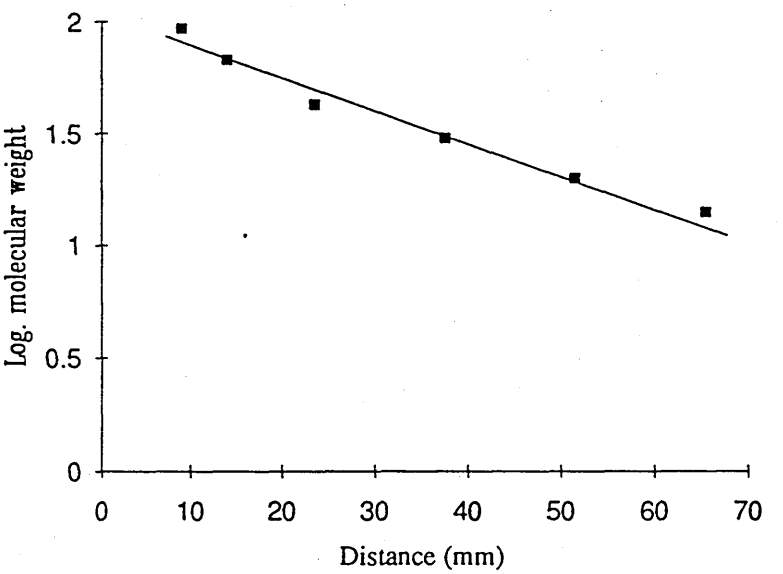


Figure 4.8A 2-dimensional PAGE of total leaf protein extracted from leaves of different ages taken from a single control biennial sugar beet plant.

Leaves at different stages of development were harvested (in duplicate) from control biennial plants growing in warm SDs (22°C, 8 hours fluorescent light), and the total protein extracted (section 2.6.1). 100µg of each sample was separated by IEF/SDS PAGE (section 2.7.3 and 2.7.4) and stained with silver (section 2.8.2).

The figure shows silver stained 2-dimensional gels of protein extracted from **a** : leaf 1, **b :** leaf 2 and **c :** leaf 3 (see Figure 4.8B).

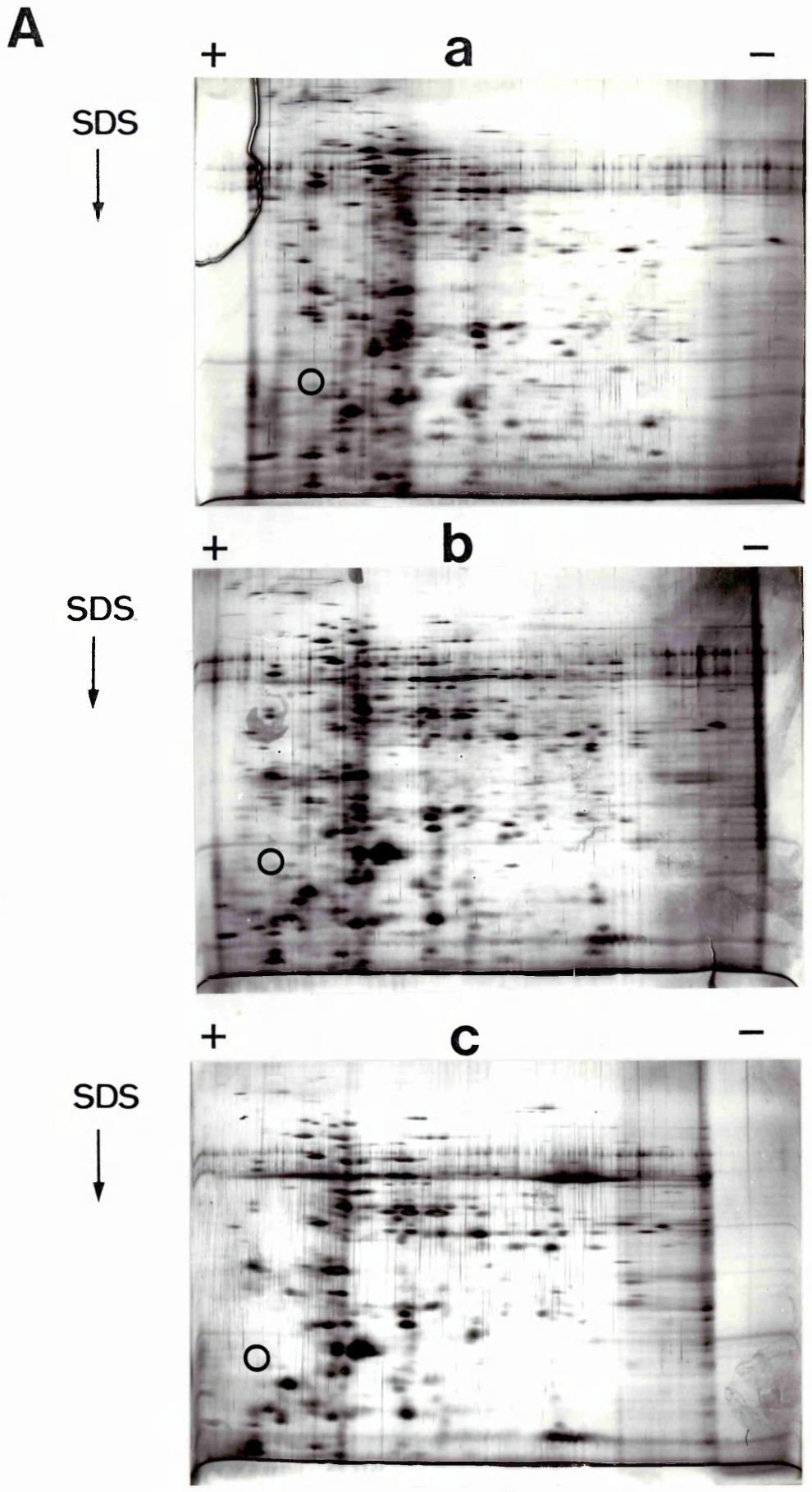
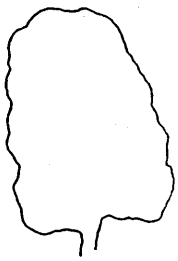
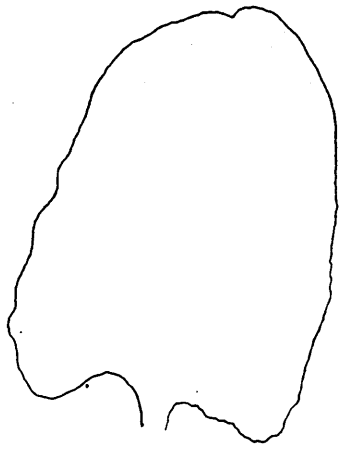

A
+
a
–
SDS
+
b
–
SDS
+
c
–
SDS

Figure 4.8 B **Tracings of leaves of different sizes from which total protein was extracted.**

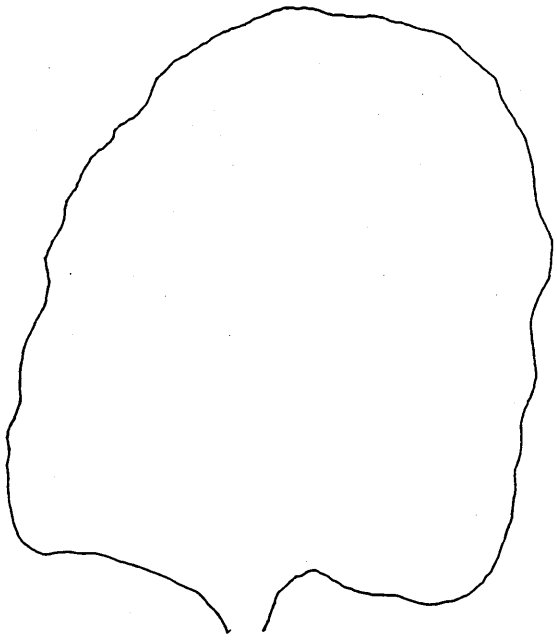
The figure shows the actual sizes of leaves (**a**,**b** and **c**) from which total protein was extracted. This protein was separated by 2-D IEF/PAGE (see figure 4.8A).



a



b



c

4.6 Time course of accumulation of the cold-induced polypeptides

In most of the previous experiments leaves were sampled after plants had been grown in low temperatures for two months. In studies of cold acclimation in several species new mRNAs and proteins have been detected in days rather than weeks (Tseng and Li, 1990; Hughes and Pearce, 1988; Mezo-Basso *et al.*, 1986). A time course experiment was therefore set up to investigate when the cold-induced polypeptides started to accumulate.

Figure 4.9 shows the result of such a time course experiment. Again proteins from individual newly expanded leaves were extracted. Plants were exposed to low temperatures for 0 days, 1 day, 2 days, 4 days, 6 days and 1 month. Two separate experiments gave the same result. The cold-induced polypeptides accumulated to detectable levels within 2 days of exposure to low temperatures and persisted for the duration of the experiment.

4.8 Response to wounding

Some cold-induced proteins are also induced by other stimuli e.g. ABA (Kurkela and Frank,1990), water stress (Hajela *et al.*, 1990) or heat shock (Guy *et al.*, 1989). It was therefore decided to investigate whether the 22 and 23kDa polypeptides appeared in response to stresses other than low temperature. In this experiment the leaves of 2 control biennial sugar beet plants grown in warm SDs (22°C, 8 hour fluorescent light) were wounded by scoring their leaf surface with a razor blade several times (section 2.4.4). After 2 days a wounded leaf from each plant was collected and protein extracted and separated by IEF/SDS-PAGE. Figure 4.10 shows one of the silver stained gels. Polypeptides of very similar molecular mass and pI to the cold-induced polypeptides are present.

4.8 Quantification of the cold-induced proteins

In previous experiments proteins were stained with silver. Silver staining is much more sensitive (100 times) than coomassie blue staining but unlike coomassie

Figure 4.9 2-dimensional PAGE of total proteins extracted from mature leaves of biennial plants exposed to low temperatures for different times.

Fully expanded leaves were harvested from a control biennial plant and from plants exposed to low temperature for 1, 2, 4, 6 and 30 days. Total protein was extracted as described in section 2.6.1 and 100µg of each sample separated by IEF/SDS PAGE (section 2.7.3 and 2.7.4). The gels were then silver stained (section 2.8.2).

The figure shows only the region of the gel containing acidic low molecular mass polypeptides after: **a** 0 days, **b** 1 day, **c** 2 days, **d** 4 days, **e** 6 days, **f** 30 days exposure to low temperature. Protein molecular mass markers are indicated to the left and arrows point to the cold-induced polypeptides.

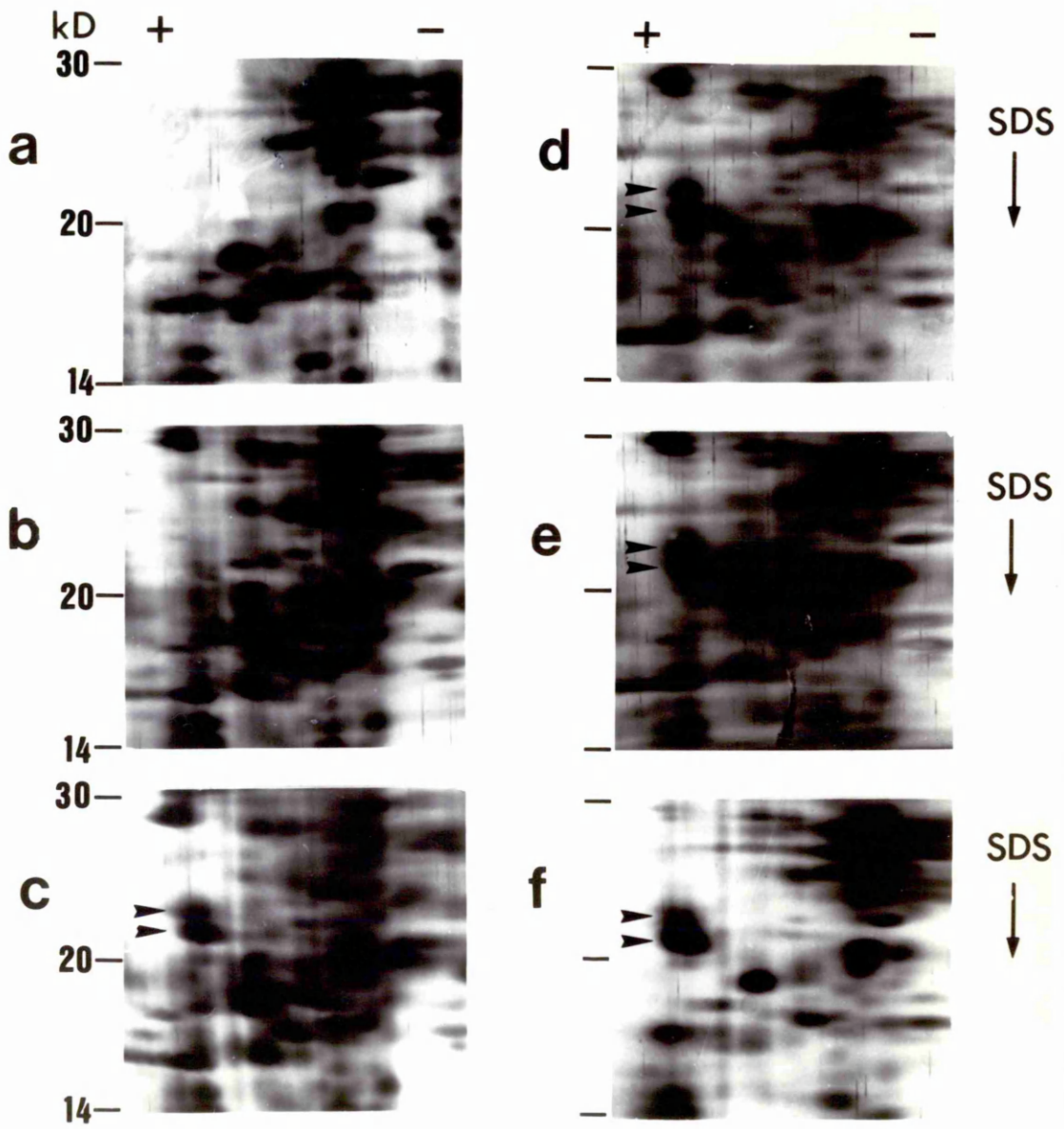
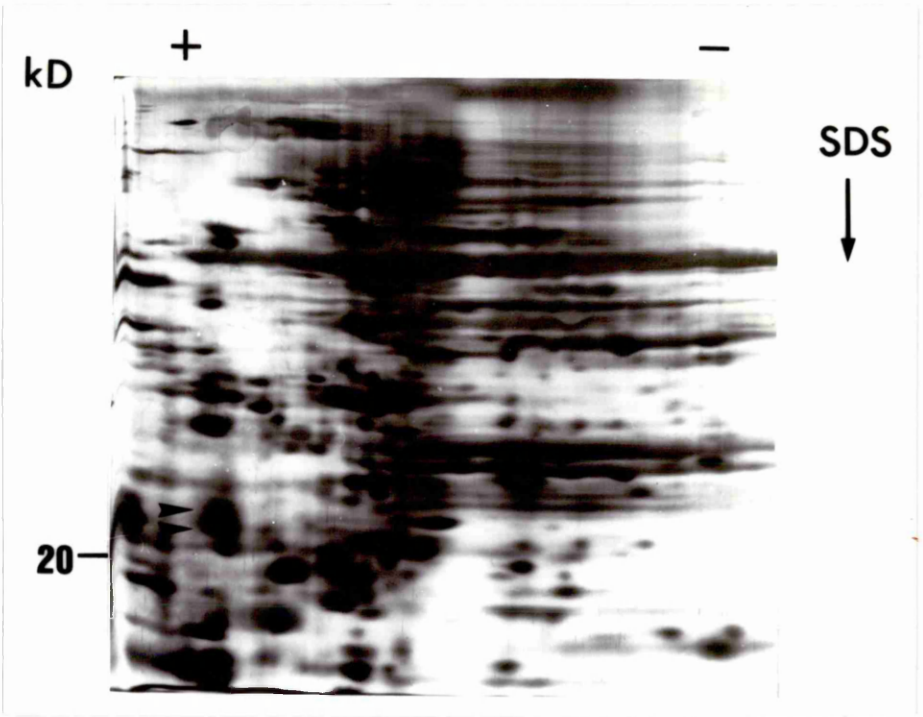

kD
+
−
30
20
14
a
b
c
d
e
f
SDS
SDS
SDS

Figure 4.10 2-dimensional PAGE of total protein extracted from wounded unvernalized biennial leaf tissue.

Mature expanded leaves of a control biennial sugar beet plant grown in the warm SD room (22°C, 8 hours fluorescent light) was wounded by scoring the leaves several times with a razor blade (section 2.4.4). Two days later the leaves were harvested and total protein extracted (section 2.6.1). 100µg of each sample was separated by IEF/SDS PAGE (section 2.7.3 and 2.7.4) and stained with silver (section 2.8.2).

The figure shows the pattern of proteins extracted from the wounded leaf tissue. Protein molecular mass is indicated on the left. Arrows point to the two polypeptides of interest.


kD
+
−
SDS
20

blue the silver does not bind quantitatively. Approximately 50 ng of protein is required for detection with coomassie blue staining (Reisner *et al.*,1975).

In order to get an estimate of the amount of the cold-induced polypeptides in relation to total protein, 2-D gels of protein extracted from the leaves of biennial plants grown at low temperatures were stained with coomassie blue. Aliquots containing 300µg and 400µg of total protein were loaded on the first dimension gels. The cold-induced polypeptides were only visible on coomassie blue stained gels when 400 µg of total protein was loaded (Figure 4.11). However, it is obvious that only a small amount of the protein loaded entered the first dimension IEF gel. The black vertical line on the right hand side of the 2-D gel corresponds to protein which has not separated in the first dimension. Thus, quantitatively, all that can be concluded is that at least 50 ng of each of the cold-induced polypeptides was present in the 400 µg sample. In relative terms, the polypeptides appear to be in the medium range of abundance.

4.9 Separation of proteins extracted with different buffers

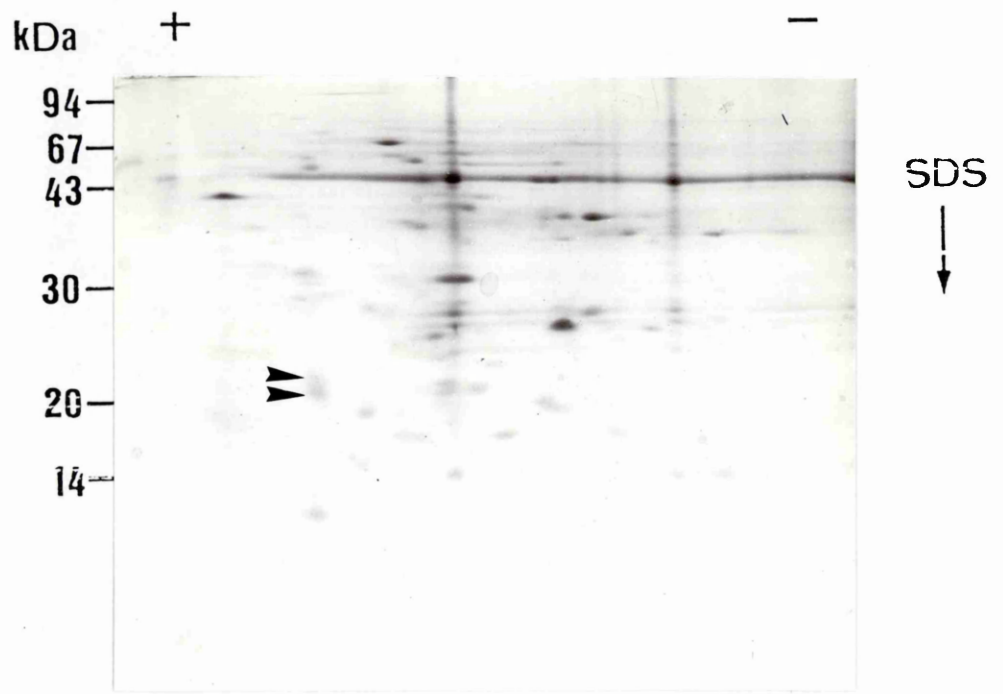
Proteins can be extracted from plant tissue using either denaturing or non-denaturing buffers. Denaturing extraction allows the recovery of both soluble and membrane bound proteins. Non-denaturing extraction allows only the extraction of soluble proteins; or, possibly, extrinsic membrane proteins; integral membrane proteins are pelletted during centrifugation. In the experiments described so far a denaturing extraction buffer containing the detergent SDS, the protease inhibitors PMSF and leupeptin, 2-mercaptoethanol a reducing agent, and PVP a phenol complexing agent was used. Both the SDS and the protease inhibitors should limit the activity of proteases released from the cell vacuoles during extraction.

The efficiency of protease inhibitors is however seldom 100% (Wu and Wang, 1984), and there are several reports of residual protease activity in the presence of SDS (Granier, 1988). This is perhaps due to the increased availability of cleavage sites revealed as the proteins are denatured. Although the protease activity is lost after

Figure 4.11 Quantification of coomassie blue stained cold-induced polypeptides.

400 µg of total protein extracted from vernalized leaf tissue was separated by IEF/SDS PAGE and stained with coomassie blue (section 2.8.3).

Arrows point to the polypeptides of interest. Molecular mass markers are shown on the left.


kDa
+
−
94
67
43
30
20
14
SDS

boiling in the presence of SDS, some protein degradation could still occur prior to this stage of the extraction procedure (see section 2. 6.1).

It is feasible that the presence of the cold-induced polypeptides is due to increased amounts and or activities of proteases present in cold-treated tissue which results in degradation of protein during the extraction procedure. Hence the 22 and 23 kDa polypeptides could be degradation products. On the other hand, this seems unlikely because of the general similarity in the protein profiles of cold-treated and control extracts, and the absence of any obvious loss of high molecular weight polypeptides. Nevertheless, it is possible that if protein present at a high concentration was degraded for a short while in the samples from cold-treated plants the loss would go unnoticed. Alternatively, higher protease activity in control tissue might selectively cause degradation of the 22 and 23 kDa polypeptides, accounting for their absence.

To investigate these possibilities an experiment was set up in which different extraction procedures were used. Proteins from control and cold-treated biennial leaf tissue were extracted using 1) SDS 2) phenol and 3) a non-denaturing Tris buffer. Figure 4.12 shows the acidic low molecular mass proteins extracted from control and vernalized plants using the different methods. In all the samples from cold-treated plants at least one of the cold-induced polypeptides is present. It seems likely that both polypeptides are present but have not been resolved by these gels, since even in the samples extracted with SDS only one protein spot is present. Detection of the polypeptides in cold-treated tissue, but not control tissue, which was extracted with phenol make it unlikely that the presence of these polypeptides in cold-treated tissue is the result of degradation during extraction. The polypeptides were extracted to a similar extent with a non-denaturing Tris buffer and this is a good indication that they are not integral membrane proteins.

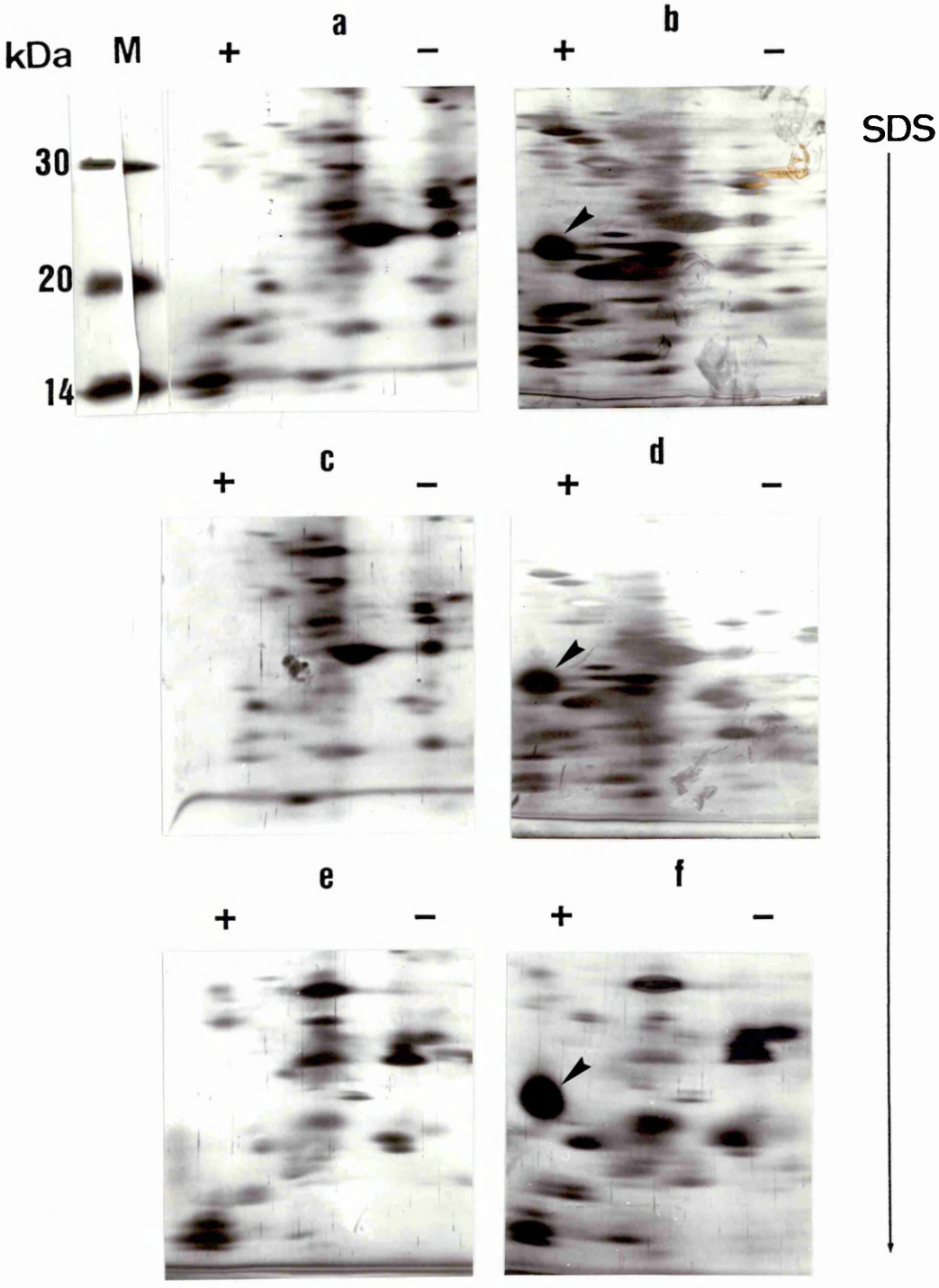
4.10 Total protein from control and cold-treated leaf tissue probed with antibodies against the large and small subunits of RUBISCO and the LHCP.

Two of the most abundant chloroplast proteins, the small subunit (SSU) of RUBISCO and the light-harvesting chlorophyll a/b-binding protein (LHCP), have

Figure 4.12 2-dimensional PAGE of proteins extracted using different method

Mature leaves were harvested from control and cold-treated biennial pl Total protein was extracted using either SDS, phenol or Tris buffer (see sections 2 2.6.2 and 2.6.3). 100 µg of each protein sample was separated by IEF/SDS P/ (section 2.5.3 and 2.5.4) and the gels stained with silver (section 2.6.2).

The figure shows the region of the gels containing acidic low molecular proteins. Protein profiles from; **a,** control tissue extracted with SDS; **b,** cold-tr tissue extracted with SDS; **c,** control tissue extracted with phenol; **d,** cold-treated t extracted with phenol; **e,** control tissue extracted with Tris buffer and, **f,** cold-tr tissue extracted with Tris buffer. Molecular mass markers are shown on the left The 22 and 23 kDa polypeptides are indicated by arrows.


kDa
M
a
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SDS
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f
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−

molecular masses (approximately 14 kDa and 26 kDa respectively) which would place them in the region of the gel containing the cold-induced polypeptides. The prevalence of the cold-induced polypeptides suggested that they could in some way be related to these proteins or to others involved in photosynthesis. Mezo-Basso *et al.* (1986) reported the presence of two *in vitro* translation products of 22 and 23 kD from warm-grown rape seedlings which co-migrated with products immunoprecipitated by an anti-RUBISCO SSU serum. Although SSU mRNA abundance has been reported to decrease at low temperatures (Meza-Basso *et al.*, 1986) it was thought prudent to check the possiblity that the cold-induced polypeptides might be related to the SSU of RUBISCO or LHCP.

Total protein was extracted from the leaves of control and cold-treated biennial plants. The cold-treated plants had been exposed to low temperatures for one week. 100 µg of each sample was separated in duplicate on a 1-D SDS gel. One lane of each sample was stained with coomassie blue, while the remaining one was electroblotted onto nitrocellulose membrane. The membane was then incubated with antisera against RUBISCO (recognising both SSU and LSU) and LHCP (see Figure 4.13). None of the proteins recognised by the antibodies are 22 or 23 kDa and therefore the polypeptides detected in cold-treated tissue are very unlikely to be related to these chloroplast proteins.

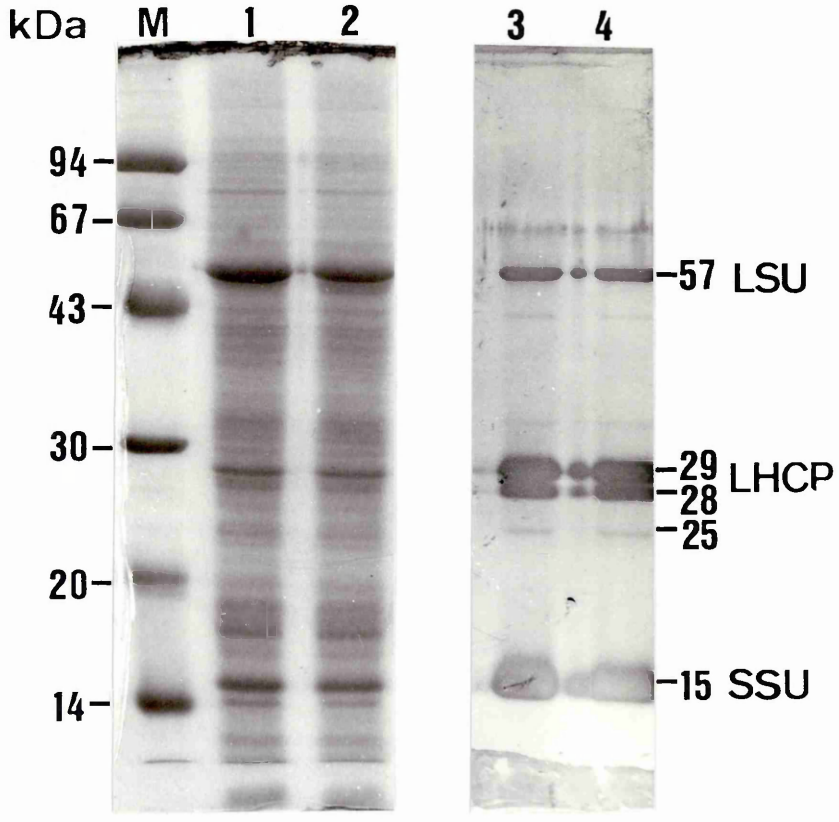
4.11 Summary

No consistent major differences were seen on comparison of 2-D separations of total protein extracted from annual and biennial sugar beet plants. Two acidic polypeptides of 22 and 23 kDa accumulated in both annual and biennial plants grown at low temperatures. These polypeptides could not be detected at the same level on 2-D gels of proteins extracted from leaves of different ages taken from control biennial plants. The polypeptides accumulated in cold-treated leaves of different ages and could be seen on silver stained gels 2 days after transfer to low temperatures. Two polypeptides of similar size and pI accumulated when the leaves of biennial plants were wounded.

Figure 4.13 **Analysis of RUBISCO (SSU and LSU) and LHCP antisera activities against total protein extracted from control and cold-treated biennial sugar beet leaf tissue using immuno- blotting procedures.**

Total protein was extracted (section 2.6.1) from the leaves of control and cold-treated biennial sugar beet plants and each sample was separated in 2 lanes of a 12% SDS-PAGE gel. One of the lanes was stained with coomassie blue (section 2.8.3). Western blotting (section 2.9) was carried out on the other and the nitrocellulose membrane was incubated with antisera against RUBISCO (LSU and SSU) and LHCP and as described in section 2.5.9.

The figure shows coomassie blue stained proteins from: **1**, control and **2**, cold-treated leaf tissue. Molecular mass markers are in lane **M** and are indicated on the left. On the right are blots of protein extracted from: **3**, control and **4**, cold-treated tissue with bands corresponding to RUBISCO LSU and SSU and LHCP indicated. Molecular masses of the proteins recognised by the antisera were calculated (section 2.2.6) and are shown on the right.


kDa
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1
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4
94
67
43
30
20
14
57 LSU
29
28
25 LHCP
15 SSU

The polypeptides were present in proteins extracted from the leaves of cold-treated plants with a non-denaturing Tris buffer and are therefore probably not integral membrane proteins. Probing with antisera against RUBISCO and LHCP showed that the polypeptides are not precursors or degradation products of these proteins.

Chapter Five: Comparisons of mRNA in control, cold-treated, and ABA-treated leaf tissue

5.1 Introduction

Data presented in the previous section (4.3) indicated that there were changes in gene expression detectable at the level of accumulated proteins in leaves of annual and biennial plants exposed to low temperatures. Further experiments were therefore carried out to investigate whether changes in gene expression could be observed at the mRNA level using *in vitro* translation. Whereas comparison of total protein from control and vernalized tissue will detect large changes in accumulated protein, comparison of *in vitro* translation products allows identification of differences in the mRNA populations of control and cold-treated plant tissue. Although *in vitro* translation products are not necessarily those translated *in vivo*, the technique is useful in that small changes in protein concentration can be detected which may be obscured if total accumulated proteins are compared. Comparison of 2-D separations of radioactively labelled *in vitro* translation products might detect (a) differences between control and cold-treated annual and biennial plants and (b) translation products corresponding to the cold-induced polypeptides. The latter would only be detected if the cold-induced polypeptides are not modified in any way after translation. That is, if a precursor is cleaved off or if the polypeptides are phosphorylated the translation products would have different sizes and pI's to the mature proteins detected on silver staned gels of total leaf protein.

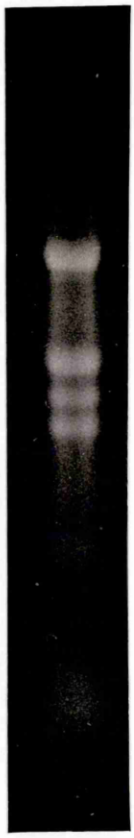
5.2 Patterns of *in vitro* translation products of RNA extracted from young unexpanded biennial control and cold-treated leaf tissue.

Biennial plants were grown in SDs at 22°C and thereafter transferred to the cold SD growth room (6-11°C, 8 hours fluorescent light). Young leaf tissue was sampled after 0, 1, 2 and 7 days in the cold. To minimize variation between samples, leaf tissue from three individual plants was pooled and the RNA extracted from this tissue treated as one sample. Figure 5.1 shows an ethidium bromide stained agarose-formaldehyde gel containing a sample of RNA extracted from leaf tissue. After extraction the

Figure 5.1 Visualization of RNA extracted from plant tissue.

RNA was extracted from leaf tissue (section 2.5.1) and its purity and concentration determined (section 2.2.4). 10µg aliquots were prepared for electrophoresis through a 1.3% agarose-formaldehyde gel (section 2.7.1). Once electrophoresis was complete the RNA was stained with ethidium bromide (section 2.8.1).

The figure shows RNA extracted from leaf tissue separated through a formaldehyde gel . The prominent bands are rRNA molecules.



concentration and purity was determined (section 2.2.4). Amino acid and mRNA concentration were then optimized for the *in vitro* translation system.

Figure 5.2 shows autoradiographs of *in vitro* translation products separated by 2-D gel electrophoresis. From comparison of the gels it is evident that differences do exist. For instance protein 1 changes in intensity, protein 2 while present in gel **a** is not detected in gel **c**. However, comparison of gels from three separate experiments showed that none of the observed differences were consistent. Moreover, no products could consistently be identified which might correspond to the cold-induced polypeptides of 22 and 23 kDa.

In studies with other plant species many clear and reproducible differences in translation products have been observed following exposure to low temperatures (Guy, 1990). It was thought such changes might occur within 24 hours in sugar beet and therefore would not have been detected in the above experiment. In the experiments which follow, where leaf samples were taken from plants exposed to low temperatures for less than 24 hours, the plants were transferred to the cold SD room (6-11°C, 8 hours fluorescent light) at various times thoughout the day so that all samples could be taken at 3pm on one day. This lowered the possibility that any changes seen would be due to diurnal variation in gene expression, as has been shown for other proteins, such as the chlorophyll a/b binding protein (Kloppstech, 1985). Leaves taken from three individual plants in each treatment were pooled before extracting RNA.

Figure 5.3 shows the *in vitro* translation products of RNA samples extracted from young unexpanded leaves of biennial plants exposed to low temperatures for 0, 6, 12, 24 and 48 hours. Unfortunately, the synthesis of larger molecular mass translation products was not consistent between samples. One protein band of approximately 16 kDa increased in intensity in the samples from plants exposed to low temperatures. It is possible that this translation product is derived from cold-induced mRNA. However, tracings of the leaf samples used in this experiment showed that the young leaves taken from the control plants were slightly smaller than the leaves taken from plants exposed to low temperatures. This 16 kDa translation product may therefore be induced at different stages of leaf development rather than by low temperatures.

Figure 5.2 2-dimensional PAGE of *in vitro* translation products synthesized from RNA isolated from the leaves of biennial sugar beet exposed to low temperatures for 0, 1, 2 and 7 days.

Young leaves with leaf blades <1cm in length were harvested from biennial sugar beet plants exposed to low temperatures (6-11°C, 8 hours fluorescent light) for 0, 1, 2 and 7 days. Total RNA was extracted (section 2.5.1) and translated *in vitro* (section 2.5.3). Equal amounts ($1x10^6$ cpm) of TCA- precipitable counts were separated by IEF/SDS-PAGE (sections 2.7.3 and 2.7.4) followed by fluorography (section 2.8.5) and autoradiography (section 2.8.4).

The figure shows autoradiographs of 2-dimensional gels of *in vitro* translation products synthesized from RNA extracted from the young leaves of biennial plants exposed to **a**: 0 days, **b**: 1 day, **c**: 2 days and **d**: 7 days of low temperatures. Protein molecular mass is indicated on the left. Numbered proteins referred to in the text are arrowed. The circle in (**c**) shows the position where spot 2 would have been expected.

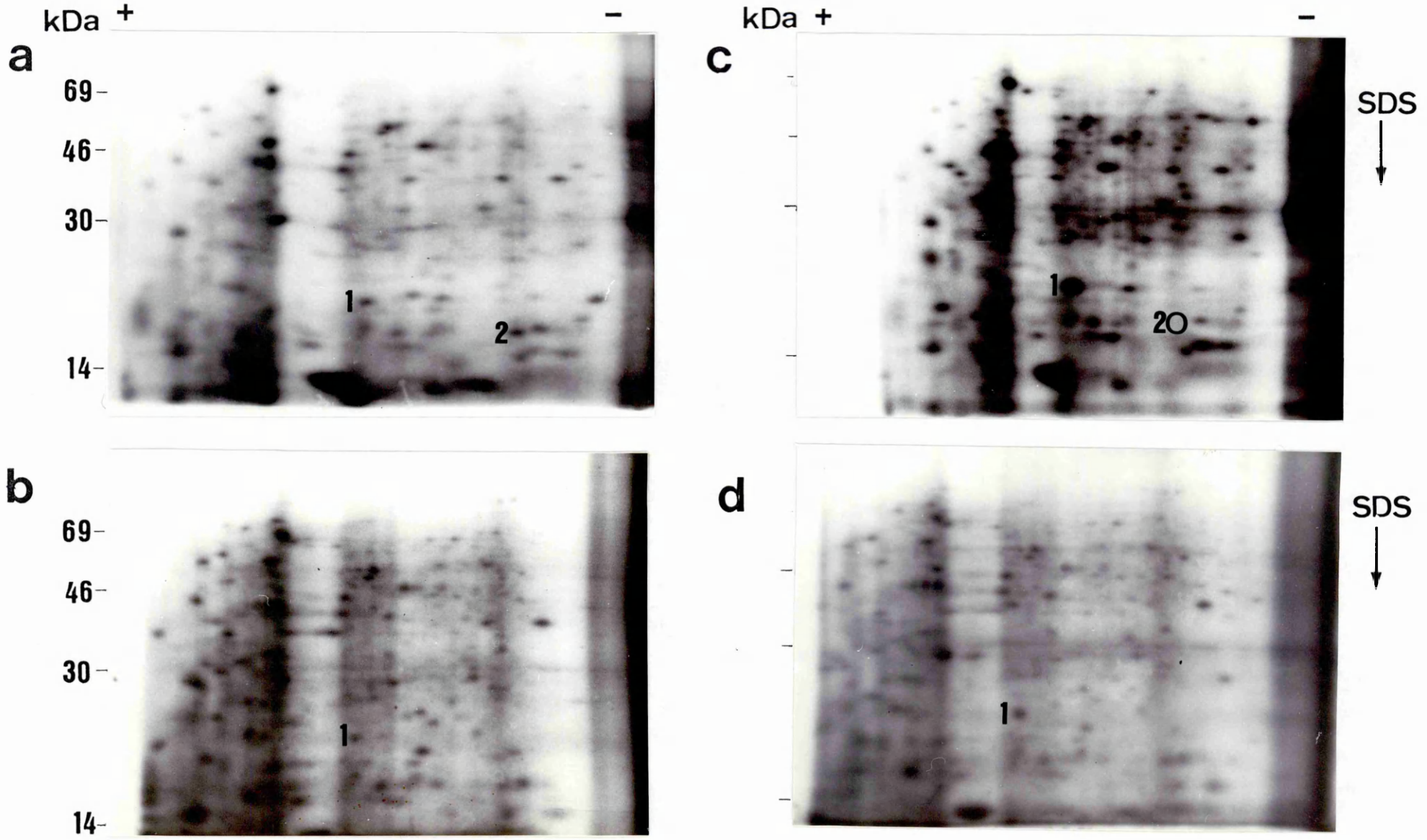

kDa
+
–
a
69
46
30
14
1
2
b
69
46
30
14
1
kDa
+
–
c
SDS
1
2O
d
SDS
1

Figure 5.3 **1-dimensional PAGE of *in vitro* translation products of RNA extracted from young unexpanded leaves of biennial sugar beet plants exposed to low temperatures for 0, 6, 12, 24 and 48 hours.**

Young leaves with leaf blades <1cm in length, were harvested from biennial sugar beet plants exposed to low temperatures (6-11°C, 8 hours fluorescent light) for 0, 6, 12, 24 and 48 hours. Total RNA was extracted (section 2.5.1) and translated *in vitro* (section 2.5.3). Equal amounts ($2x10^5$cpm) of TCA-precipitable counts were separated by SDS-PAGE (section 2.7.4). The times of exposure to low temperatures are indicated above the lanes. The lane labelled WG (wheat germ) is a control sample to which no RNA was added. The figure shows an autioradiograph of the *in vitro* translation products. The positions of the molecular mass markers are shown on the right hand side. A band of approximately 16 kDa which changes in intensity is marked with an arrow.

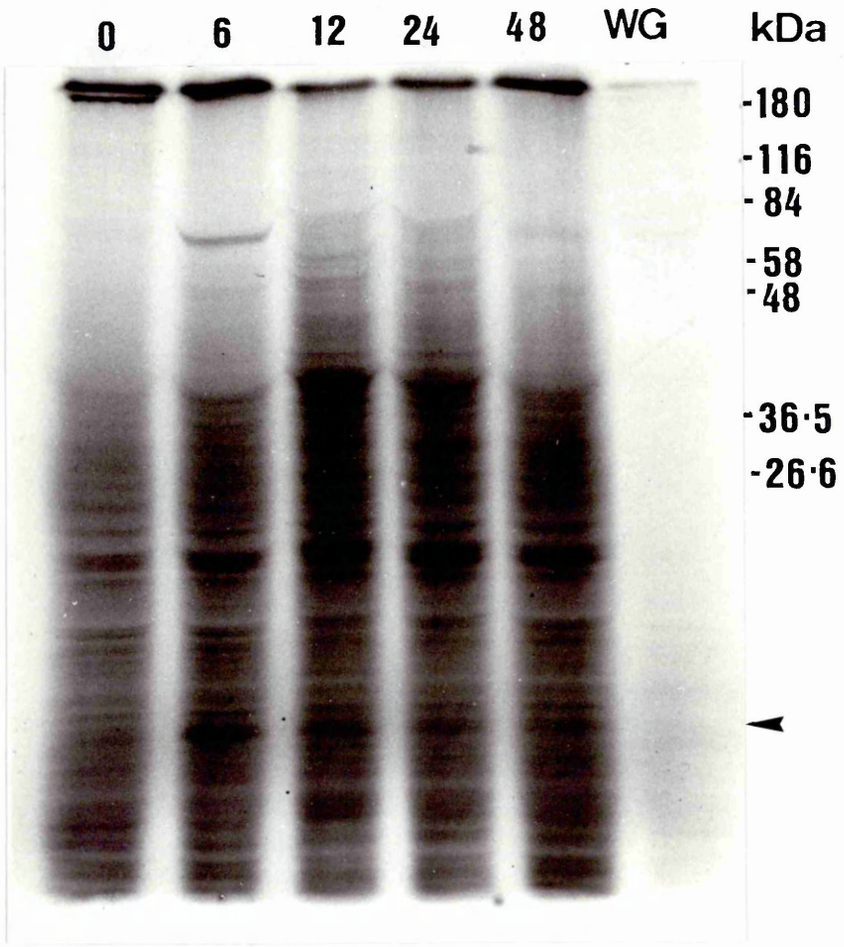

0
6
12
24
48
WG
kDa
-180
-116
-84
-58
-48
-36·5
-26·6

Figure 5.4 shows the result of a similar experiment with samples taken after the plants had been exposed to low temperatures for 0, 1, 3, 6, 9 and 24 hours. In this experiment a protein of approximately 20 kDa increased in intensity in leaf tissue from plants exposed to low temperatures for 1 and 3 hours.

5.3 Patterns of *in vitro* translation products from RNA extracted from mature leaves of biennial sugar beet.

In the 1 and 2-D gels of *in vitro* translation products of mRNA extracted from small unexpanded leaves, protein patterns were quite variable. It was thought that this might be due to the use of leaves of slightly different sizes, resulting in the visualization of developmental changes in translation products. Although an effort was made to harvest leaves of the same size (<1cm in length) the availability of such leaves made this difficult. It was decided therefore to look for changes in mature leaves. Although changes due to vernalization were not expected in these leaves, from previous experiments it was known that cold-induced proteins accumulated in mature leaves and therefore these and other changes due to low temperature might be visualized.

RNA was extracted from mature fully expanded leaves from plants exposed to low temperatures for 0, 6, 12 and 24 hours. Figure 5.5 shows the *in vitro* translation products separated on an SDS gel. Several protein bands show increases and decreases in intensity, though none are of 22 and 23 kDa.

Figure 5.6 shows the result of a similar experiment in which samples were taken from plants exposed to low temperatures for 0, 1, 3, 6, 9 and 12 hours. In this experiment no differences between *in vitro* translation products in the different samples were detected on the 1-D gel. *In vitro* translation products of these same RNA samples were also separated by IEF/SDS-PAGE. Figure 5.7 shows autoradiographs of these 2-D gels. Two polypeptides of similar size and pI to the cold-induced polypeptides are present in samples from plants exposed to cold for 3 and 6 hours. Only one of these polypeptides is clearly resolved in samples from control plants and plants given 1 hour cold treatment. Close examination with respect to other spots on the gels suggests that this is the upper one of the two translation products. Further repetition of this

Figure 5.4 1-dimensional PAGE of *in vitro* translation products of RNA extracted from young unexpanded leaves of biennial sugar beet plants exposed to low temperatures for 0, 1, 3, 6, 9 and 24 hours.

Young leaves with leaf blades <1cm in length, were harvested from biennial sugar beet plants exposed to low temperatures (6-11°C, 8 hours fluorescent light) for 0, 1, 3, 6, 9 and 24 hours. Total RNA was extracted (section 2.5.1) and translated *in vitro* (section 2.5.3). Equal amounts ($2x10^5$cpm) of TCA- precipitable counts were separated by SDS-PAGE (section 2.7.4). The figure shows an autioradiograph of *in vitro* translation products. The times of exposure to low temperatures are indicated above the lanes. The molecular mass of marker proteins is indicated to the left. The polypeptide which changes in intensity, referred to in the text, is marked with an arrow.

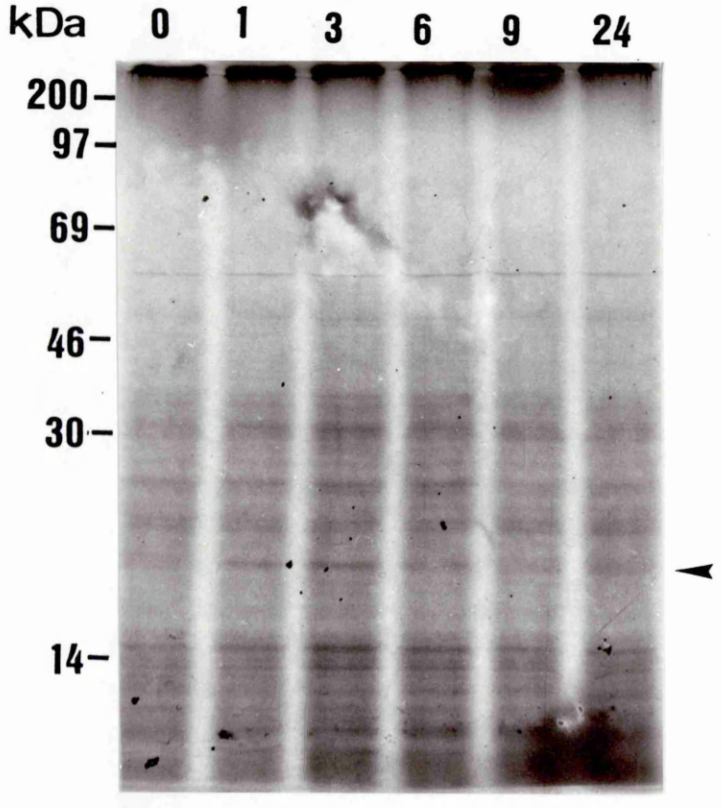

kDa
0
1
3
6
9
24
200
97
69
46
30
14

Figure 5.5 1-dimensional PAGE of *in vitro* translation products of RNA extracted from mature leaves of biennial sugar beet plants exposed to low temperatures for 0, 6, 12 and 24 hours.

Mature fully expanded leaves were harvested from biennial sugar beet plants exposed to low temperatures (6-11°C, 8 hour fluorescent light) for 0, 6, 12 and 24 hours. Total RNA was extracted (section 2.5.1) and translated *in vitro* (section 2.5.3). Equal amounts of TCA-precipitable counts were separated by SDS-PAGE (section 2.7.4).

The figure shows an autoradiograph of the *in vitro* translation products. The times of exposure to low temperatures are indicated above the lanes. Lane **M** contains ^{14}C-methylated protein markers the molecular masses of which are shown on the right. Translation products which change in intensity are indicated by arrows on the left.

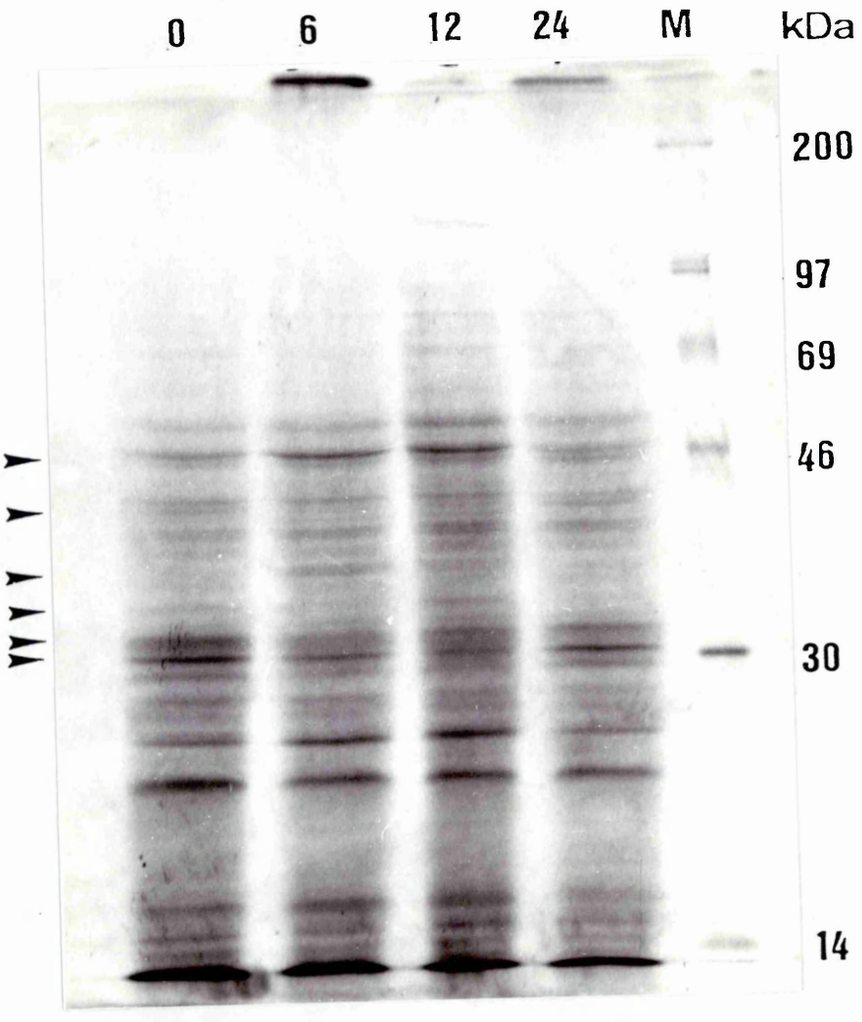
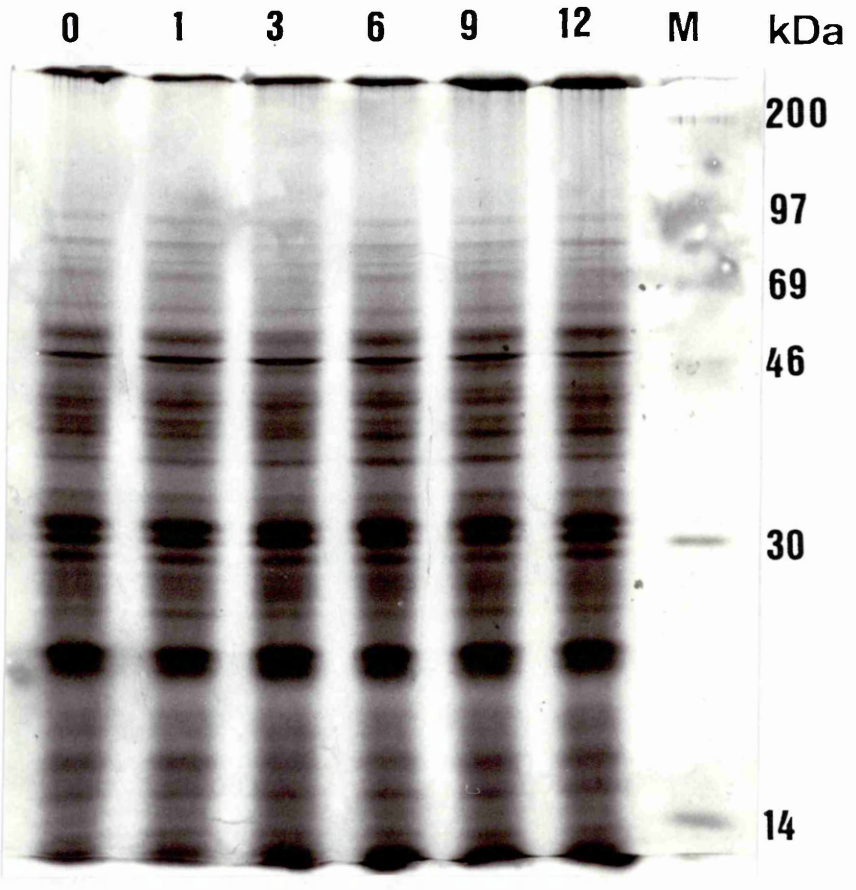

0
6
12
24
M
kDa
200
97
69
46
30
14

Figure 5.6 1-dimensional PAGE of *in vitro* translation products of RNA extracted from mature leaves of biennial sugar beet plants exposed to low temperatures for 0, 1, 3, 6, 9 and 12 hours.

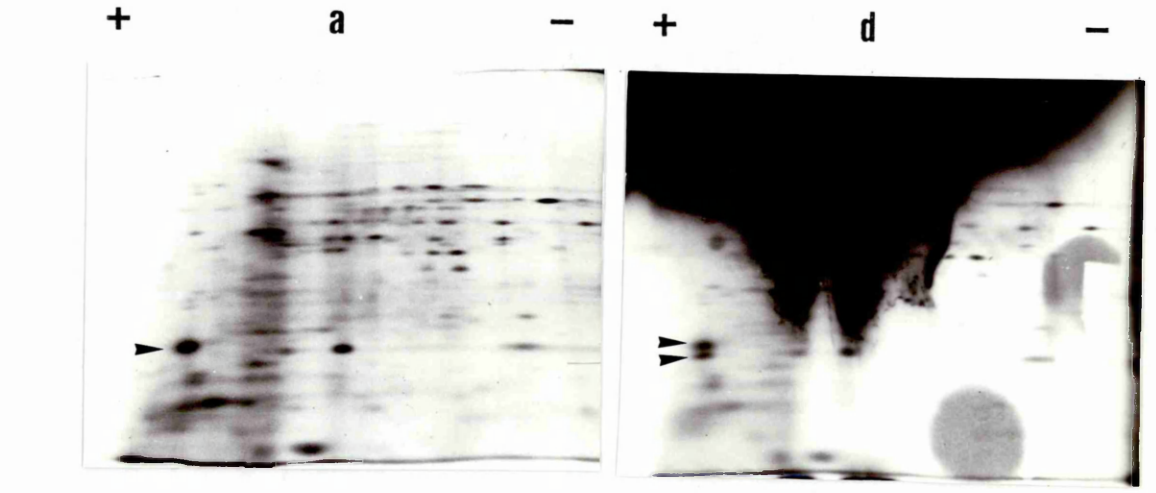
Mature fully expanded leaves were harvested from biennial sugar beet plants exposed to low temperatures (6-11°C, 8 hours fluorescent light) for 0, 1, 3, 6, 9 and 12 hours. Total RNA was extracted (section 2.5.1) and translated *in vitro* (section 2.5.3). Equal amounts of TCA-precipitable counts were separated by SDS-PAGE (section 2.7.4). The figure shows an autoradiograph of the translation products. The times of exposure to low temperatures are indicated above the lanes. Lane **M** contains ^{14}C-methylated protein markers the molecular masses of which are indicated on the right.

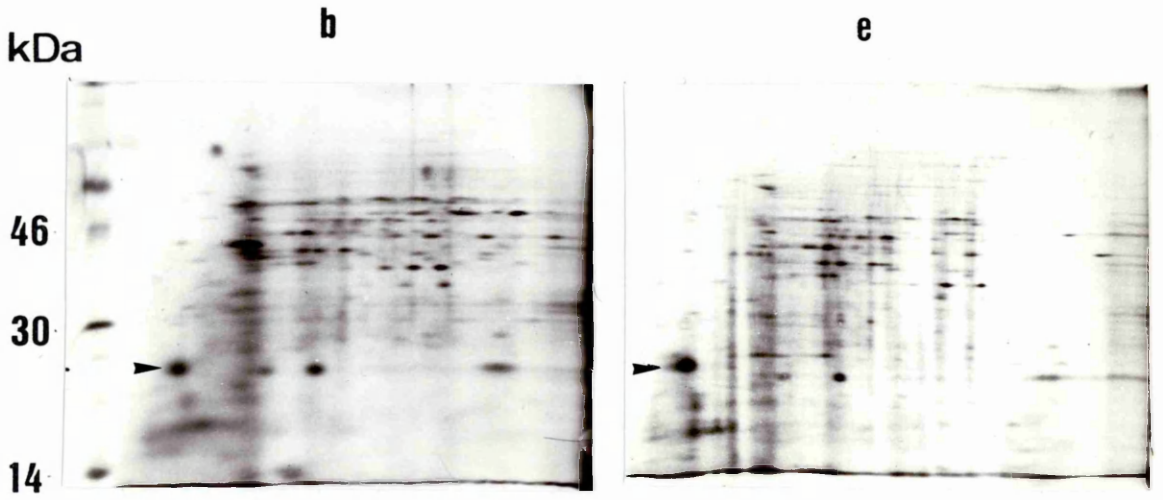

0
1
3
6
9
12
M
kDa
200
97
69
46
30
14

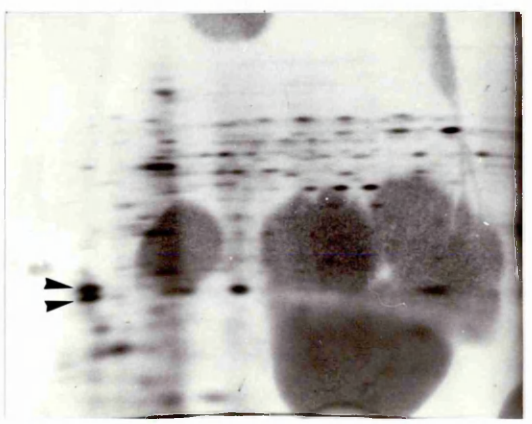
Figuire 5.7 2-dimensional separation of *in vitro* translation products synthesized from RNA extracted from the mature leaves of biennial sugar beet plants exposed to low temperature for 0, 1, 3, 6 and 9 hours.

Mature fully expanded leaves were harvested from biennial sugar beet plants exposed to low temperatures (6-11°C, 8 hours fluorescent light) for 0, 1, 3, 6 and 9 hours. Total RNA was extracted (section 2.5.1) and translated *in vitro* (section 2.5.3). Equal amounts of TCA precipitable counts were separated by IEF/SDS-PAGE (sections 2.7.3 and 2.7.4).

The figure shows autoradiographs of the 2-dimensional gels of translation products of plants exposed to low temperatures for **a**: 0 hours, **b**: 1 hour, **c**: 3 hours, **d**: 6 hours, and **e**: 9 hours. The molecular masses of ^{14}C-methylated protein markers are indicated on the left. Arrows point to the polypeptides of interest.


+
a
−
+
d
−


kDa
b
e
46
30
14


c

experiment is required, but these data suggest that one of the 22/23 kDa translation products appears following cold treatment.

5.4 Patterns of *in vitro* translation products of RNA extracted from mature leaves of biennial plants exposed to 0 and 3mM ABA for 1 day

ABA has been shown to induce cold acclimation in some species (Guy, 1990) and some proteins induced by low temperatures are also induced by ABA (Lang *et al.*, 1989; Mohapatra *et al.*, 1989; Kurkela and Franck, 1990). An experiment was therefore carried out to investigate whether ABA application to mature leaves of sugar beet caused changes in the translation products of RNA and whether any such changes could be correlated with the 22 and 23 kDa cold-induced polypeptides.

In this experiment plants were grown in the warm SD room (22°C, 8 hours fluorescent light) until they had reached the 10 - 12 leaf stage. Two control plants were sprayed with water, another 2 plants were sprayed with 3 mM ABA (in water) to run off. Samples of the leaf tissue were taken after 1 day and pooled for each treatment. RNA was extracted and translated *in vitro*. Figure 5.8 shows a 2-D separation of the translation products from control plants and those exposed to 3mM ABA for 1 day. One translation product of similar mass (22-23 kDa) and pI to those seen in Figure 5.7 is present on the 2-D gel of translation products of RNA from plants exposed to ABA for 1 day. A protein spot can be seen in the control in the same position but at a much reduced intensity.

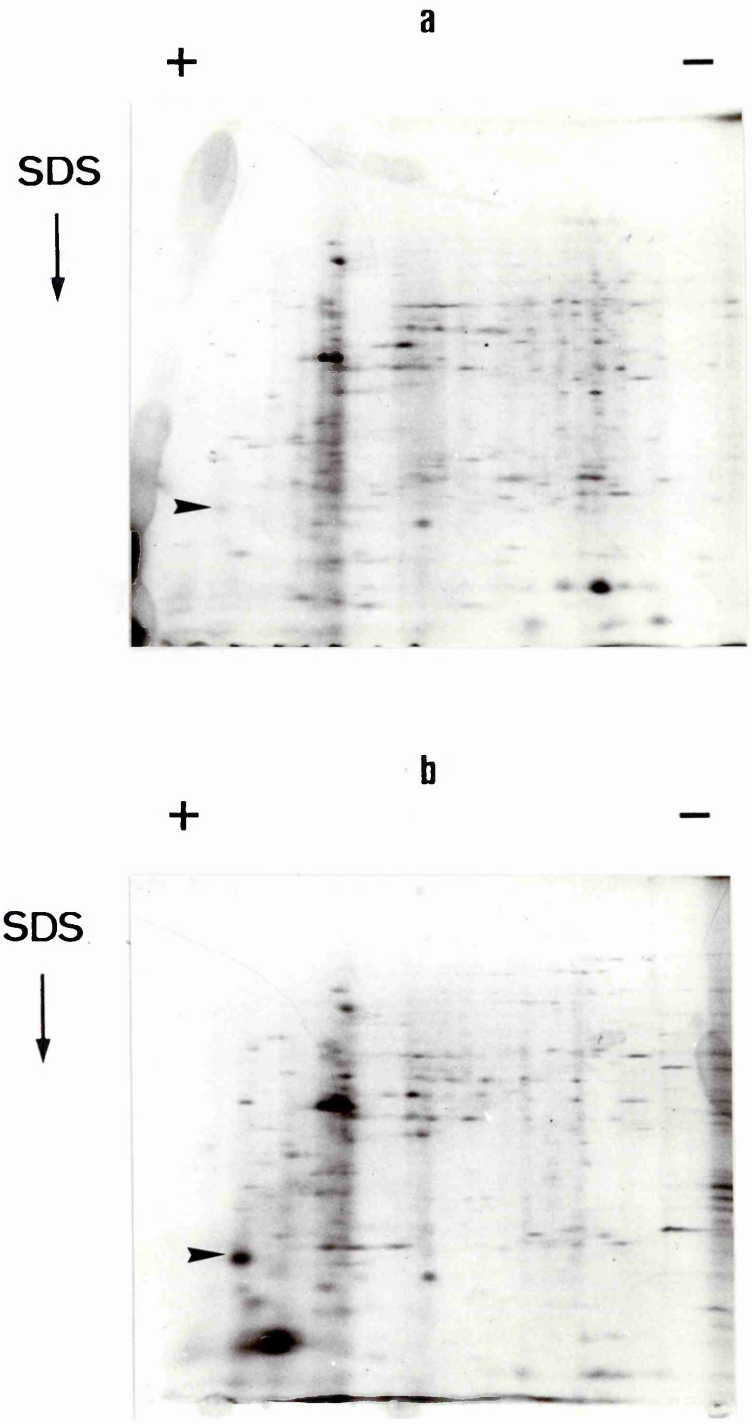
5.5 Summary

Several differences between the profiles of *in vitro* translation products of RNA from control and low temperature-treated plants were seen on 1-D and 2-D SDS gels. It is possible that some of these differences represent cold-induced mRNAs. However, even though the overall pattern of polypeptides was similar, translation products found in different samples from plants given the same treatment and in samples from plants given different treatments were quite variable, and without further repetition of the experiments nothing conclusive can be drawn from the results.

Figure 5.8 2-dimensional separation of *in vitro* translation products synthesized from RNA extracted from mature leaves of biennial sugar beet exposed to 0 and 3mM ABA for 1 day.

Mature fully expanded leaves were harvested from biennial sugar beet plants sprayed with water or 3mM ABA and left for 1 day. Total RNA was extracted (section 2.5.1) and translated *in vitro* (section 2.5.3). Equal amounts of TCA-precipitable counts were separated by IEF/SDS-PAGE (sections 2.7.3 and 2.7.4).

The figure shows autoradiographs of *in vitro* translation products synthesized from RNA extracted from **a**: control plants and **b**: plants exposed to 3mM ABA for 1 day. Arrows point to the polypeptides of interest.


a
+
−
SDS
b
+
−
SDS

The 2-D gels shown in Figure 5.7 are interesting because they show that RNA extracted from mature leaves includes transcripts for polypeptides of similar size and pI to the cold-induced polypeptides seen on the 2-D gels of total protein extracted from the leaves of cold-treated plants (see Chapter 4). It is possible that one of these translation products increases in intensity following exposure to cold, although further experiments are required to investigate this point. A polypeptide of similar size (22-23 kDa) and pI was detected in 2-D gels of *in vitro* translation products of mRNA extracted from the leaves of mature biennial plants exposed to 3mM ABA for 1 day. It is also present in the sample taken from the control which was sprayed with water, but at a much reduced intensity.

Chapter Six: Discussion

6.1 Sites of perception and response to vernalization and daylength.

Investigations of the responses of several species to low temperatures have indicated that the presence of oxygen, carbohydrate and actively dividing cells are prerequisites for vernalization (Lang, 1965). These studies have led to the general belief that exposure of meristematic tissue alone is sufficient to cause vernalization (Curtis and Chang, 1936; Curtis, 1960; Schwabe, 1954 and Wellensiek, 1964). However, in some other species, low temperatures have been shown to affect non-meristematic tissue and result in the promotion of flowering (Barendse, 1985; Metzger, 1988 see section 1.4.6).

By circulating cold water through fine rubber tubing wound around the base of leaf petioles of sugar beet, Chrobozek (1934) showed that lowering the temperature of the growing point to approximately 12°C resulted in vernalization. However, this localized cooling experiment did not distinguish between vernalization of the apical meristem and vernalization of the young leaf tissue, nor was the possibility that vernalization might affect mature leaves investigated. Grafting experiments with sugar beet indicate that unvernalized biennial meristems are competent to respond to floral stimulus translocated from induced or flowering annual and biennial donor plants (Margara, 1968; Stout, 1945; Curtis and Hornsey, 1964; Curtis, 1964). Moreover these experiments showed that unvernalized leaves are incompetent to produce or transport such a signal in response to inductive LDs. Thus the site of perception of vernalization in sugar beet and the nature of the changes induced by low temperature is not entirely resolved in these studies.

The physiological experiments reported here were devised to investigate 1) if only those leaves initiated from a vernalized meristem are able to respond to LDs or if leaves which experience low temperatures become competent and, 2) whether an inhibitor of flowering is present in the old leaves. The results of the defoliation experiments confirmed previous work showing that annuals have a facultative and biennial plants an obligate requirement for vernalization. The most significant findings

from the defoliation experiments are that 1) sugar beet leaves lose their ability to respond to inductive daylengths as the leaves age and 2) that leaves initiated before plants are exposed to low temperatures can become competent to respond to LDs.

None of the plants from which all the young leaves were removed flowered. Lack of flowering of annual plants in this group suggests that in sugar beet leaves lose their capacity to respond to inductive daylengths with age. Loss of sensitivity and reduced responses to inductive photoperiods as leaves age have been noted in several plant species, although it is not a universal trait (Lang, 1965). For example, fully expanded leaves of *Lolium temulentum* continue to respond to inductive daylengths (Vince-Prue, 1975). Another explanation for the failure of bolting and flowering of the annuals with the youngest leaves removed is that sugar beet may go through a juvenile phase and that the first few leaves produced cannot respond to inductive daylengths. These would correspond to the oldest leaves in the plants used in the defoliation experiments. Several investigators have reported that there is no juvenile stage with regard to vernalization in sugar beet (Margara, 1968; Lexander, 1975) and no juvenile phase has been reported for induction of flowering. However, seeds can only be partially vernalized, and young plants require longer periods of low temperature treatment than old plants. If a juvenile state does occur when tissue cannot respond to inductive daylengths it may have gone unnoticed in these published experiments where it is likely that the short juvenile phase is completed whilst the plants are being vernalized. However, the existence of a short juvenile phase could account for the longer low temperature treatment required by young plants. After a longer period at low temperatures the plants could complete the juvenile stage of growth and would be competent to respond to inductive daylengths. Clearly, more work needs to be done to investigate whether there is a juvenile phase in sugar beet. In any case, it seems likely from studies with other species, that a decrease in sensitivity to photoperiod in older leaves is likely to be important.

Several observations make it unlikely that an inhibitor of flowering is present in the leaves of sugar beet. Comparison of intact plants with those from which old leaves present before vernalization commenced were removed showed no significant

difference in the time to bolting or the time of the first flower opening. Moreover, complete defoliation of control and vernalized plants did not result in bolting or flowering, and annual and biennial plants with intact leaves produced significantly longer seed-stalks than those with the old leaves removed. Nevertheless, these results do not exclude the possibility that an inhibitor is produced in non-inductive daylengths or that an inhibitor is produced in unvernalized leaves of the biennial which may be broken down at low temperatures.

Caution should be exercised when interpreting these results. The criteria used to assess the effect of the treatments do not distinguish clearly between whether the treatments solely affect flowering or whether their main effect was on the rate of vegetative growth. This is immaterial when one is interested in an all or nothing response, that is, whether plants are vegetative or reproductive. But if one is looking for the subtle effects of an inhibitor substance through the acceleration or delay of flowering in different treatment groups, it would be more satisfactory to use additional criteria which do not rely on time. For instance, the number of leaves formed when the first floral primordia were produced could have been recorded. If this had been noted it may have been found that intact plants, although flowering at the same time as those with old leaves removed, had produced many more leaves. The number of leaves produced before the first floral primordia appear could be important if, for instance, leaves which are not vernalized produce an inhibitor which is counteracted by some promotive substance produced by vernalized leaves in LDs. It would also have been useful to score other parameters such as the final number of flowers and seed weight.

The results of the second defoliation experiment indicate that leaves initiated before the cold treatment commenced were vernalized. In this experiment plants on which only one leaf remained, which was initiated but not expanded before the start of the cold treatment, bolted and flowered. These leaves were less than 1cm in length when the plants were transferred to low temperatures and some cell division as well as cell expansion is likely to have occurred. No previous studies with sugar beet have addressed the question of whether leaves themselves can become vernalized.

Vernalization describes the promotion of flowering by exposure to low temperatures. Although it has been concluded that young leaves of the biennial initiated before the start of vernalization can become competent to produce a floral stimulus as a result of low temperatures, they have not been pinpointed as the primary site of action of low temperature. It is still not known whether in sugar beet 1) both leaves and meristem are vernalized, 2) whether the meristem only is vernalized and sends a signal to the leaves which causes a developmental change allowing them to respond to LDs, or 3) whether the leaves are vernalized and send a signal to the meristem allowing it to respond to the floral stimulus. Further experiments need to be conducted to produce valid information on these alternatives. Two approaches could be taken. Firstly, localised cooling of different leaves on an intact plant would indicate whether low temperatures act directly on leaves and, if so, which leaves can be vernalized. Localised cooling of the meristem would show whether or not this tissue responds to low temperatures. The second approach would involve vernalization of excised leaves. If these leaves were subsequently grafted onto non-vernalized biennial recipients flowering would show that exposure of leaf tissue alone to low temperatures is sufficient to cause vernalization. If these leaves were kept in the dark and the recipients in inductive daylengths the result might provide evidence for the presence of vernalin. But this is doubtful since for translocation of such a stimulus a food deficit in the recipient would have to be produced and this usually involves defoliation of the receptor. If this approach was followed, resistant bolting varieties should be used as recipients since low vernalizing temperatures would have to be maintained until the graft took, and this might also cause sufficient vernalization of the donor. Lack of stabilization of the vernalized state in sugar beet precludes attempts to prove vernalization of mature leaf tissue by vernalizing excised leaves *in vitro* followed by regeneration since low temperatures would have to be maintained and might cause vernalization of the meristematic cells.

If the sensitivity to inductive daylengths is a transient attribute in annual sugar beet leaves which is lost as leaves age, it would be interesting to find out if the same is true of vernalized leaves of both annual and biennial plants. Whether or not a juvenile

stage exists could easily be determined by exposing the first few leaves produced to LDs and removing all subequent leaves as they emerge.

6.2 Changes in protein content induced by low temperature

The annual phenotype in sugar beet is conferred by the presence of a dominant *B* gene. This gene affects the competence of the leaves to respond to inductive daylengths. To understand vernalization in sugar beet two questions must be answered. The first is: what is the function of the *B* gene? Does it code for a protein which itself promotes competence in the leaves to respond to LDs and produce a floral stimulus or does it prevent the action of an inhibitor substance? The second question relates to the process/action of vernalization. Does vernalization induce expression of the *B* gene in the biennial, does it act directly on the *B* gene product to change its activity, or does it produce a change in other substances which affect the flowering process essentially mimicking the action in the biennial of the *B* gene product? Without information on the nature of the *B* gene product it is impossible to answer this question. However, it is clearly important to look for changes in gene expression concerned with vernalization as it may be possible to identify the *B* gene product or genes likely to be differentially expressed during vernalization.

Differences in the protein profiles of annual and biennial varieties of sugar beet may exist due to the presence of the *B* gene in the annual and its presumed absence in the biennial. It is always possible, however, that single amino acid substitutions cause the *B* gene product to be inactive in the biennial, and such changes might not change the profile of accumulated proteins. Nevertheless, if protein differences are present they should be apparent at normal temperatures if the *B* gene is expressed throughout development. Alternatively, the *B* gene product might be induced during exposure to low temperatures, or to inductive daylengths when it is known that the leaves of the annual produce a floral stimulus.

Of course, it is possible that important changes not involving gene expression are part of the process of vernalization. For instance, changes in the properties of membranes might alter ion fluxes or signal perception in cold-treated tissues. Moreover,

changes in the activity of enzymes could result in the accumulation of different metabolites at low temperatures. Nevertheless, the hypothesis made at the start of this project was that changes in gene expression are likely to occur during vernalization. By vernalizing plants in SDs it was hoped that the processes of vernalization occurring at low temperatures and induction of

flowering by LDs could be separated. As discussed in section 1.8.2, it is reasonable to investigate changes associated with vernalization in young unexpanded leaves as well as the shoot meristem. Comparison of total protein from young leaves of annual and biennial plants grown in warm SDs or cold SDs might uncover differences due to expression of the *B* gene and/or other genes concerned with vernalization. However, cold-induced changes in gene expression would not necessarily be related to vernalization but might equally be due to the expression of genes required for growth and survival of the plants at low temperatures. Isolation of genes encoding the proteins showing altered expression at low temperatures would be necessary to elucidate their function and significance in developmental processes.

No consistent differences were detected at normal or low temperatures between the protein profiles of annual and biennial leaf tissue. One possible explanation for this is that the chosen method of analysis is not sensitive enough to detect all the proteins present in the tissue and thus differences between proteins present at a lower concentration could not be resolved. Goldberg (1986) claims that approximately 25,000 genes are expressed in each tobacco organ system. The same is probably true in sugar beet and if this is the case it is evident that only 1-2% of the proteins present in the leaf tissue were visualized on the 2-D silver stained gels. Faced with this limitation in sensitivity it might be suggested that a more sensitive technique such as the screening of a subtracted cDNA library would have been more productive. However, the literature is full of examples where this simple technique of 2-D PAGE has detected important changes in gene expression. Much of the data on the regulation of gene expression in higher plants originates from comparisons of mRNA and proteins in induced and non-induced cells or tissues at different stages of development; e.g. light-induced proteins (de Vries *et al.*, 1982), seed storage proteins (Gatehouse *et al.*, 1986), proteins

synthesized during fruit ripening (Grierson *et al.*, 1986) and heat shock proteins (Ougham, 1987). Although screening a library may have resulted in the selection of clones of mRNA expressed differentially, proteins regulated at a translational or post-translational level would not have been identified. In any case it is important to look for changes in accumulated proteins as it is the proteins *per se* whose activities are likely to be important in responses to low temperatures.

The 2-D PAGE technique was found to be highly reproducible and to give reliable indications of changes in specific polypeptides when carried out on replicate samples. Although several polypeptides were found to vary between 2-D separations, only two polypeptides of 22 and 23 kDa and acidic pI, were found consistently to accumulate in the plants exposed to low temperatures. They accumulated in the young leaves of annual plants, and in both the young and mature leaves of biennial plants grown at low temperatures. Moreover the polypeptides could not be detected at the same levels in control leaves of any age.

Until the genes encoding these polypeptides are isolated and their expression investigated in detail there is no way of telling if they are involved in vernalization. The fact that they accumulate in both the annuals and biennials does not necessarily rule out this possibility because the data presented in section 3.1 show that the annual is responsive to vernalizing treatments. If vernalization results in the accumulation of proteins required for the induction of flowering it may be that these proteins could also be detected in the leaves of unvernalized annuals exposed to inductive LDs, but this has not been investigated.

Many studies of cold acclimation have identified quantitative and qualitative changes in protein or mRNA, usually by *in vivo* labelling of proteins and *in vitro* translation of mRNAs. Changes in 1-D and 2-D separation of proteins stained with coomassie blue (Mohapatra *et al.*, 1987) and with silver (Guy and Haskell, 1988) have been observed. Proteins of similar molecular masses (i.e. 22 - 25 kDa) have been reported to accumulate in cold-treated *Oryza sativa* (Kuznetsov *et al.*, 1987), *Solanium commersoni* (Tseng and Li, 1990) and *Bromus inermis* (Robertson *et al.*, 1988). Where available, the data indicate that these proteins are of different pI to those observed in

this study.

Some proteins induced at low temperatures belong to a group of stress-induced proteins which are not specific to cold stress. Proteins with similar electrophoretic mobilities to cold-induced proteins on 1- and 2-D gels have been induced by ABA (Kawata and Yoshida, 1988 see section 1.5.3), and heat shock (Kawata and Yoshida, 1988, Guy *et al.*, 1985). That some of these proteins are induced by more than one stress is now beyond doubt. Hajela *et al.* (1990) have shown that the transcript levels of four COR genes increased in response to low temperature, ABA and water stress. In this study polypeptides of similar size and pI were seen to accumulate when leaf tissue was wounded. It is likely that these polypeptides are the same as the cold-induced polypeptides though further work will need to be carried out to investigate their relationship.

The time course experiment which monitored the accumulation and persistence of the two polypeptides in mature leaf tissue from biennial plants showed that the polypeptides accumulated to detectable levels within two days of the plants being exposed to low temperatures. This is in agreement with other studies in which cold-induced proteins in alfalfa have been detected after two days (Mohapatra *et al.*, 1987). Until recently it was thought that the response of plants to low temperatures was much slower than their response to heat shock. However, now that cDNA clones of cold-induced proteins are available some transcripts have been found to increase within one hour of exposure, in for example *Arabidopsis* (Kurkela and Franck, 1990). In the present study, the two polypeptides were found to be present in the young leaf tissue of both annual and biennial plants after two months exposure to low temperature, the longest time point at which proteins were extracted. This implies that either the polypeptides are very stable in the cold or that the genes encoding them continue to be expressed such that the polypeptides are constantly synthesized in the cold.

All the features of the accumulated 22 and 23 kDa polypeptides are consistent with a possible function in cold acclimation to protect the plants against damage by low temperature. That is, they appear quite quickly in response to cold treatment, they persist throughout the cold period and are present in leaves of different ages. The

wounding suggests that they have a role in protecting the plants against more than one stress. Cold acclimation involves metabolic changes as well as changes which confer freezing tolerance. Other workers have suggested that cold-induced proteins might correspond to cold tolerant isozymes induced to replace cold labile enzymes, enzymes which are up-regulated to compensate for decreased activity at low temperatures, or to meet an increased demand for certain products which are important in protecting cells against the effects of low temperatures. Non-enzymatic cryoprotectant proteins may also be important in cold acclimation. The functions of the COR genes isolated over the last 2-3 years by differential hybridization is still unknown. However, *kin1* isolated from Arabidopsis (Kurkela and Franck, 1990) has sequence similarity to fish anti-freeze proteins.

It is interesting to note that, along with many other substances, the regulation of stress proteins has been correlated with the flowering process. Lotan *et al.* (1989) reported PR (pathogenesis related) protein accumulation during flower development, and Meeks-Wagner *et al.* (1989) isolated five gene families from tobacco thin cell layer explants initiating floral development, four of which contain genes encoding the stress related proteins: chitin, ß-1,3-glucanase, extensin and osmotin. Neale *et al.* (1990) looked at the spatial and temporal expression of these genes both in explants and whole tobacco plants and suggested that the hydrolytic activity of some of these proteins may play a secondary role by mediating the release of oligosaccharides from plant cell walls. The oligosaccharides have been shown to control the levels of phytohormones which may induce flowering (Albersheim and Darvill, 1985).

6.3 Changes in *in vitro* translation products induced by low temperature

mRNAs present in the leaves of biennial sugar beet transferred to the cold were compared with those present in leaves from control plants using the technique of *in vitro* translation. ^{35}S-methionine was used to label the translation products. The intensity of the signal detected by autoradiography for each polypeptide synthesized depends on the number of methionines in each molecule of the polypeptide. It is

depends on the number of methionines in each molecule of the polypeptide. It is generally assumed that the presence of the translation product is correlated with the availability of its mRNA for translation *in vitro*. This could in principle be affected by modification of the mRNA during extraction, and, it is possible that the translatability *in vitro* does not necessarily correspond with translatability *in vivo*. Nevertheless, changes in the relative intensity of labelling of translation products in different samples provide a strong indication of the relative abundance of their mRNAs in total RNA.

In the experiments reported in Chapter 5, the *in vitro* translation assay gave good incorporation of ^{35}S-methionine into protein and the translation products usually included high molecular mass polypeptides. Hence the assay was working satisfactorily.

No consistent differences were detected between the translation products of control and cold-treated young leaf tissue separated by 2-D PAGE (Figure 5.2). One-dimensional PAGE separations of translation products from RNA extracted from young and mature leaf tissue were equally inconsistent. In one experiment a polypeptide of 16 kDa increased in intensity in samples from young leaf tissue exposed to low temperatures for 6, 12, 24 and 48 hours. In a similar experiment a polypeptide of 20 kDa increased in intensity, reaching a maximum in leaf samples which had been exposed to low temperatures for 1 and 3 hours. These mRNAs may be cold-induced, but the experiments need to be repeated especially since the 16 and 20 kDa translation products did not increase in intensity in both experiments. The variability in protein patterns may have been due, in part, to the use of leaves of slightly different sizes and, therefore, the detection of developmental rather than cold-induced gene expression.

In an attempt to solve this problem RNA from mature leaf tissue was extracted. By using mature leaf tissue it was thought that variability due to development would be much reduced. Nevertheless, the results of two experiments in which RNA from mature leaves was translated differed. In the first experiment several differences were observed in the relative intensities of polypeptides separated on 1-D SDS gels. In the second experiment no differences were observed, but when the translation products were separated in 2-D some differences were evident (Figure 5.7). Significantly,

polypeptides were detected of similar size and pI to the cold-induced polypeptides seen on silver stained gels. Only one of these polypeptides was detected in the control tissue and tissue exposed to low temperatures for 1 hour. In samples taken from plants exposed to low temperatures for 3 and 6 hours both polypeptides were resolved. There is no evidence from examination of the gels that the differences were due to incomplete resolution of the two polypeptides. Although these experiments were repeated, technical problems prevented visualization of the translation products. Unfortunately this means that there is insufficient evidence to be certain whether or not the mRNA for these two polypeptides is actually induced by low temperatures or whether it is always present in control leaf tissue. Because the molecular mass and pI of these translation products is very similar to that of the cold-induced polypeptides seen on the silver stained gels of total accumulated proteins, it seems likely that they are the same polypeptides. If this is correct the results of this preliminary experiment raise the possibility that the synthesis of only one of the cold-induced polypeptides is regulated at the level of mRNA accumulation. In addition, one translation product was detected of similar mass and pI to the cold-induced polypeptides when plants were treated with ABA (Figure 5.8). This polypeptide is also present in the control but at a much reduced intensity. This is consistent with several previous reports of cold-induced gene products also being induced by ABA (Guy, 1990; Thomashow, 1990).

There are many differences between the polypeptide profiles seen in Figures 5.7 and 5.8 compared to previous gels (Figure 5.2). In these later experiments mRNA was extracted from mature rather than young leaf tissue, but this does not explain the absence of translation products similar to the cold-induced polypeptides in some of the RNA samples from young unexpanded leaf tissue. Translation products which may correspond to the 22-23 kDa polypeptides are present in Figure 5.2 c. This shows the need for repetition in these experiments in order to obtain consistent results and reliable conclusions.

To further investigate the cold-induced polypeptides and the control of the genes that encode them it would be important to obtain cDNA clones. If control is at the level of mRNA accumulation, the genes could easily be cloned by differential screening of a

cDNA library. If, however, control is post-transcriptional purification of the polypeptides directly from 2-D gels should be possible. The polypeptides can be stained with coomassie blue which indicates that more than 50ng of each can be separated on a single 2-D gel, making feasible the elution of enough protein for microsequencing or for the raising of antibodies. 25-100 pmols of a protein is required for microsequencing corresponding to approximately 5-10 µg of a 50 kDa protein. Protein spots from several gels can be pooled, blotted onto Immobilon-P-transfer membrane and sequenced on a gas-phase sequenator (Charbonneau, 1989). Experiments described in section 4.9 showed that the cold-induced polypeptides in sugar beet leaves could be extracted with a non-denaturing buffer. Therefore extraction of proteins in for example, Tris buffer should increase the proportion of these two polypeptides relative to total protein and by electrophoresis through a 15% SDS gel adequate separation should be achieved.

Close *et al.* (1989) found that the late embryogenesis abundant (LEA) proteins in barley are not precipitated by boiling in an SDS - free buffer. Hajela *et al.* (1990) reported cold-induced proteins of 160, 47, 24 and 17 kDa in *Arabidopsis* which were not denatured upon boiling. The possibility that the cold-induced polypeptides discovered in this project might also have this property should be investigated. If they are not precipitated on boiling large quantities could be purified quickly and easily.

The possibility that control of the level of at least one of the polypeptides is post-transcriptional highlights the advantage of the approach used here. These two polypeptides accumulate to become medium abundance proteins in tissue exposed to low temperatures, yet cloning and screening a cDNA library would probably not have isolated clones encoding both the polypeptides.

6.4 Concluding remarks

The results presented in this thesis have extended our present knowledge of vernalization and cold-induced gene expression in sugar beet. The physiological experiments show that young unexpanded leaves become competent to respond to inductive daylengths after exposure to low vernalizing temperatures. Hence the hypothesis that only the apical meristem is vernalized and only leaves subsequently

initiated are competent to produce a floral stimulus is clearly too simplistic. The experiments reported here do not rule out the possibility that the apical meristem is the primary site of perception of low temperatures. However, if this is true a more complex model of vernalization in sugar beet involving signal transmission is required. The possibility that it is the young leaves alone which respond to the low temperatures is likewise unresolved and these alternatives merit further investigation.

The second principle finding is that two polypeptides are induced by low temperatures in sugar beet leaf tissue. The induction of these polypeptides is not confined to unexpanded leaves nor to the biennial genotype and it seems most likely that these polypeptides are involved in cold acclimation rather than vernalization. The regulation of the synthesis of these cold-induced polypeptides has several interesting features which deserve further study.

Returning to the points raised in the Introduction, it is clearly very important to identify genes concerned with the control of developmental processes such as vernalization. However it is clear from the work described here that the isolation of such genes is far from straightforward. When the method employed to detect such genes relies on their differential expression an appreciation of the relevant physiology is essential. Moreover, although comparisons of proteins and mRNAs can be utilized to identify differentially expressed genes, many differences may be identified and it may not be immediately apparent which differences are pertinent to the process under study. With this in mind perhaps an alternative approach should be considered. It might be possible in due course to isolate the *B* gene using RFLP and genetic maps to initiate a chromosome walk to the gene. However, this approach will also not be straightforward because of the size of the sugar beet genome. By which ever means potentially useful genes are isolated, the successful transformation and regeneration of transformed sugar beet will be required. To date sugar beet has been transformed using T-DNA (Lindsey et al., 1990) and ultrasound (Joersbo and Brundstedt, 1990). Where these methods are unsuccessful electroporation of protoplasts, microprojectile bombardment, microinjection (Schweiger, Dirk and Koop, 1987) and shoot tip transformation (Ulian *et al.*, 1988) methods can be attempted. Optimal conditions for the regeneration of

transformed sugar beet cells have still not been found, but the likelihood that more efficient methods will be found is high. Successful conditions have recently been found for the regeneration and transformation of many ecconomically important recalcitrant plants eg. rice (Shimamoto *et al.*, 1989), cotton (McMullen *et al.*, 1990 transformed only), apple, strawberry (James *et al.*, 1990), and maize (Gordon-Kamm *et al.*, 1990).

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