# Circadian regulation of gene expression and carbon dioxide fixation in *Bryophyllum fedtschenkoi*

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To Steph with love,

for helping me with the final onslaught

and to the one piece of work that you will not want to read.

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## Abbreviations

Abbreviations not described in 'Instructions to Authors', The Biochemical Journal (1992) 281: 1-19 are listed below, with their definitions.

| | |
|---|---|
| **CAM** | Crassulacean acid metabolism |
| **cpm** | counts per minute |
| **DEPC** | diethyl pyrocarbonate |
| **ds** | double stranded |
| **EtBr** | Ethidium bromide |
| **kb** | kilobases |
| **kbp** | kilobasepairs |
| **MDH** | Malate dehydrogenase |
| **NAD** | Nicotinamide adenine dinucleotide |
| **OAA** | Oxaloacetate |
| **%** | per cent |
| **PEP** | phosphoenolpyruvate |
| **PEPc** | phosphoenolpyruvate carboxylase |
| **pH** | hydrogen ion concentration, $-\log_{10}$ of |
| **TEMED** | N,N,N',N',-tetramethylene diamine |
| **UV** | ultraviolet radiation |
| **(v/v)** | (volume/volume) |
| **(w/v)** | (weight/volume) |

**Summary**

Many plants are known to exhibit circadian rhythms in the expression of the genes for chlorophyll a/b binding proteins (*CAB* genes). Detached leaves of the plant *Bryophyllum fedtschenkoi* show circadian oscillations in carbon dioxide ($CO_2$) metabolism and in the regulation of phosphoenolpyruvate carboxylase (PEPc) by PEPc kinase. The aim of this work was to investigate the relationship between circadian rhythms of *CAB* gene expression and $CO_2$ metabolism in *Bryophyllum fedtschenkoi*. A *CAB* cDNA from *Bryophyllum fedtschenkoi* has been cloned and sequenced. This was shown to be one member of a *CAB* multigene family. When detached leaves of the plant *Bryophyllum fedtschenkoi* (adapted to a 8 h light/16 h dark cycle) are placed in constant darkness and $CO_2$ free air, or constant light and normal air, at a constant temperature, the *CAB* gene transcript level shows circadian regulation. If the leaves are placed in constant darkness and normal air, one period of *CAB* gene expression still occurs, but a continuous cycling rhythm is not observed. The leaves that show maximal *CAB* expression are smaller than those which show the clearest rhythms in $CO_2$ release/uptake. In the larger leaves, *CAB* gene expression was detected but at a much lower level of expression than in the smaller leaves. The *Bryophyllum fedtschenkoi* leaves, which show circadian cycling in *CAB* gene expression, show some differences in the pattern of $CO_2$ release/uptake compared to larger leaves. The smaller leaves (2.5 cm to 4.0 cm in length) show a circadian rhythm in $CO_2$ metabolism but the amplitude is greatly reduced and the rhythm dampens out more rapidly than in larger leaves. *CAB* gene expression is affected by temperature. In constant darkness and normal air, the pattern of *CAB* gene expression is altered by extremes of temperature (5°C and 35°C). *CAB* gene expression was totally inhibited

at low temperatures (5°C) whereas at high temperatures (35°C) *CAB* gene expression was detectable but the expression showed no clear time-dependent pattern. The data indicate that *Bryophyllum fedtschenkoi* leaves show rhythms of both $CO_2$ metabolism and *CAB* gene expression. Possible physiological and mechanistic links between these rhythms are discussed.

# Chapter 1. Introduction

## 1.1 Circadian Rhythms

Rhythmical patterns are present in a vast array of systems in the world around us. One only needs to look at what is shown in the high number of reviews that discuss biological rhythms (Hillman, 1976; Feldman, 1982; Nongkynrih and Sharma, 1992; Wilkins, 1992; Piechulla, 1993; Beator and Kloppstech, 1994). Hall and Rosbash (1993) have argued, due to the wide range of organisms that show circadian rhythmicity, that such clocks probably arose early in evolution. Obviously, following such pre-determined patterns is only advantageous if the system has the ability to change in response to the particular circumstances which are surrounding it. Therefore the major questions are, what is the mechanism and function of such rhythms?

What is a circadian rhythm? Circadian rhythms have been known since 1729, when de Mairan stated "the sensitive plant senses the sun without seeing it in any way" (Satter and Galston, 1981). If the word is broken down into its constituent parts of 'circa' and 'diem' the meaning is "about a day". So these rhythms are biological phenomena which show repetitive cycles of about 24 hours. Consequently circadian rhythms are often referred to as biological clocks.

### 1.1.1 Definition and characteristic features of circadian rhythms

The basic characteristic features of circadian rhythms are as follows (Hall and Rosbash, 1987; Dunlap, 1990):

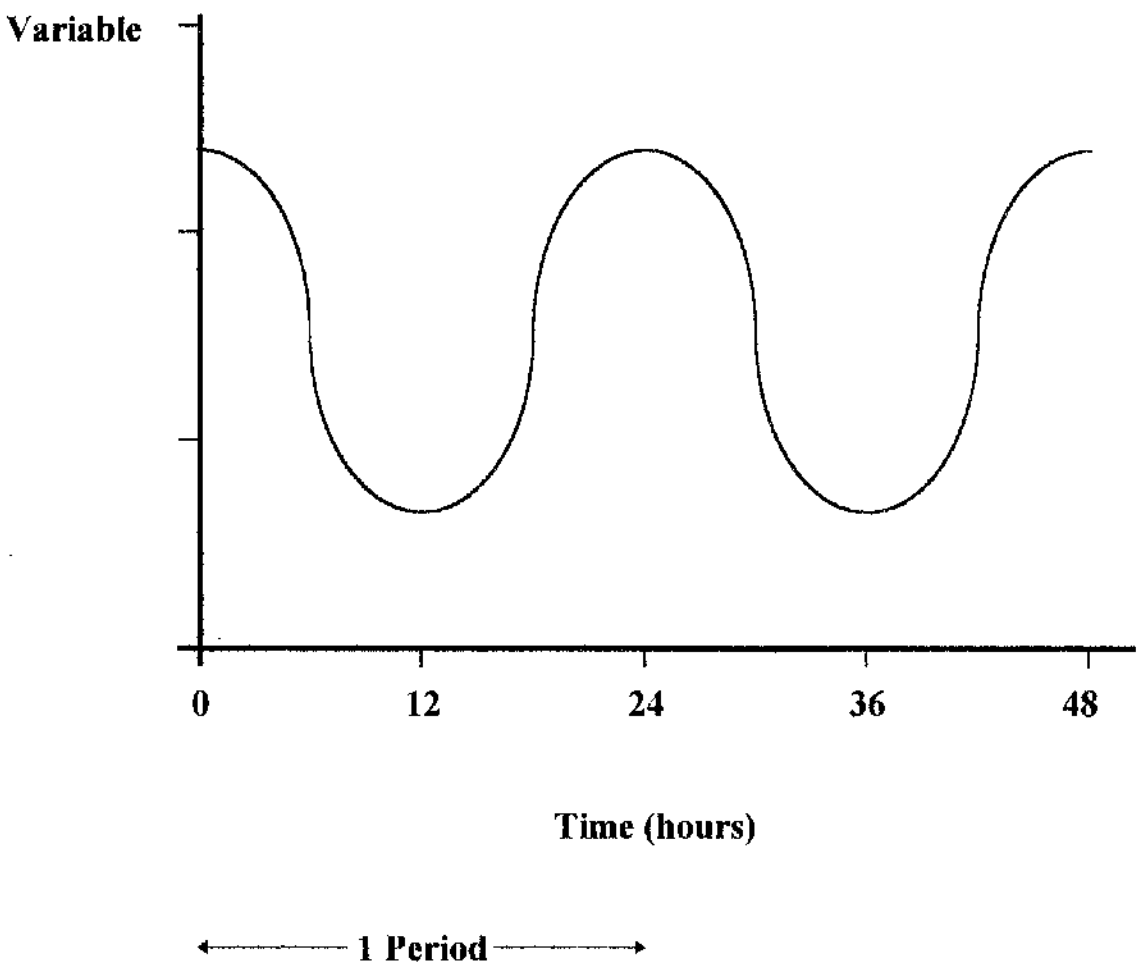
1. The oscillation of a specific parameter is found to have a period length of about 24 hours.
2. The oscillation will persist under uniform conditions still maintaining the period length.
3. The period of this oscillation shows a high degree of temperature compensation.
4. The rhythm is entrained by environmental cues or "Zeitgeber".

The details of circadian rhythms (period etc.) depends on the organism and variable parameter studied. One illustration of this is that the period length varies among different organisms between 19 hours and 27 hours. Circadian rhythms can be termed biological clocks because of the factor of temperature compensation. At temperatures within the physiological range there is little influence of temperature on the period length. Figure 1.1 shows the ideal case of a circadian rhythm in which the variable parameter shows a constant amplitude and 24 hour period length on a never ending scale. In many circadian rhythms a degree of amplitude dampening is seen.

The production of circadian rhythms is the result of a complex system, such as that seen in a clock mechanism. A pathway of control must therefore exist in producing these rhythms. A source will set up the entrainment, for example light/dark, which will link to a central oscillator which in turn is coupled to produce the circadian rhythms that are

**Figure 1.1 Qualitative representation of a circadian rhythm**

Figure 1.1 is a representation of a circadian rhythm, where an unknown variable is showing a repetitive cycle with a period length of about 24 hours. This pattern is continuing in constant conditions.



identified. The implication is that biological functions become entrained to environmental variables. In the case of endogenous rhythmic functions, the synchronization is by environmental stimuli, the rhythms will then persist when the stimuli are absent.

### 1.1.2 Occurrence of circadian rhythms

All eukaryotic organisms appear to show the existence of circadian rhythms. The classical rhythmical cases are in nyctinastic leaf movements (Bünning, 1956) of *Phaseolus multiflorus*, *Albizzia julibrissin* and *Samanea saman* (Satter and Galston, 1981), eclosion (adult emergence) and locomotor activity in *Drosophila pseudoobscura* and *Drosophila melanogaster* (Hall and Rosbash, 1987; Dunlap, 1990; Hardin *et al.*, 1990, 1992), of conidiation (asexual spore formation) in the fungus *Neurospora crassa* (Sargent *et al.*, 1966; Dunlap, 1990), bioluminescence of the living cells of the marine algae *Gonyaulax polyedra* (Walz *et al.*, 1983; Morse *et al.*, 1990) and the rhythms of carbon dioxide metabolism in the plant *Bryophyllum fedtschenkoi* (Wilkins, 1959, 1962, 1983). In fact in *Gonyaulax polyedra* circadian rhythms are also displayed in cell division, photosynthetic capacity and in patterns of motility. Circadian rhythms have also been found in man, with sleep patterns (Folkard *et al.*, 1985; Winfree, 1987, 1991; Honma *et al.*, 1992) and in a range of physiological and biochemical processes, and in hamsters (*Mesocricetus auratus*) with wheel running (Mrosovsky and Salmon, 1987). In plants circadian rhythms may help coordinate metabolic functions with periods of photosynthesis and may provide a mechanism to achieve detection of season in photoperiodism. A number of higher plants exhibit a circadian rhythm in the transcription of *CAB* genes (Taylor, 1989; Giuliano *et al.*, 1988; Kloppstech, 1985). The

photoreceptor involved in the entrainment of these rhythms, which involve light/dark cycles, is probably phytochrome.

The occurrence of circadian rhythms in the functioning of chloroplasts implied that a circadian clock like pattern might also occur in prokaryotic organisms. The simple reasoning behind this was that these organelles were believed to have evolved from prokaryotic ancestors. Indeed, a circadian rhythm was recently found in a prokaryote. Grobbelaar *et al.* (1986, 1992) showed that exposure of the cyanobacterium *Synechococcus* RF-1 to a light/dark regime resulted in an endogenous rhythm in nitrogenase activity that persisted for several days under continuous illumination. Since this discovery circadian rhythms of cell division and in amino acid uptake (Chen *et al.*, 1991) have been identified in this cyanobacterium. The fact that a circadian rhythm was only recently identified in a prokaryote was believed to be due to the fact that original studies followed the bacterial cell cycle under logarithmic growth conditions and subsequently the cell cycle seen was likely to be much shorter than 24 hours. Kondo *et al.* (1993) transformed a genetically tractable cyanobacterium (*Synechococcus* PCC7942) with bacterial luciferase genes, which functioned as a reporter of clock controlled expression of the endogenous *psb*AI gene. In this way they demonstrated that cyanobacteria exhibit circadian behaviour that was fundamentally similar to circadian rhythms of eukaryotes. A similar reporter system was used by Millar *et al.* (1992) with firefly luciferase and the plant *Arabidopsis thaliana*. Since circadian clocks were found in a prokaryotic organism it suggested that the circadian mechanisms must have evolved earlier than was previously assumed.

All of these cases show the presence of circadian rhythms in a large number of different organisms. Each case has been studied for different reasons, but the ultimate goal is to gain insight into the operation of the circadian clock. What is the oscillator itself, and where, how and why is it controlled? Further work on a wide range of organisms should reveal details of the operation of circadian clocks.

### 1.1.3 Analysis of circadian rhythms

How can a circadian rhythm be followed? The form of analysis will depend upon the factor to be measured for the periodic oscillation. A vast array of circadian rhythms have been identified, and because of this a number of methods are used to measure them. The approaches can use morphological, physiological, genetic/molecular or biochemical methods of study.

When investigating circadian rhythms Lumsden (1991) considers that the components of the oscillatory system must be assessed. He also states that three experimental approaches could be adopted in the analysis of circadian rhythms; inhibit the oscillator and locate the site of action of the inhibitor; identify the components of the actual rhythm and work backwards along the transduction pathway to locate the oscillatory mechanism; or identify the photoreceptor, which is responsible for light entrainment, and work forwards along the transduction pathway to locate the oscillatory mechanism.

On a biochemical level the assessment of circadian rhythms may be a rather complex process. However, biochemical studies on circadian rhythms have basically used two approaches. The investigator has

assayed a biochemical parameter, which showed a circadian pattern, and then investigated the mechanisms that control this parameter. The transduction pathway affecting these mechanisms are then studied with the hope of eventually finding the primary oscillator. For example the occurrence of rhythms in *Gonyaulax polyedra* has been extensively studied on a biochemical level (Morse *et al.*, 1990), as it has in *Bryophyllum fedtschenkoi* (Wilkins, 1992) and in the occurrence of rhythms in higher plants gene expression (Beator and Kloppstech, 1994; Piechulla, 1993). An alternative approach uses various inhibitory mechanisms, such as drugs, on the organism. Their effects on the rhythm are determined. It is hoped that this will allow identification of a cellular component that is closely linked to the oscillator. Winfree (1986) discussed the effects of benzodiazepines, in relation to the discovery of an elusive jet-lag pill, on the rhythm of wheel running in hamsters and found that it affected the phase and period of the rhythm. There are a number of interpretation problems with this approach, such as the possibility that the drug affects a system that blocks the rhythm's expression rather than a component of the oscillator.

The use of genetic systems for analysing circadian clocks was developed to complement the work of biochemical studies. The study of mutants has become an important tool in scrutinizing biological systems and has also become important as increasing attention focussed on molecular events in the signal transduction processes of numerous organisms. The genetic approach to understanding the control of circadian rhythms has resulted in the isolation of a number of circadian mutants. Clock mutants show changes in the free running period length, phase, or sensitivity to environmental factors. Consequently, the study of clock mutants has led to the analysis of the rhythms on a genetic level, a physiological level and

on a molecular level. A high proportion of clock mutants have appeared at the same few genetic loci, in particular the *frq* locus in *Neurospora* and the *per* locus in *Drosophila* (Dunlap, 1990; Hall and Rosbash, 1987). The use of clock mutants in neurobiological studies has shown that the expression of a specific gene is required for the production of a circadian rhythm in a specific organism. Moses and Ashburner (1985) discussed how the transplantation of brains between *Drosophila* flies, which differed in their genotype at the *per* locus, determined the clock periodicity of the phenotype. In wheel running hamsters, Mrosovsky (1989) discussed how the circadian-activity rhythms of the wild type was shortened. This was achieved by replacing the suprachiasmatic nucleus (SCN), in the brain, with the SCN of a mutant, known as the *tau* mutant, that had an abnormally short free running circadian period. Therefore the cells or tissues were responsible for the particular behaviour shown. Bargiello *et al.* (1984) were able to restore circadian rhythmicity of eclosion and locomotor activity in the *Drosophila melanogaster* $per^{o}$ mutant fly, which was arrhythmic, simply by introducing a specific DNA fragment from a wild type *Drosophila melanogaster* into the genome of the arrhythmic mutant. Other mutants that have been isolated include biochemical mutants and drug resistant mutants. In these cases the alterations of the rhythmical functions analysed have been related to the effects that a metabolic or chemically induced inhibition have in the operation of the clock mechanism. Feldman (1982) has reviewed the circadian clocks and mutants of the organisms *Neurospora crassa*, *Chlamydomonas reinhardtii* and *Drosophila* from which most of the information has been gained.

## 1.2 *Bryophyllum fedtschenkoi* and the CAM cycle

The original work on the plant *Bryophyllum fedtschenkoi* was carried out by Wilkins (1959). In the study of endogenous rhythms of $CO_2$ metabolism Wilkins required a slowly growing tissue that had sufficient food reserves, which would sustain the tissue for several days in darkness, and consequently he focussed his attention upon the excised leaves of succulent plants. On account of its leaf size and perennial habit *Bryophyllum fedtschenkoi* proved to be the most suitable out of all the plant species examined.

### 1.2.1 Crassulacean acid metabolism

*Bryophyllum fedtschenkoi* (Figure 1.2) is a member of the plant family *Crassulaceae*. It is a succulent plant and is found growing in semi-arid regions with a distinct day and night temperature variation. In this environment it has evolved an adaptation to save water in which the stomata open only at night. Its own appearance shows this by its tough fleshy-like leaves. This plant uses the photosynthetic process of crassulacean acid metabolism (CAM). There are 25 to 30 flowering plant families that have species showing CAM. In the family *Crassulaceae* nearly all are CAM plants whereas in other plant families the trend is that only the succulent species show the utilisation of CAM. However, there are cases of plants that grow in aquatic environments (*Isoetes*), which are low in $CO_2$, that still utilise CAM. Therefore, CAM is not just an adaptation that is only related to arid environments (water saving). CAM is defined as a massive diurnal fluctuation of titratable acidity that is normally accounted for by malic acid (Ting, 1985). In general CAM plants show diurnal rhythms of carbon dioxide fixation (Osmond and

**Figure 1.2 Photograph of the plant *Bryophyllum fedtschenkoi***

The plant *Bryophyllum fedtschenkoi* shown in the photograph was grown in the conditions described in section 2.2.

Holtum, 1981; Kluge *et al.*, 1992). At night the carbon dioxide is fixed into malate via phosphoenolpyruvate carboxylase (PEPc) and during the day the carbon dioxide is released from the malate and refixed photosynthetically (Figure 1.3b). The malate produced during the nocturnal $CO_2$ fixation is transported to storage vacuoles which accumulate malate as malic acid. During the day the vacuole store releases the malic acid as malate which is decarboxylated producing free $CO_2$ and pyruvate (or another 3-carbon fragment). The pyruvate is eventually returned to form storage carbohydrate in the plant. In CAM plants, as the respiratory $CO_2$ is recycled through the dark fixation pathway and photosynthesis, water is retained, photoinhibition is avoided and, even though there is no net $CO_2$ exchange, an active photosynthetic system is still maintained.

The enzyme phosphoenolpyruvate carboxylase (PEPc) is an important site in the regulation of CAM, as it catalyses the key step of $CO_2$ fixation at night. It accounts for the organic acid fluctuation. There are distinct day and night reactions that occur in CAM and the PEPc must be inactive during the day in order to prevent the occurrence of futile cycling. The enzyme is subject to feedback inhibition by malate. As PEPc is a cytosolic enzyme it was proposed that the regulation of the enzyme was by the periodic accumulation of malate in the cytoplasm (Osmund and Holtum, 1981). Ting (1985) argued that the changing properties of PEPc through a diurnal cycle might regulate CAM metabolism.

The biochemical basis of CAM involves a complex interplay of a number of enzymes. These control the metabolism of $CO_2$ such that specific reactions occur at particular periods of the day/night cycle. Therefore, it

is of no surprise that a number of rhythmical cycles occur in CAM plants such as *Bryophyllum fedtschenkoi*.

### 1.2.2 The circadian rhythms of *Bryophyllum fedtschenkoi*

#### 1.2.2.1 The rhythms of $CO_2$ metabolism

Previous analytical studies found that detached leaves of the plant *Bryophyllum fedtschenkoi* display circadian rhythms of carbon dioxide metabolism under two types of constant environmental conditions. Wilkins (1959, 1960) originally discovered that in continuous darkness and a $CO_2$ free air stream at temperatures between 10°C and 30°C, a rhythm of $CO_2$ gaseous exchange continued for about 3 to 4 days. Subsequent work of Wilkins (1984), and Anderson and Wilkins (1989a, 1989b) showed that in continuous light and a stream of normal air within the same temperature range a rhythm of $CO_2$ gaseous exchange continued for about 10 days. However, the period length of this rhythm was shorter than that seen in darkness and a $CO_2$ free air stream. The two rhythms showed different responses to temperature, in that under continuous light the period increased with temperature (Anderson and Wilkins, 1989c) whereas in continuous darkness and a stream of $CO_2$ free air the period decreased as the temperature rose (Wilkins, 1962). In each case these results indicated that a temperature compensating mechanism was functioning although not perfectly. The occurrence of the rhythms of $CO_2$ metabolism has been attributed to changes in flux through the enzyme PEPc (Bollig and Wilkins, 1979). This enzyme was responsible for the fixation of internal $CO_2$ that would otherwise be released.

A number of crassulacean plants have shown such rhythms. Wilkins (1959) found that detached leaves of the crassulacean plants *Bryophyllum daigremontianum*, *Bryophyllum calycinum* and *Sedum praealtum* when placed in continuous darkness and $CO_2$ free air also showed clearly defined circadian rhythms in their $CO_2$ gaseous exchange. Buchanan-Bollig (1984) showed that a rhythm of $CO_2$ assimilation occurred in leaves of *Kalanchoë blossfeldiana* that were kept in continuous light and normal air.

The circadian rhythms of $CO_2$ metabolism in *Bryophyllum fedtschenkoi* were inhibited when the leaves were studied at extreme high or low temperatures. In the case of the rhythm of $CO_2$ gaseous exchange of detached *Bryophyllum fedtschenkoi* leaves kept in darkness and $CO_2$ free air, Wilkins (1962) showed that the rhythm was inhibited by exposure to extreme temperatures (2°C or 36°C). The rhythm returned when the temperature was reduced to 26°C or 16°C, with the first peak occurring after a specific time had passed. Wilkins (1962) found that 36°C treatments of a few hours' duration shifted the phase of the rhythm when given at peaks of $CO_2$ fixation whereas low temperature stimuli of 2°C induced phase shifts at the peaks of $CO_2$ output. The rhythm of $CO_2$ gaseous exchange in leaves kept in $CO_2$ free air and continuous darkness were also inhibited by prolonged exposure to light (Wilkins, 1959, 1960) and showed phase shifts when applied at peaks of $CO_2$ fixation. Therefore, light and high temperature treatments had similar effects on the oscillating mechanism.

Anderson and Wilkins (1989a, 1989b) showed that the rhythm of $CO_2$ gaseous exchange in continuous light and normal air at 15°C was inhibited by prolonged exposure to high or low temperature, darkness or 5 % $CO_2$.

In these environmental conditions it was found that the rhythm was inhibited in such a way that the oscillator was driven to and held at fixed phase points in the cycle. A prolonged exposure of the leaves to 40°C and 2°C fixed the phases of the rhythms at points in the cycle that differed by 180°. When the leaves were removed from the inhibitory condition and returned to constant conditions the rhythm began again, but the phase of the rhythm was determined by the time the transfer was made since the first peak occurred a specific time after the transfer. Anderson and Wilkins (1989a, 1989b) found that exposures to 2°C, darkness and 5 % $CO_2$ held the oscillator to the same fixed phase point in the cycle. This was characterised by the leaves having a high malate content whereas a low malate content was found in leaves after exposure to 40°C.

Anderson and Wilkins (1989a, b, c) attributed phase control in continuous light and normal air by the stimuli high or low temperature, darkness and high $CO_2$ concentrations to changes induced in the rate of synthesis and metabolism of malate. However, Wilkins (1983, 1989) argued that phase control in continuous darkness and $CO_2$ free air, by light or high temperature was due to the redistribution of malate between the vacuole and the cytoplasm in the leaves caused by the opening of gates in the tonoplast. Mechanisms for the generation of the rhythms in $CO_2$ gaseous exchange in *Bryophyllum fedtschenkoi* in the constant conditions of continuous light and normal air or continuous darkness and $CO_2$ free air were suggested by Bollig and Wilkins (1979), and Wilkins (1984). They proposed that the generation of the rhythm was due to the periodic synthesis of malate in the cytoplasm and its removal, either to the vacuole in continuous darkness or by metabolic breakdown in continuous light.

It was discovered that the phase of the rhythms of *Bryophyllum fedtschenkoi* could be reset by varying a specific environmental factor at a specific point of the cycle. In a number of organisms which express circadian rhythms, the phase of a rhythm can be altered (advances or delays) by the same kinds of cues that affect entrainment (Sargent *et al.*, 1966; Chen *et al.*, 1991). In *Bryophyllum fedtschenkoi* leaves kept in $CO_2$ free air and continuous darkness the phase of the rhythm could be altered by exposing the leaves to a number of treatments; by raising or lowering the temperature for a few hours (Wilkins, 1962) and by exposure to light (Wilkins, 1960; Harris and Wilkins, 1978). In the case of leaves kept in continuous light and normal air phase shifts in the rhythm were induced by brief exposures to 40°C, 2°C, darkness and 5 % $CO_2$ (Anderson and Wilkins, 1989c). In both the constant conditions of $CO_2$ free air and continuous darkness, and normal air and continuous light, the size and direction of the shift induced were dependent on a number of factors. The length of the treatment, the position in the cycle at which it was applied and, in the case of light treatments, the wavelength used were important in this control. This was similar to circadian rhythms in other organisms. Anderson and Wilkins (1989c) found that maximum phase shifts occurred when *Bryophyllum fedtschenkoi* leaves, kept in continuous light and normal air, were exposed to 40°C for a period of 4 hours at points when $CO_2$ fixation was greatest. There was no effect on the phase of the rhythm, from this latter treatment, when it was applied at a period of peak $CO_2$ output. However, in the case of 4 hour treatments with darkness, 5 % $CO_2$ or a temperature of 2°C, there was no effect on the rhythm when the application was at a peak of $CO_2$ fixation but they showed maximum phase shifts when this fixation was at a trough. Therefore, treatments

applied at one position in the cycle induce no phase shift, while those applied at all other positions induce phase shifts of varying size.

The rhythm of $CO_2$ gaseous exchange seen in *Bryophyllum fedtschenkoi* leaves kept in $CO_2$ free air and continuous darkness when exposed to light for a period of 3 hours showed maximum phase shifts if the application was at a point of maximum $CO_2$ fixation (Wilkins, 1960). Wilkins (1962, 1983) found that the effects of varying the temperature (either 35°C or 2°C treatments) for periods of 3 to 4 hours, on the phase of the rhythm showed similar results to those seen in *Bryophyllum fedtschenkoi* leaves kept in continuous light and normal air. Wilkins (1960, 1989) and Harris and Wilkins (1978) discovered that the photoreceptor involved in the regulation of the rhythm in *Bryophyllum fedtschenkoi* leaves was the chromoprotein phytochrome. Initially Wilkins (1962) found that the rhythm of $CO_2$ gaseous exchange in leaves kept in continuous darkness and $CO_2$ free air was completely reset by a 6 hour red light treatment applied at a point of maximum $CO_2$ fixation. This result was similar to the effects of high temperature and light on the rhythm of $CO_2$ gaseous exchange. Blue light was found to have no effect on the rhythm. Harris and Wilkins (1978) found that entrainment of the rhythm, in continuous darkness and $CO_2$ free air, was most effective in the red region of the spectrum when leaves were exposed to 15 minutes of red light every 24 hours. They also found that the effects of red light were reversed by far red light. Experiments in which *Bryophyllum fedtschenkoi* leaves were exposed to a single red light pulse each day followed by an exposure to far-red light prevented the clear entrainment of the rhythm that was obtained with the red light alone. These results implied that phytochrome was involved in the photocontrol of the rhythm in *Bryophyllum fedtschenkoi*. Simon *et al.* (1976) and Roenneberg and

Hastings (1991) have shown that red light pulses affected the circadian rhythms seen in *Samanea saman* and *Gonyaulax polyedra*, respectively.

Wilkins (1991) examined the role of the epidermis in the generation of the circadian rhythm of $CO_2$ gaseous exchange in *Bryophyllum fedtschenkoi* leaves. When small pieces of *Bryophyllum fedtschenkoi* mesophyll, with their upper and lower epidermis removed, were placed in conditions of $CO_2$ free air and continuous darkness at a temperature of 26°C, Wilkins (1959) found that a circadian rhythm of $CO_2$ gaseous exchange occurred. The response of the rhythms of mesophyll to light was identical to that seen in whole leaves. However, Wilkins (1991) later concluded that the circadian rhythm of $CO_2$ exchange in leaves of *Bryophyllum fedtschenkoi* was dependent on the presence of the epidermis, except in the particular conditions mentioned above. Wilkins (1991) found that the stomata were essential for the rhythm of $CO_2$ gaseous exchange in conditions of constant light and normal air and suggested that the rhythm may be controlled by the stomatal guard cells.

### 1.2.2.2 The biochemical analysis of the control of $CO_2$ metabolism in *Bryophyllum fedtschenkoi*

Regulation of PEPc plays a central role in the control of CAM. It is the key enzyme in the nocturnal fixation of carbon dioxide and to avoid futile cycling must be inactive during the day. It has been mentioned that the rhythm of $CO_2$ metabolism was attributable to the periodic activity of PEPc, and that the enzyme can be inhibited by malate in a negative feedback mechanism. Bollig and Wilkins (1979) showed that the activity of freshly prepared crude extracts of PEPc from leaves of *Bryophyllum fedtschenkoi* was inhibited by malate. Therefore, the periodic

accumulation of malate in the cytoplasm was believed to regulate the PEPc enzyme activity. Subsequently, a periodic change in the sensitivity of PEPc to malate, the end product of the dark $CO_2$ fixation reactions, was found to occur. Nimmo *et al.* (1984, 1986) showed that the properties of purified PEPc from *Bryophyllum fedtschenkoi* went through marked changes between the night and day. The "night" form, which had a $K_i$ of 2.5-3.0 mM for malate, was found to be much less sensitive to feedback inhibition by malate as compared to the "day" form that had a $K_i$ of 0.3 mM for malate. Both interconversions between the night and day forms of PEPc occurred during the dark period, about 4-5 hours after the end of one light period and 1-2 hours before the start of the next. Nimmo *et al.* (1986) found that both purified forms of PEPc contained a minor protein of subunit $M_r$ 123 000 and a major protein of subunit $M_r$ 112 000. Immunoprecipitation and labelling studies of *Bryophyllum fedtschenkoi* leaves with $^{32}P$ showed that only the night form of PEPc contained $^{32}P$. The phosphate present in the night form of PEPc was covalently bound to serine. The implication of these results were that the activity of the *Bryophyllum fedtschenkoi* enzyme PEPc was likely to be controlled by a covalent modification mechanism and not solely by a diurnal malate rhythm.

There is a degree of evidence which indicated that the reversible phosphorylation of PEPc was involved in the generation of the rhythms of $CO_2$ gaseous exchange in *Bryophyllum fedtschenkoi* and controlled by an endogenous clock. In the normal diurnal cycle the period during which PEPc was phosphorylated, as judged by a high $K_i$ for malate, was the same as the period during which malate was accumulated. As noted above, Nimmo *et al.* (1984) showed that this was not just a light controlled response as the night form of PEPc was found to be

dephosphorylated 1 to 2 hours before the beginning of the light period and phosphorylation started several hours after its end. Nimmo *et al.* (1987) found that in the constant conditions of continuous darkness and $CO_2$ free air, and continuous light and normal air, the phosphorylation state of the PEPc showed a pronounced circadian rhythm. In continuous darkness and $CO_2$ free air the periods where PEPc was phosphorylated correlated with the periods when there was highest flux through the enzyme.

The regulation of the kinase and phosphatase which phosphorylate and dephosphorylate the PEPc clearly plays a key role in the regulation of CAM, and of the diurnal and circadian rhythms of PEPc activity in *Bryophyllum fedtschenkoi*. Carter *et al.* (1990, 1991) found that *Bryophyllum fedtschenkoi* leaf tissue contained protein phosphatase and kinase activity. When the partially purified plant protein phosphatase, which consisted of type 1 and type 2A phosphatases, was incubated with the purified night form of PEPc, there was an increase in the sensitivity of the enzyme to inhibition by malate. Carter *et al.* (1990) showed, through the use of inhibitors of type 1 and type 2A phosphatases (inhibitor 2 and okadaic acid respectively), that protein phosphatase type 2A was responsible for dephosphorylating the night form of *Bryophyllum fedtschenkoi* PEPc. A partially purified protein kinase from *Bryophyllum fedtschenkoi* leaves phosphorylated PEPc (day form) *in vitro* and caused a decrease in the sensitivity of the PEPc to inhibition by malate. The $K_i$ of the PEPc for malate was found to increase from 0.48 mM to 2.7 mM. Carter *et al.* (1991) prepared *Bryophyllum fedtschenkoi* leaf extracts throughout the normal diurnal cycle and with leaves that were placed in continuous darkness and $CO_2$ free air. The leaves were assayed for PEPc kinase activity and for PP2A. The activity of the kinase was controlled in

a circadian fashion; during the diurnal cycle the kinase activity appeared 4-5 hours after the start of darkness and disappeared 2-3 hours before the end of darkness, while in constant environmental conditions the kinase activity displayed circadian oscillations. The activity of the protein phosphatase type 2A showed no such oscillation. Therefore, it was likely that the endogenous rhythm in the phosphorylation state of PEPc, in *Bryophyllum fedtschenkoi* leaves, was due to the periodic activities of the PEPc kinase and the uniform activity of the PP2A during the 24 cycle.

Bollig and Wilkins (1979) showed that cycloheximide and 2,4-dinitrophenol inhibited the rhythm of $CO_2$ gaseous exchange in *Bryophyllum fedtschenkoi* leaves kept in constant darkness and $CO_2$ free air. They found that cycloheximide inhibited $CO_2$ fixation and malate accumulation. Lumsden (1991) stated that the application of inhibitors such as cycloheximide indicated that protein synthesis on 80S ribosomes was important for the function of some circadian oscillators. Bollig and Wilkins (1979) interpreted the effect of cycloheximide on the abolition of circadian rhythms of $CO_2$ gaseous exchange in terms of an effect on the properties of the tonoplast. However, Carter *et al.* (1991) showed that both cycloheximide and puromycin (both protein synthesis inhibitors) blocked the nocturnal appearance of PEPc kinase activity, and the rhythms of $CO_2$ metabolism when taken up by detached leaves of *Bryophyllum fedtschenkoi*. The PEPc remained in its malate sensitive dephosphorylated day form. The inhibitors did not drastically disrupt the leaf cell metabolism as judged by the fact that the leaves were still capable of photosynthesis. This implied that protein synthesis was required to produce the circadian rhythms of this plant. The PEPc kinase may be periodically synthesised and degraded in a circadian oscillation under constant environmental conditions. It should be noted that the use of

protein synthesis inhibitors does not always reset or block circadian rhythms. Dunlap and Feldman (1988) found that in the *Neurospora* strain *frq*-7, which is a clock mutation, treatment with cycloheximide inhibited protein synthesis without affecting the circadian clock. They suggested that inhibition of protein synthesis caused phase shifts because it lowered the concentration of a labile protein(s) that was required at specific phases of the circadian cycle and not because synthesis of such proteins was part of the basic feedback mechanism.

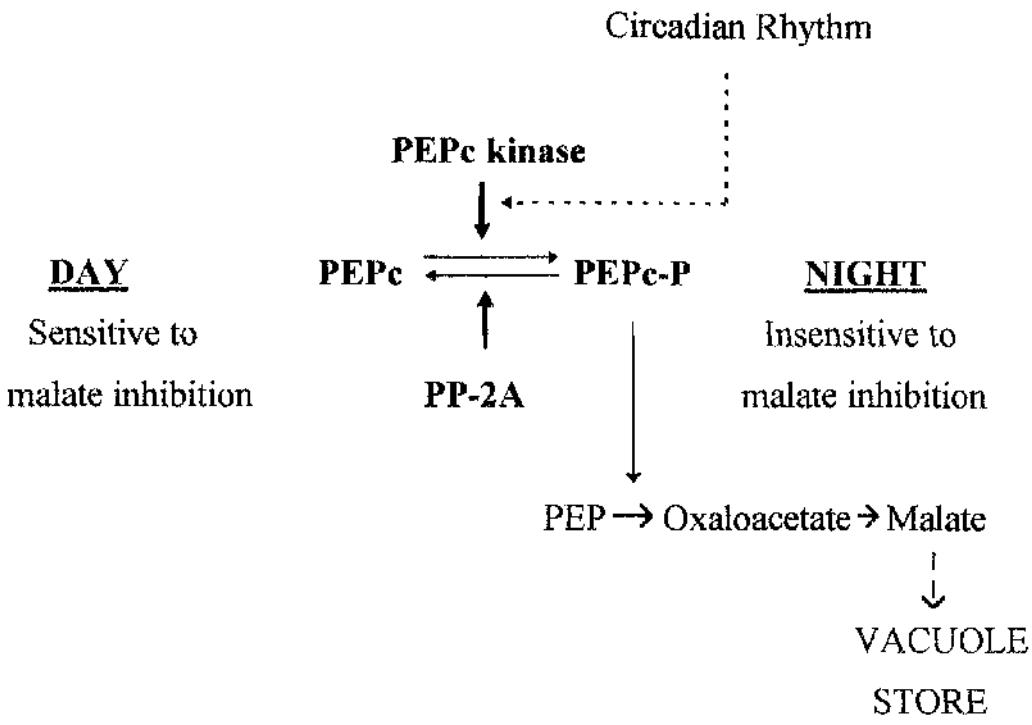
In summary, PEPc is an allosteric enzyme, which is subject to feedback inhibition by malate, where phosphorylation reduces its sensitivity to malate. Therefore, the evidence links the rhythms of carbon dioxide metabolism in the plant *Bryophyllum fedtschenkoi* with the rhythms of PEPc phosphorylation. In *Bryophyllum fedtschenkoi* the PEPc is regulated by reversible phosphorylation in response to a circadian rhythm. This enzyme's regulation plays a central role in the control of CAM. The enzyme is controlled by feedback inhibition by malate, the degree of sensitivity depending upon its state of phosphorylation. An endogenous clock controls these rhythms of phosphorylation, by affecting the activity of a protein kinase. Figure 1.3a summarises this picture.

Similar control of PEPc has been found in other systems. A parallel system of PEPc control was found in the $C_4$ photosynthetic plants *Zea mays* and *Sorghum*. McNaughton *et al.* (1991) found a PEPc kinase in *Zea mays* leaves that could phosphorylate and reduce the malate sensitivity of PEPc. The *Zea mays* PEPc was dephosphorylated by a type 2A phosphatase. The activity of this kinase appeared after illumination and this led to the belief that the phosphorylation state of PEPc was controlled by the light-stimulated activation of the PEPc kinase. In the

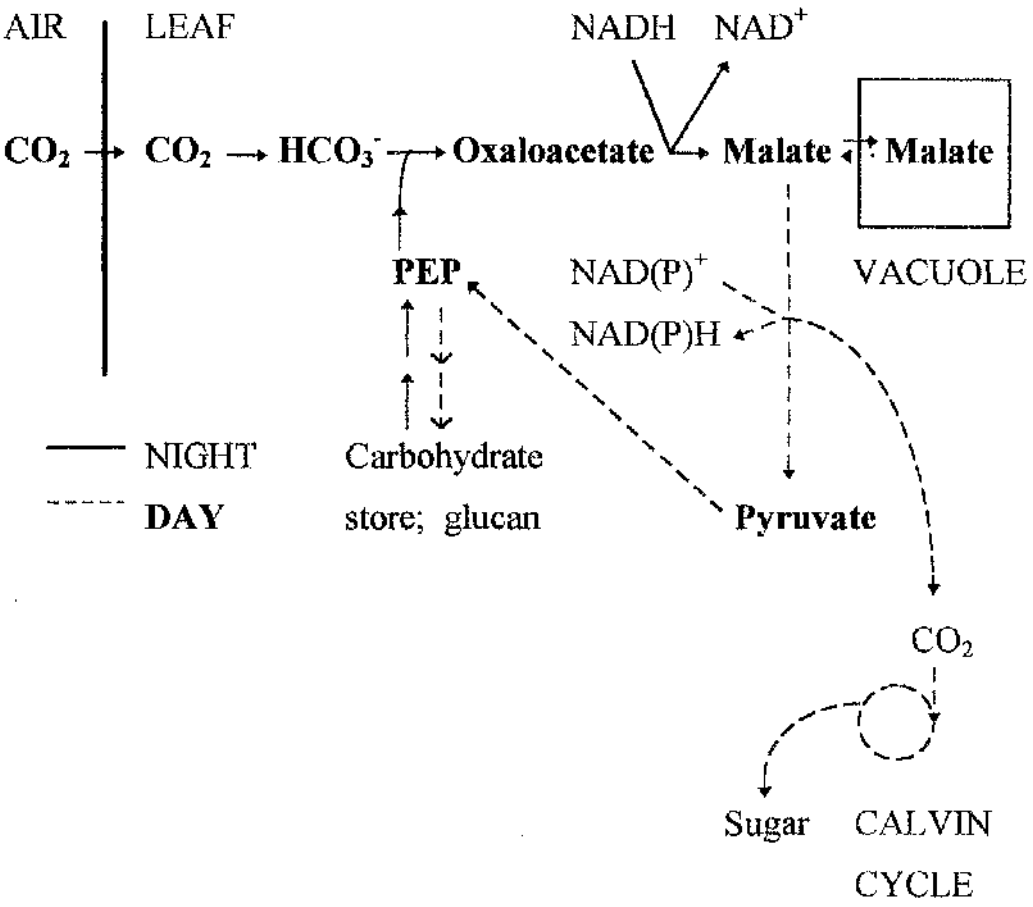
**Figure 1.3 Sequence of events occurring within Crassulacean acid metabolism (CAM) of *Bryophyllum fedtschenkoi***

Phosphoenolpyruvate carboxylase (PEPc) is the central enzyme in carbon dioxide fixation. Figure 1.3a shows the PEPc covalent modification via its state of phosphorylation. The state is controlled by a PEPc kinase, which follows a circadian rhythm, and a phosphatase (PP-2A). Figure 1.3b represents the network of reactions in the day/night cycle in CAM. The night and day reactions are labelled accordingly.

**a.**


Circadian Rhythm
PEPc kinase
DAY
Sensitive to
malate inhibition
PEPc
PEPc-P
PP-2A
NIGHT
Insensitive to
malate inhibition
PEP → Oxaloacetate → Malate
VACUOLE
STORE

**b.**


AIR
LEAF
NADH
NAD+
CO2
CO2
HCO3-
Oxaloacetate
Malate
Malate
VACUOLE
PEP
NAD(P)+
NAD(P)H
NIGHT
DAY
Carbohydrate
store; glucan
Pyruvate
CO2
Sugar
CALVIN
CYCLE

case of PEPc isolated from *Sorghum* leaves and protoplasts, which were from mesophyll cells, Bakrim *et al.* (1992) and Pierre *et al.* (1992) found a similar system of control as occurred in the *Zea mays* leaves. Light activated a *Sorghum* PEPc kinase that phosphorylated the PEPc. Jiao and Chollet (1992) found that the light induced phosphorylation of C4 PEPc occurred at a specific N-terminal seryl residue (Ser-15 or Ser-8 in the *Zea mays* or *Sorghum* enzymes, respectively) and this also resulted in a decrease in malate inhibition. Cycloheximide and a number of photosynthesis related inhibitors were found to block the light activation of the PEPc kinases in *Zea mays* and *Sorghum* (Jiao *et al.*, 1991; Jiao and Chollet, 1992; Bakrim *et al.*, 1992; Pierre *et al.*, 1992). The effects of these inhibitors implied that protein turnover was involved as part of the mechanism that controlled the reversible light activation of the PEPc kinase. It was also thought that some photosynthesis-related signal, which originated from the Calvin cycle, interacted with the protein synthesis system and ultimately brought about the light activation of the PEPc kinase. In comparison to *Bryophyllum fedtschenkoi*, the components of the signal transduction pathway seemed to be the same, but they responded to light/darkness rather than an endogenous clock.

Recently Carter *et al.* (1995a, 1995b) have assessed the role of temperature in the regulation of the circadian rhythm of $CO_2$ gaseous exchange in *Bryophyllum fedtschenkoi*. It was found that temperature could affect the activity of *Bryophyllum fedtschenkoi* enzyme PEPc in three ways (Carter *et al.*, 1995a); increasing temperature increased the catalytic activity of the enzyme; temperature altered the activity of the PEPc kinase and subsequently affected the phosphorylation state of the PEPc enzyme; the allosteric properties of the enzyme were affected in that increasing temperature increased the sensitivity of the enzyme to

malate inhibition. They suggested that these effects of temperature were important in the control of CAM in *Bryophyllum fedtschenkoi* in that the cold nights and warm days favour the patterns of malate accumulation and breakdown required for CAM. The circadian rhythms of $CO_2$ gaseous exchange in leaves of *Bryophyllum fedtschenkoi* showed temperature compensation within the range of 10°C to 30°C, but outside this range the rhythm was inhibited. This occurrence clearly indicated that there was a complex role of temperature in regulating the processes involved in the generation of the rhythm. In order to achieve temperature compensation in a circadian rhythm there must be one enzyme the *in vivo* activity of which can compensate for changes in temperature, such that the overall system is relatively unaffected. In *Bryophyllum fedtschenkoi* the integrated activities of the PEPc, PEPc kinase and protein phosphatase 2A, and the allosteric, feedback inhibition by malate, were likely to control the periodicity of the $CO_2$ rhythm and could account for it being independent of temperature. Carter *et al.* (1995a) suggested that there were compensating effects of temperature on the carboxylating and decarboxylating reactions that synthesise and degrade malate. Carter *et al.* (1995b) also showed that *Bryophyllum fedtschenkoi* leaves kept in normal air and continuous darkness at a constant temperature of 30°C exhibited a circadian rhythm in their $CO_2$ exchange. At this temperature there was no PEPc kinase activity. This implied that the oscillation of PEPc activity was due to the rhythmic changes in the cytosolic malate levels. Therefore, the theory of Wilkins (1962, 1983) that the tonoplast in *Bryophyllum fedtschenkoi* leaf cells became increasingly permeable to malate at temperatures above 30°C must

account for the loss of circadian rhythmicity as the temperature increased above this value.

## 1.3 Chlorophyll a/b binding proteins and the biological clock

Many genes in higher plants are expressed in a highly regulated fashion (Beator and Kloppstech, 1994; Kuhlemeier, 1992; Thompson and White, 1991). Certain genes are expressed only at specific stages of development and only in certain cell types and their expression is regulated by various environmental stimuli. Light controls plant development at various levels of gene expression. A result of this is that photosynthesis specific genes have been used as model systems to study gene regulation in higher plants. Consequently, many investigators have focussed on understanding the mechanisms that regulate *CAB* gene responsiveness to light. Other studies have focussed on the expression of *CAB* genes during plant development and the organisation and evolution of the *CAB* gene families. These studies can be used in furthering the analysis of circadian rhythms, phytochrome-regulated gene expression and promoter function in plants.

There are a number of plants that are known to exhibit circadian rhythms in the expression of the genes for chlorophyll a/b binding proteins (*CAB* genes). A few examples, which show circadian rhythms in mRNA accumulation, are *Lycopersicon esculentum* (Piechulla *et al.*, 1988, 1989; Piechulla and Gruissem, 1987), *Triticum aestivum* (Nagy *et al.*, 1988, 1990), *Pisum sativum* (Kloppstech, 1985; Adamska *et al.*, 1991), *Nicotiana tabacum* (Paulsen and Bogorad, 1988), and *Arabidopsis thaliana* (Millar and Kay, 1991). The list is ever growing, for instance; Reimann and Dudler (1993) identified a novel light

regulated gene (*lir*1), from rice, whose expression pattern showed similarities to certain members of CAB proteins in that it was controlled by light and a circadian clock, but they have not been able to classify this gene or identify its biological function. Circadian regulation of expression has been demonstrated for other photosynthetic genes encoding products required for carbon fixation, such as *RBCS*, which encodes the Rubisco small subunit (Kloppstech, 1985; Spiller *et al.*, 1987; Giuliano *et al.*, 1988; Otto *et al.*, 1988; Adamska *et al.*, 1991; Pilgrim and McClung, 1993), and *RCA*, encoding Rubisco activase (Martino-Catt and Ort, 1992; Pilgrim and McClung, 1993; Watillon *et al.*, 1993). However, the circadian expression of mRNA from *RBCS* genes has been found to be dependent upon the plant, since no such rhythms have been found in *Triticum aestivum*, *Petunia* (Mitchell) and *Nicotiana tabacum*. In the plant *Pisum sativum*, Otto *et al.* (1988) and Adamska *et al.* (1991) also found that the level of mRNA for the early light inducible protein (ELIP) was regulated in a circadian fashion.

In some plants circadian expression has been demonstrated for genes whose products have non-photosynthetic roles; for example, nitrate reductase, catalase and heat-inducible proteins (Otto *et al.*, 1988). Cheng *et al.* (1991) and Pilgrim *et al.* (1993), and Deng *et al.* (1990), showed that the levels of mRNA, protein and activity of nitrate reductase were mediated by a circadian rhythm in *Arabidopsis thaliana* and *Nicotiana tabacum*, respectively. Pilgrim *et al.* (1993) argued that the rhythms of nitrate reductase, in *Arabidopsis thaliana*, were controlled by a "gate" that opened and closed rhythmically and so governed the timing of light-regulated phenomena. Nagy *et al.* (1988) originally proposed this theory in relation to a phytochrome-regulated plant gene expression. Zhong *et al.* (1994) found that when etiolated *Arabidopsis thaliana*

seedlings were transferred to continuous light or dark, *CAT2* mRNA (catalase) showed circadian oscillations in its levels of abundance. These circadian oscillations of nitrate reductase and catalase expression still appeared to show a connection to the rhythms of the photosynthetic genes by the involvement of light and the circadian clock in their rhythms.

The circadian rhythms in mRNA levels are not unique to higher plants, but are also found in other organisms, such as *Neurospora crassa* (Loros *et al.*, 1989), *Drosophila melanogaster* (Hardin *et al.*, 1990, 1992), *Acetabularia mediterranea* (Driessche *et al.*, 1969), *Gonyaulax polyedra* (Walz *et al.*, 1983) and in lower vertebrates (Korenbrot and Fernald, 1989). One good example is the *per* gene in *Drosophila*. Hardin *et al.* (1990, 1992) and Takahashi (1992) showed that, in *Drosophila melanogaster*, the fluctuations in *per* gene mRNA abundance were influenced by its own translation product that also cycled in abundance. Therefore, they proposed that a feedback loop was in operation, where the *per* gene product regulated its own mRNA level and they predicted that a circadian clock-regulated transcription factor was responsible for the mRNA cycling. The *per* feedback loop had all the properties necessary to be a component of a circadian oscillator in *Drosophila*.

### 1.3.1 The structure and function of CAB proteins

All photosynthetic organisms that evolve oxygen have two photosystems (PSI and PSII). In green plants these systems consist of chlorophylls, which absorb light energy, that are non-covalently bound to specific proteins (chlorophyll-binding proteins) to form chlorophyll-protein complexes. In green plants two kinds of chlorophyll are found (chlorophyll a and chlorophyll b) and the chlorophyll-protein complexes

that form can either have chlorophyll a or both chlorophyll a and b attached. The light-harvesting chlorophyll a/b binding (CAB) protein of photosystem II (LHC II) is one of the most abundant proteins found in higher plants. In fact LHC II is the major chlorophyll a/b light harvesting complex of green plants, accounting for up to 50 % of the total chlorophyll in the thylakoid membrane (Green *et al.*, 1992).

Chlorophyll a/b binding proteins are encoded by the nuclear genome. Initially they are synthesised as precursor polypeptides in the cytoplasm. These pre-proteins are post-translationally modified upon transport into the chloroplast and the mature proteins are embedded within the thylakoid membrane. Post-translational modifications such as the attachment of pigments are critical for CAB protein accumulation and function (Thompson and White, 1991). Mature CAB proteins noncovalently bind with chlorophyll a, chlorophyll b and carotenoid pigment molecules to form pigment-protein complexes. The carotenoid molecules are thought to protect the chlorophyll-protein complexes from the damaging effects of light (Green *et al.*, 1991). A number of the post-translational steps in CAB protein biosynthesis are directly or indirectly regulated by light acting through a number of photoreceptors, such as phytochrome, and the process of chlorophyll synthesis.

The functions of these chlorophyll a/b binding protein-pigment complexes are to set up an effective system of harvesting light energy and to transfer this energy to the photosystem reaction centres (Chang and Walling, 1992a, 1992b). The chlorophyll a/b binding proteins are essentially acting as light antennae. Grana stacking is also mediated by these complexes. This connection to light suggests that these proteins may only be required during the light periods (Thompson and White, 1991). Light

is believed to regulate this process of transferal of energy by controlling phosphorylation and dephosphorylation of LHC II, a photoinhibitory system or by another form of regulation. Phosphorylation of certain CAB polypeptides found in LHC II redirects absorbed light energy between the reaction centres of photosynthetic membranes (Thompson and White, 1991; Allen, 1992). Therefore, the reversible phosphorylation observed can be looked upon as a short-term adaptive response that results in optimal energy capture and utilization under conditions of varying light intensities and wavelengths. In this way the function of photosynthesis is controlled.

The structure of a *Lemna gibba* chlorophyll a/b protein-pigment complex within the thylakoid membrane was proposed by Karlin-Neuman *et al.* (1985). They used a number of factors to predict the conformation of the mature polypeptide (LHC II) in the thylakoid membrane. They proposed that the amino terminus was located in the stroma, there were three membrane spanning domains (α-helices) and consequently, that the carboxyl terminus was located on the opposite side of the membrane. In the model proposed by Karlin-Neuman *et al.* (1985) there was a pattern of charged residues, which consisted of a series of negatively charged amino acids on the lumenal side and positively charged ones on the stromal side, around the hydrophobic membrane spanning sequence nearest to the carboxyl terminus. This pattern was believed to play a role in the initial insertion of the protein into the membrane and to lock it in the correct orientation. This model matched the three-dimensional structure of *Pisum sativum* LHC II (Allen, 1992) that had three membrane spanning helices but this structure also showed how 15 chlorophylls were interlinked into the conformation.

### 1.3.2 The multigene family of chlorophyll a/b binding proteins

The group of evolutionary and structurally related genes known as *CAB* genes encode the chlorophyll a/b binding proteins for the light harvesting systems in plant thylakoids. These proteins are encoded by a diverse, evolutionary related family of nuclear genes. These extended multigene families encode the polypeptides of LHC I and LHC II, the light harvesting photosystems I and II. In addition, a number of minor chlorophyll a/b binding proteins, CP29 and CP24 are encoded by *CAB* genes. Recently it has been discovered that other proteins not known to bind chlorophyll, the early light-inducible proteins (ELIP), are also related and could be considered as part of this family (Green *et al.*, 1991). All of these proteins, other than ELIP, form complexes with chlorophyll and arrange themselves within the thylakoid membrane so that an effective photosynthetic system operates.

CAB proteins have been isolated and characterised in several dicot (*Petunia*, *Lycopersicon esculentum*, *Nicotiana tabacum* and *plumbaginifolia*, *Arabidopsis thaliana* and *Pisum sativum*) and monocot (*Lemna gibba*, *Zea mays* and *Triticum aestivum*) species. A vast range of *CAB* genes have been sequenced. This showed that all of the chlorophyll a/b binding proteins shared some common amino acid sequences, which folded into the proposed structural model of Karlin-Neumann *et al.* (1985) for LHCII, and subsequently confirmed that CAB proteins comprise an extended family (Green *et al.*, 1991). This recent proliferation of reported *CAB* gene sequences confirmed a number of beliefs. First, these studies showed that *CAB* gene families range from 3 to 16 members (Walling *et al.*, 1988). Second, all members of the *CAB* gene family, with the exception of the LHC II Type I *CAB* genes, contain introns.

Third, at least 10 recognisably distinct types of CAB proteins throughout angiosperms and gymnosperms were identified (Jansson *et al.*, 1992; Green *et al.*, 1991). This latter finding has led to a new proposal, by Jansson *et al.* (1992), for a nomenclature for the genes encoding the chlorophyll a/b binding proteins of the light harvesting complexes of PSI and PSII. The genes encoding LHCI and LHCII proteins have been named *Lhca*1 to *Lhca*4, and *Lhcb*1 to *Lhcb*6, respectively. This system followed the number of distinct types of proteins that were identified in the different light harvesting antenna systems.

The comparison of the nucleotide sequences of the *CAB* multigene families has led to the discovery of family relations between the differing members of the *CAB* family in one particular plant species, and also of the phylogenetic relatedness between the CAB proteins of different plant species. Green *et al.* (1991), Demmin *et al.* (1989) and Walling *et al.* (1988) discuss this organisation and evolutionary relatedness of the *CAB* gene families. Walling *et al.* (1988) found that the *CAB* multigene family of *Glycine max* consisted of at least 11 genes. They characterised three members of this family, *CAB* 3, *CAB* 2 and *CAB* 1, that were associated with PSII. Comparison of the predicted amino acid sequences showed that *CAB* 3 and *CAB* 2 were 95 % similar. When these sequences were compared to other known CAB protein sequences the indication was that *CAB* 3 coded for the major protein of LHC II, *CAB* 2 for a novel protein and *CAB* 1 was determined to be a pseudogene. Demmin *et al.* (1989) went on to compare the predicted nucleic acid sequences of all of the PSII *Glycine max CAB* genes isolated (*CAB* 1 to *CAB* 5) and then further investigated the molecular evolution of the *CAB* gene family by comparing the nucleotide sequences from 25 different *CAB* genes from diverse phylogenetic taxa (monocot and dicot species). They concluded that, in

*Glycine max*, *CAB* 3 was an evolutionary distant member within that family and that the interspecies comparisons of *CAB* sequences conformed to existing morphological phylogenies as all the species within one family clustered together. They also showed that *CAB* type I and II genes originated before the divergence of the monocotyledon and dicotyledon plants. Green *et al.* (1991) summarised this information by stating that the two major LHC II polypeptides (Type I and II) were the most closely related members of the family, although the transit peptides tended to show far greater diversity than the mature polypeptides. The LHC I polypeptides, CP29 polypeptide and CP24 polypeptide shared a few characteristics with the LHC II polypeptides but were all distinctly different. Green *et al.* (1991) went on to conclude that the ELIPS were even more distant relatives of the *CAB* gene family, which may be involved in the synthesis of carotenoids, and that, due to the presence of conserved transmembrane helices, all of the members of the present *CAB* gene family were descended from an ancestral gene.

### 1.3.3 Sequence analysis of *CAB* genes

Many *CAB* genes have been sequenced and certain characteristic features identified. As has been mentioned the CAB proteins are initially synthesised as a precursor polypeptide that consists of the transit sequence, which is 30-35 amino acid residues in length, and the mature CAB protein of 235-240 amino acid residues (Brandt *et al.*, 1992; Knight *et al.*, 1992; Zhang *et al.*, 1991). Thylakoid membranes contain two light harvesting complexes, LHC I and LHC II, that are in close association with photosystems I and II respectively. At present four distinct types of CAB proteins have been identified in LHC I, three types in the major LHC II complex and also three minor components of PS II

(Green *et al.*, 1991; Jansson *et al.*, 1992). As has been previously mentioned, all Type I LHC II *CAB* nucleotide sequences have no introns.

Obviously, *CAB* gene sequences consist of a number of parts; the untranslated regions, and the sequences encoding the transit peptide and the mature polypeptide. Leutwiler *et al.* (1986) sequenced 3 *CAB* genes (AB165, AB180, AB140), which encoded the CAB protein of PSII, of *Arabidopsis thaliana*. All 3 genes contained an open reading frame, which started with a methionine, that contained 267 amino acid residues up to a translation termination codon (TGA). This was similar to other sequenced CAB proteins (Knight *et al.*, 1992; Cashmore, 1984). In the coding sequence, all three amino acid sequences were 100 % identical, except for the substitution of an asparagine for a lysine in the transit sequence of AB140. The genes were 96 % identical at the nucleotide level in the translated region. Demmin *et al.* (1989) found that, within the mature coding region, the amino acid sequence for *Glycine max CAB* 4 and 5 genes showed 98 % identity. In comparison to the findings of Leutwiler *et al.* (1986), the transit sequences of most *CAB* genes tended to show a greater degree of sequence divergence compared to the mature *CAB* sequences. This was mentioned by Knight *et al.* (1992) in reference to the *Zea mays CAB* genes sequenced. AB165 and AB140 also showed a high degree of homology in the noncoding region. This was not usually found in other sequenced *CAB* genes. The sequence divergence of the noncoding region was seen in a number of *Zea mays CAB* genes by Becker *et al.* (1992). Upstream of the transcription initiation site all 3 *Arabidopsis thaliana CAB* genes contained sequences that were characteristic of many eukaryotic promoters, and of many promoter regions of the *CAB* genes of other plant species. A TATA box and CAAT box were found. In contrast, Walling *et al.* (1988) and Demmin *et al.*

(1989) found the presence of more than one set of the above consensus sequences in members of the *Glycine max CAB* gene family.
This shows a general picture of the sequence of *CAB* genes.

### 1.3.4 The circadian rhythms of *CAB* gene expression

The expression of genes for chlorophyll a/b binding proteins under diurnal and circadian conditions has been thoroughly studied in a number of plant species. Kloppstech (1985) demonstrated that the mRNA levels of the nuclear genes *CAB*, *RBCS* and *ELIP* in *Pisum sativum* followed a diurnal and circadian pattern of expression. This phenomena was found to occur in a number of other plant species. The diurnal patterns of *CAB* gene expression, in both monocotyledonous and dicotyledonous plants (Meyer *et al.* 1989), followed a similar level of expression over the cycle. Maximum levels of *CAB* mRNA were detected in the middle of the light period or a few hours after the transition from dark to light, this level proceeded to decline as the dark period approached and then increasing levels were detected either a couple of hours before or right after the transition from dark to light. The presence of *CAB* mRNA levels before the start of the light period implied that some form of endogenous mechanism of control was in operation.

Piechulla and Gruissem (1987), and Piechulla (1988, 1989) found that circadian rhythms of *CAB* gene expression occurred in *Lycopersicon esculentum* fruits and leaves when placed in conditions of continuous light or dark. However, under conditions of continuous darkness the amplitude of the rhythm was found to dampen rapidly. This characteristic was also found to occur in a number of other plant species. Paulsen and Bogorad (1988) found that no rhythm of *CAB* mRNA

oscillation occurred in *Nicotiana tabacum* plants that were placed in continuous darkness. Nagy *et al.* (1988) showed that a circadian rhythm of a *CAB* 1 gene expression occurred in *Triticum aestivum* when plants were placed into constant conditions of continuous light or dark. The same characteristic pattern of a dampening amplitude, as seen in *Lycopersicon esculentum* plants, occurred with the rhythm expressed in continuous darkness. They showed that by stimulating the plant, previously kept in darkness, with a pulse of red light the *CAB* 1 transcript abundance increased. This effect was reversed by a pulse of far-red light. Therefore, Nagy *et al.* (1988) concluded that *CAB* 1 gene expression was regulated by light via a phytochrome photoreceptor. As a circadian rhythm of expression still occurred in continuous light the clock was considered to operate as a "gate" that opens and closes rhythmically, so allowing phytochrome to induce expression of the *CAB* 1 gene at a specific time. Stayton *et al.* (1989) believed that a similar operation of control, which involved phytochrome, was occurring in the *CAB* rhythms expressed in *Petunia* (Mitchell). Millar *et al.* (1992) showed that a 320 bp fragment of the *Arabidopsis thaliana CAB* 2 promoter, fused to the firefly luciferase (*Luc*) gene, showed transcriptional regulation mediated by both phytochrome and the circadian clock.

Different types of *CAB* genes have been found to show circadian rhythms of *CAB* gene expression. Millar and Kay (1991), Stayton *et al.* (1989) and Kellman *et al.* (1993) all assessed the level of expression of a number of *CAB* genes within a particular plant species. The most comprehensive study (Kellman *et al.*, 1993) followed gene expression in nineteen members of the *CAB* gene family of *Lycopersicon esculentum* under conditions of continuous darkness and light. This analysis was of nine different types of LHC polypeptides. All the different *CAB* genes were

found to be under the control of a circadian rhythm and only showed differences in the amplitudes of the oscillations not in the basic rhythmical pattern. This pattern was the same as had been previously shown in *Lycopersicon esculentum* (Piechulla, 1988, 1989). However, Millar and Kay (1991) found that the expression patterns, in conditions of continuous darkness, of three *Arabidopsis thaliana CAB* genes were not identical. *CAB* 1 mRNA levels exhibited no rhythmical cycling. However, when they analysed *CAB* promoter fusions and carried out *in vitro* nuclear run-on transcription assays, transcription of the *CAB* 1 gene was found to show circadian regulation. They suggested that a post-transcriptional mechanism of control regulated *CAB* 1 mRNA levels in *Arabidopsis thaliana.* They proposed that the cycling of *CAB* mRNA was controlled by a mechanism in which the synthesis of the *CAB* apoprotein could be coupled to light regulated chlorophyll biosynthesis. This compared to the feedback loop seen in *Drosophila melanogaster* (Hardin *et al.*, 1992). Nagy *et al.* (1988) and Fejes *et al.* (1990) identified a transcriptional pattern of control in the *Triticum aestivum CAB* 1 gene and identified a small promoter region that was responsible for the circadian clock regulated expression. Transcriptional control of circadian rhythms was first postulated by Ehret and Trucco (1967). As well as the work discussed above, Taylor (1989), Giuliano *et al.* (1988) and Nagy *et al.* (1988) all showed, through the use of nuclear run-on transcription assays, that the circadian oscillations of *CAB* mRNA in *Lycopersicon esculentum, Zea mays* and *Triticum aestivum* were controlled at the level of transcription.

The circadian rhythms of *CAB* genes show a number of characteristic features that have been identified in other organisms. Light and temperature regimes have been found to affect these rhythms. It has been

mentioned that phytochrome is involved in the control of the circadian rhythms of *CAB* gene expression. For example Tavladoraki *et al.* (1989) found that a single red light pulse induced an increase in the level of *CAB* mRNA, which was reversed by a far-red light treatment, in the etiolated tissue of *Phaseolus vulgaris*. They found that this single red light pulse produced a circadian rhythm, both in continuous darkness and light, and that if a second red light pulse was applied 36 hours after the initial application the rhythm was phase shifted. The implications were that light induced the rhythm, and the oscillator was under phytochrome control. The effects of red light and phase shifting on a number of circadian rhythms in different organisms have been well documented (Roenneberg and Hastings, 1991). Different wavelengths and intensities of light have been found to have differing effects in plant gene expression. For instance, *ELIP* transcription was induced by blue light in developing *Pisum sativum* plants and not red or far-red light (Adamska *et al.*, 1992).

In a number of plants, circadian rhythms of *CAB* gene expression have been synchronized by the application of cyclic heat shock treatments in the dark. Beator *et al.* (1992, 1993) showed that the effect of daily heat shock treatments on etiolated *Hordeum vulgare* led to the circadian oscillation of *CAB* gene expression. The synchronization of the circadian clock and *CAB* gene expression by heat shock treatments, which could be viewed as a substitute for a light signal, has also been observed in *Lycopersicon esculentum* (Piechulla and Riesselmann, 1990). Extremes of temperature have been found to totally inhibit a number of circadian rhythms. Martino-Catt and Ort (1992) found that a low temperature treatment inhibited the rhythm of *CAB* gene expression in *Lycopersicon esculentum* such that when ambient conditions were returned a displaced rhythm began.

The occurrence of the circadian rhythms in *CAB* gene expression was argued to be an anticipation of the light. The natural environment dictates a continual cycle and in plants photosynthesis is a repeatedly occurring process. Therefore, mechanisms are present which ensure that CAB proteins will accumulate when the plant begins to accumulate chlorophyll, since light energy absorbed by free chlorophyll can not be utilised in photosynthesis and will damage the plastids. Evidence has started to accumulate in support of this theory. Busheva *et al.* (1991) showed a correlation between the diurnal cycle of the level of *CAB* mRNA in the cytosol and the accumulation of LHC II in the thylakoid membranes in developing *Triticum aestivum* leaves. Spiller *et al.* (1987) showed that in *Pisum sativum*, which were recovering from induced iron stress, the levels of chlorophyll and *CAB* mRNA increased upon the addition of iron. They suggested that the changes observed occurred in parallel. Beator *et al.* (1992, 1993) showed that greening or thylakoid membrane assembly was under the control of a circadian oscillator. They found, in *Hordeum vulgare*, that the extent of greening, as measured by the quantification of chloroplast pigments and the apoprotein LHC II, varied in a circadian manner in parallel with the oscillations of *CAB* gene expression. Riesselmann and Piechulla (1992) found that the *in vivo* synthesis of a CAB polypeptide was under the control of a circadian rhythm in *Lycopersicon esculentum*. The synthesis of the protein correlated well with the circadian rhythms of *CAB* mRNA levels. Adamska *et al.* (1991) also found that circadian oscillations occurred in the levels of CAB proteins. Therefore, this evidence suggested that there was a correlation between the rhythm of *CAB* gene expression and in the synthesis of the components of the thylakoid membrane, and that they were under the control of a circadian oscillator.

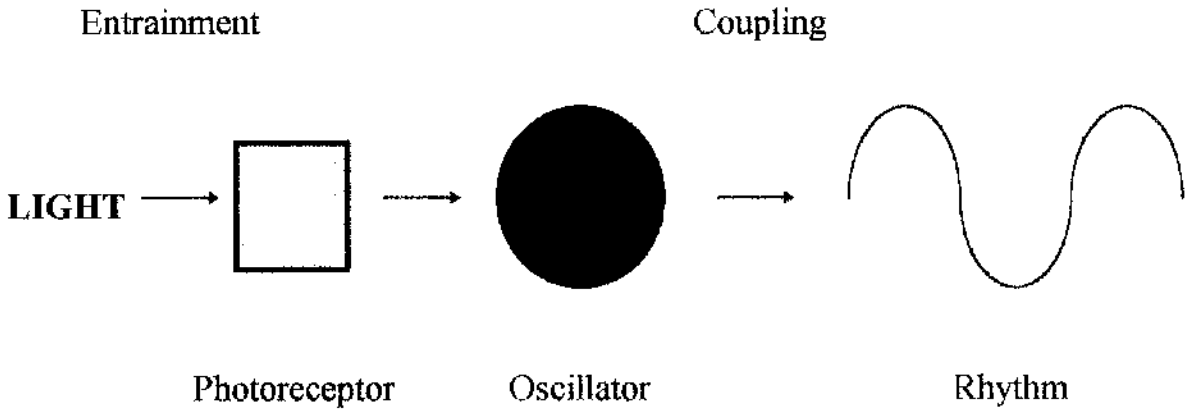
### 1.3.5 Phytochrome and its link to the circadian rhythm

Higher plants contain several major light receptors that respond to light intensity, direction, duration and spectral quality. These receptors can absorb photons over a wide range of wavelengths. There are three classes of photoreceptors that absorb photons in the ultraviolet, blue/near ultraviolet and red/far-red spectral ranges and mediate the effects of light on plant development (Thompson and White, 1991; Li *et al.*, 1993). One of these is the chromoprotein phytochrome. Its involvement as a light receptor is well documented (Quail, 1991). The signal transduction cascade leading to light induced responses begins with photoreceptor excitation. In this way phytochrome can interact with some component of the circadian oscillator, which controls the expression of a circadian rhythm, to bring about its entrainment (Figure 1.4).

Phytochrome is a regulatory photoreceptor that functions as a molecular switch that controls plant gene expression in response to light signals from the environment. Two types of response to light, involving phytochrome, have been identified. One is an inductive response and the other is the high irradiance response (Lumsden, 1991). The photoreceptor molecule is reversibly photo-interconvertible between its inactive ($P_r$, absorption maxima 660 nm) and active ($P_{fr}$, absorption maxima 735 nm) forms by the sequential absorption of red and far-red photons of light respectively. Signal perception initiates transduction processes that culminate in altered expression of selected genes and ultimately in altered growth and development appropriate for the prevailing light environment (Quail, 1991).

**Figure 1.4 The generation of a circadian rhythm**

This represents a model for the generation of circadian rhythms; from the oscillator's initial entrainment by light (environmental time cue) acting on the photoreceptor and coupling production of the rhythm to the oscillator itself.



A number of differing circadian rhythms have been identified that involve phytochrome; in the movement of ions with leaf and leaflet movement in *Samanea* (Simon *et al.*, 1976) and *Phaseolus* (Satter and Galston, 1981); with metabolic rhythms in initial entrainment and in the control of the phase of rhythm of *CAB* gene expression in *Phaseolus vulgaris* (Tavladoraki *et al.*, 1989); in the bioluminescence of *Gonyaulax polyedra* (Roenneberg and Hastings, 1991). Lumsden (1991) believed that the interaction of phytochrome with circadian rhythms led to three possible types of response; the control of phase was achieved by light acting on the oscillator itself; light had a direct effect on the rhythmic function by only affecting specific phases of the cycle; and removing the active form of phytochrome led to a general effect that was not phase dependent. Essentially, circadian rhythms interacted with photoreceptor systems to control gene expression. The phase and amplitude of *CAB* transcript rhythms were determined by light signals (Giuliano *et al.*, 1988). Experiments with red or far-red light given at the end of the photoperiod indicate that phytochrome interacts with the circadian clock to control the amplitude of *CAB* transcript accumulation (Nagy *et al.*, 1988).

Jewett *et al.* (1991) showed that just a few exposures of bright light affected the endogenous rhythms in humans. Roenneberg and Hastings (1988, 1991) postulated that, as blue and red light effected the expression of the circadian rhythm of bioluminescence in *Gonyaulax polyedra*, two photoreceptors controlled the circadian clock. In plants, as phytochrome was one of the photoreceptors, it was involved in the entrainment of the rhythms (Lumsden, 1991). The expression of the genes for CAB proteins represented the expression of the circadian rhythm (Figure 1.4). Therefore, identification of the photoreceptor "responsible for light

entrainment" in *Bryophyllum fedtschenkoi*, can hopefully lead an understanding of how the oscillator controls the rhythms. This can be achieved by working forwards along the signal transduction pathways of CAM and *CAB* gene expression.

Purified phytochrome preparations often contain protein kinase activity, which led some researchers to suggest that phytochrome might be a protein kinase (discussed in Quail, 1991). However, all of the phytochrome sequences characterised lack the strongly conserved motifs that are found in most eukaryotic kinases. Recently, Algarra *et al.* (1993) have shown that the phytochrome of the moss *Ceratodon purpureus* has a light-regulated protein kinase activity and so this implies that the phytochromes of higher plants could be protein kinases. The polycation-stimulated protein kinase that copurifies with phytochrome has also been found to phosphorylate a highly conserved serine-rich region at the amino-terminus of the phytochrome molecule (Thompson and White, 1991). The fact that the phytochrome itself may have the kinase activity is highly questionable since it can be separated from the photoreceptor molecule (Quail, 1991). However, the presence of kinase activity is interesting as the mechanisms of control (discussed in chapter 1) seen in various photo-transduction pathways, eg; CAM regulation and *CAB* gene expression, involve protein phosphorylation.

## 1.4 Conclusions

In the mass of work that has been carried out on the analysis of circadian rhythms and in the search for the oscillator mechanism some common themes have emerged. However, the molecular basis and the mechanisms involved in controlling circadian rhythmicity are still not

understood. Therefore, current research is aimed at developing our understanding of how these various biological systems operate in order to unravel the network of control that is the clock.

## 1.5 Aims of the study

The initial aim of this study was to further develop the understanding of the circadian rhythms of *Bryophyllum fedtschenkoi*. I therefore examined *CAB* gene expression in *Bryophyllum fedtschenkoi* to establish whether it is controlled in a circadian fashion. I hoped to go on to compare circadian control of *CAB* gene expression with the control of PEPc kinase gene expression that plays a major role in generation of the circadian rhythms of $CO_2$ metabolism in this plant.

# Chapter 2. Materials and Methods

## 2.1 Materials

All chemicals were obtained from BDH. (AnalaR® grade; Poole, Dorset) or Fisons. (PrimaR® grade; distributed FSA. Laboratory supplies).

Fine chemicals were obtained from Sigma (Poole, Dorset) unless indicated otherwise.

Radiochemicals were purchased from Amersham International plc. (Amersham UK).

All components for liquid and solid media/cultures were supplied by Difco Laboratories, Michigan, USA.

Agarose (ultra PURE™) was supplied by Life Technologies.

Restriction enzymes and buffers were obtained from GIBCO BRL and Promega.

Molecular size markers were a 0.25-9.5 kb RNA ladder and a 0.16-1.77 kb RNA ladder. Both were supplied by GIBCO BRL.

Chromatography media were bought from Pharmacia.

The cDNA synthesis kit, packaging extract and cDNA library construction components were obtained from Stratagene.

## 2.2 Plant material

The succulent plant *Bryophyllum* (*Kalanchoë*) *fedtschenkoi* (R. Hamet et Perrier) was propagated vegetatively from a single plant obtained from the Royal Botanic Gardens, Kew, in 1955. The stock of this plant was identical to that used by Wilkins (1959 etc.) in earlier studies. A continuous supply of plant material was maintained from cuttings grown in a glasshouse with a 16 hour photoperiod, supplied throughout the year by the use of mercury vapour lamps. Four to six month old plants were removed to controlled environment growth rooms and were allowed to adapt for at least 7 days before experiments. Two growth rooms were available and both provided an 8 hour photoperiod, either from 0800 h - 1600 h or from 1600 h - 2400 h (reverse-phase). White fluorescent lamps and twelve 100 W, tungsten lamps provided the light, giving an irradiance of 20 $W/m^2$. The temperatures during the dark and light periods were 15°C and 28°C respectively. The plants were watered every 3-5 days.

## 2.3 General experimental methods

### 2.3.1 pH calibrations

A Corning pH meter 220 and combination electrode were used to measure the pH of solutions. In the case of pH end-containing solutions Whatman Narrow Range pH paper (pH 6.0 - pH 8.0) was used.

### 2.3.2 Autoclaving

Equipment and solutions were sterilised at 15 psi for 20 min in a Laboratory Thermal Equipment Autoclave 225SE or in a Prestige Hi-Dome pressure cooker.

### 2.3.3 Filter sterilisation

Heat-labile solutions were sterilised by passing them through a Corning syringe filter (pore diameter 0.2 μm) into sterile receptacle.

### 2.3.4 Glassware

Glassware was sterilised by baking in an oven at 180°C for 8-12 hours.

### 2.3.5 Micropipetting (5 μl-5 ml)

Micropipetting was done using adjustable Gilson Pipetman pipettes (Gilson Medical Electronics, 72 Rue Gambetta, 954 00 Villiers-le-Bel, France).

### 2.3.6 Solutions and equipment used for RNA work

All solutions for RNA work were treated with 0.1 % (v/v) diethyl pyrocarbonate (DEPC; Sigma chemical Co.) overnight at room temperature and then autoclaved. DEPC-treated autoclaved water was used to make any solutions containing Tris. Glassware was soaked in DEPC-treated water for 6-8 hours and baked for 12 hours at 180°C.

### 2.3.7 Centrifugation

Centrifugation was carried out in a BECKMAN model J2-HS centrifuge using JA-20 and JS-13.1 rotors. An MSE Mistral 2L centrifuge with a swing out rotor was used in certain experimental procedures.

## 2.4 Preparation of total RNA from leaves

This method is adapted from Parish and Kirby (1966), and Hartley and Ellis (1972).

### 2.4.1 Phenol/Kirby approach

#### 2.4.1.1 Preparation of phenol and Kirby reagents

The phenol reagent was prepared in the following way; 500 g of detached crystals of phenol (Analytical reagent, Fisons, Loughborough) were melted at 65°C. The colour was checked to see if any oxidation was apparent. 0.58 g of 8-hydroxyquinoline was added to the clear phenol upon cooling. The solution was then saturated with 10 mM Tris-HCl pH 7.6, the aqueous phase was removed and replaced with 100 ml of 100 mM Tris-HCl pH 8.0. This was allowed to mix with stirring. The pH of the aqueous phase was measured and this phase was then aspirated. The phenol solution was washed with 100 mM Tris-HCl pH 8.0 until the pH of the solution of the aqueous phase was greater than pH 7.6. When this was achieved the aqueous phase was aspirated and replaced with 100 ml of Tris-HCl pH 7.6 and the phenol reagent was stored at 4°C

The Kirby reagent was prepared in the following way; six grams of 4-aminosalicyclic acid (4-AS, Na-salt; Sigma Chemical Co.) was dissolved in 10 ml of DEPC-treated distilled water. 25 ml of 40 mM Tris-HCl, 200 mM KCl pH 7.6 was added, followed by 10 ml of 10 % (w/v) tri-isopropylnaphthalenesulphonic acid (TNS; Na-salt; Eastman/Kodak chemicals) which turns the solution cloudy. 8 ml of phenol reagent was added, which dissolves the cloudy precipitate. The volume of the solution was made up to 100 ml with DEPC-treated distilled $H_2O$. The final composition of the Kirby reagent was;

| | |
|---|---|
| Kirby reagent | 6 % (w/v) 4-aminosalicyclic Acid |
| | 10 mM Tris-HCl pH 7.6 |
| | 50 mM KCl |
| | 1 % (w/v) TNS |
| | 8 % (v/v) Phenol reagent |

Kirby reagent was stored at 4°C for up to two weeks.

### 2.4.1.2 Isolation of RNA

1-3 g of frozen plant tissue was ground into a fine powder under liquid nitrogen in a mortar. The powder was transferred to a 30 ml Corex® tube containing 10 ml of Kirby reagent and 10 ml of phenol reagent. The powder was mixed in using a spatula and by inversion. The sample was put on ice between all stages. The sample was centrifuged at 3000 rpm for 10 min in an MSE mistral centrifuge at 10°C (section 2.3.7). The upper aqueous phase was removed with a baked pasteur pipette and transferred to a fresh 30 ml Corex® tube containing 5 ml of phenol reagent. After mixing, 5 ml of chloroform was added. These solutions

were mixed again and the sample was centrifuged as before, at a temperature of 4°C. After the spin the aqueous phase was transferred to a fresh Corex® tube, mixed with phenol reagent and chloroform as before and centrifuged at 4°C. The aqueous phase was transferred to an empty 30 ml Corex® tube and 16 ml of ice cold ethanol was added and the solution mixed. The tube was placed at -20°C for 6-12 hours. After this time the sample was centrifuged at 4°C. The supernatant was then discarded. The resulting pellet was washed with 2.5 M Sodium Acetate pH 5.5 and then with 80 % (v/v) ethanol, 50 mM NaCl. The final pellet was dried 'in vacuo' and resuspended in DEPC-treated distilled $H_2O$. Storage was at -80°C until required for use. An aliquot of the RNA solution was run on an agarose gel to see if the RNA was undegraded and the concentration of the RNA was estimated by using spectrophotometry.

### 2.4.2 CTAB/PVP approach

This method is adapted from Chang *et al.* (1993).

#### 2.4.2.1 Preparation of extraction buffer and solutions required

The basic composition of the extraction buffer was as follows;

CTAB extraction buffer
- 2 % (w/v) CTAB(hexadecyltrimethyl ammonium bromide)
- 2 % (w/v) PVP (K30)
- 100 mM Tris-HCl pH 8.0
- 25 mM EDTA
- 2 M NaCl
- 0.5 g/l spermidine
- 2 % (v/v) 2-mercaptoethanol

All solutions were DEPC treated.

The CTAB extraction buffer was prepared using the following protocol; 2 g CTAB, 2 g PVP (K30) and 11.7 g NaCl were dissolved in 10 ml of 1 M Tris-HCl pH 8.0, 5 ml 0.5 M $Na_2EDTA$ and 60 ml DEPC-treated distilled $H_2O$. The buffer mixture was placed on a magnetic stirrer and the components left mixing for 30 min. 0.05 g of spermidine was added and the buffer was left mixing for a further 15-30 min. The solution was left standing for 5 min and was made up to 98 ml with DEPC-treated distilled $H_2O$. The extraction buffer was autoclaved prior to use. The required volume of 2-mercaptoethanol was added, to make up the complete extraction buffer, just before use.

The basic composition of the SSTE washing solution was;

| | |
|---|---|
| SSTE | 1 M NaCl |
| | 0.5 % (w/v) SDS |
| | 10 mM Tris-HCl pH 8.0 |
| | 1 mM EDTA |

All solutions used were DEPC treated and autoclaved. 100 ml batches of SSTE were prepared. This was filter sterilised and aliquotted into sterile universals (volume of 20 ml). Storage was in a 37°C oven.

### 2.4.2.2 Isolation of RNA

2-3 g of frozen plant tissue was ground into a fine powder under liquid nitrogen in a mortar. The powder was transferred to 10 ml of pre-warmed extraction buffer in a 30 ml Corex® tube in a water bath at 65°C.

This was mixed by inverting the tube. 10 ml of chloroform/isoamyl alcohol (IAA) [24:1] was added to this, mixed by vortexing and the sample was centrifuged at 7000 rpm, 4°C in a BECKMAN centrifuge for 10 min (section 2.3.7). The upper aqueous phase was removed with a sterile Pasteur pipette and transferred to a fresh 30 ml Corex® tube containing 10 ml chloroform/IAA (24:1). The solutions were mixed and centrifuged as before. The upper aqueous phase was transferred to a fresh 30 ml Corex® tube and mixed with 10 ml of chloroform/IAA (24:1) and centrifuged again. The upper aqueous phase was transferred to an empty 30 ml Corex® tube and the solution volume was measured using a sterile plastic 10 ml pipette. A quarter of the total volume of ice cold 10 M LiCl was added to this solution and was mixed in by swirling. The sample was left at 4°C for 8-14 hours. The sample was then centrifuged at 8000 rpm, 4°C, for 20 min. The supernatant was discarded. The resulting pellet was dissolved in 500 µl of SSTE and transferred to a 1.5 ml sterile Eppendorf tube. One chloroform/IAA (24:1) extraction was carried out and the upper aqueous phase was transferred to a fresh Eppendorf tube. Two volumes of EtOH were added to this and this was mixed in by inversion. The sample was placed at -70°C for 30-120 min. The sample was centrifuged in a microfuge for 20 min, the supernatant was discarded and the resulting pellet was dried 'in vacuo'. The pellet was resuspended in DEPC-treated distilled $H_2O$ and stored at -80°C until use. The quality of the RNA was assessed as in the phenol/Kirby approach (section 2.4.1.2).

## 2.5 Isolation of poly(A)$^+$ RNA

Total RNA was extracted from plant leaf samples by using the method of Chang *et al.* (1993), as in section 2.4.2. The only variation was that after

the 8 M LiCl precipitation the resulting pellet was resuspended in 500 µl of 0.5 % (w/v) SDS and not SSTE. Total RNA extracted in this fashion was only used in the isolation of poly(A)$^+$ RNA. 40-60 mg oligo-dT cellulose (Sigma, Chemical Co.) was suspended in 1 x binding buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 0.1 % [w/v] Sodium lauryl sarcosine and 0.5 % [w/v] SDS). This suspension was poured into a plastic column to give a 1 ml oligo-dT column. The column was washed with 4 column volumes of elution buffer (10 mM Tris-HCl pH 7.5, 0.2 % [w/v] SDS). The column was then re-equilibrated by washing it with 10 column volumes of 1 x binding buffer. The RNA sample was heated to 65°C for 5 min, chilled on ice and an equal volume of 2 x binding buffer was added to the RNA sample. The RNA was then loaded onto the column. The column effluent with unbound RNA was heated to 65°C, chilled on ice and re-applied to the column. This process was carried out a further 4 to 6 times. The column was washed with 1 x binding buffer until the $A_{260}$ of the column effluent reached approximately zero. The poly(A)$^+$ RNA was eluted from the column by using elution buffer. Ten 1 ml fractions were collected in sterile 1.5 ml Eppendorf tubes and their $A_{260}$ was measured in a quartz cuvette which had been soaked in a 1:1 methanol/cHCl mixture and then washed out with DEPC-treated distilled water. The fractions containing poly(A)$^+$ RNA were pooled. The poly(A)$^+$ RNA was precipitated in 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol. After pelleting the poly(A)$^+$ RNA the pellet was washed with 80 % (v/v) ethanol and dried. The poly(A)$^+$ RNA was resuspended in DEPC-treated distilled $H_2O$ and stored at -80°C. An aliquot of the poly(A)$^+$ RNA solution was run on an agarose gel and the concentration was estimated by using spectrophotometry.

## 2.6 Preparation of total genomic DNA from leaves

This method is adapted from Draper and Scott (1988).

1-3 g of frozen leaf material was ground under liquid nitrogen to a fine powder in a mortar. The powder was transferred to a 30 ml Corex® tube and 15 ml of DNA extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 10 mM 2-mercaptoethanol) was added. The solution was mixed well. When a fine suspension was achieved 2 ml of 1 % (w/v) SDS was added, mixed in and the sample was incubated at 65°C for 12 min, with the occasional inversion of the tube. After this time 5 ml of 5 M KOAc was added, the solutions were thoroughly mixed by inversion and the sample was left on ice for 30 min. The protein/SDS precipitation was pelleted by centrifugation, in a BECKMAN centrifuge at 12,000 rpm for 30 min at a temperature of 4°C (section 2.3.7). The supernatant was poured through a filter funnel, which was packed with several layers of muslin cloth, into a clean 30 ml Corex® tube. Then 15 ml of cold (-20°C) isopropanol was added to this, mixed in and the sample was incubated at -20°C for 30 min. The DNA was pelleted by centrifugation at 8,000 rpm, 4°C for 20 min. The resulting supernatant was poured off and the pellet was left to dry. The pellet was resuspended in 200 µl Tris-EDTA buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0), transferred to a new Eppendorf and microfuged for 5 min to remove any insoluble contaminants. This new supernatant was transferred to a fresh Eppendorf, it was mixed with 75 µl of 3 M sodium acetate and 500 µl of ice cold (-20°C) isopropanol. The formation of any precipitate was examined. The sample was placed at -20°C for 10 min. The DNA was pelleted, washed with 80 % (v/v) ethanol, dried 'in vacuo' and resuspended in 80-100 µl distilled $H_2O$. An aliquot of the DNA sample

was run on an agarose gel and the concentration was estimated by using spectrophotometry.

## 2.7 Quantification and purity of nucleic acids

An aliquot (2-10 µl usually) of the nucleic acid solution to be quantified was made up to 1 ml with DEPC-treated distilled $H_2O$ in a quartz cuvette. The absorbance of the nucleic acid solution was measured between 200 nm to 300 nm against a DEPC-treated distilled $H_2O$ blank. An absorbance at 260 nm of 1 was taken to indicate the following concentrations;

| Form of nucleic acid | Concentration (µg/ml) |
|---|---|
| RNA and | |
| single stranded DNA | 40 |
| Double stranded DNA | 50 |
| Oligonucleotides | 20 |

The $A_{260/280}$ ratio was used to assess the purity of a preparation of DNA or RNA sample. A pure preparation was taken as having an $A_{260/280}$ ratio of 1.8-2.0.

## 2.8 Agarose gel electrophoresis

### 2.8.1 Electrophoresis of DNA and non-denaturing electrophoresis of RNA

Agarose gels of the appropriate concentration (0.5-2.5 % (w/v) ) were made by adding the correct amount of agarose to the specific volume of

1 x TBE (0.09 M Tris-Borate, 2 mM $Na_2EDTA$) required. A microwave oven was used to heat the agarose suspension. Once the agarose was dissolved, the heating was stopped and the agarose solution was allowed to cool to 60°C, at which point 10 mg/ml EtBr was added to a final concentration of 0.5-1.0 µg/ml. The gel solution was poured into the electrophoresis apparatus and allowed to set for approximately 30 min. When the gel was set it was submerged with just enough 1 x TBE running buffer to cover the loading wells. One-tenth the volume of DEPC-treated loading buffer (15 % [v/v] Ficoll, 0.25 % [w/v] xylene cyanol FF, 0.25 % [w/v] bromophenol blue) was added to the samples and mixed prior to loading. Samples were loaded into the wells with an automatic pipette. The gels were run at 40-100 mA until the bromophenol blue reached approximately two-thirds of the way down the gel.

### 2.8.2 Denaturing electrophoresis of RNA in formaldehyde gels

#### 2.8.2.1 Preparation of the formaldehyde gel

The required volumes of the solutions and agarose depended on the size of gel kit that was used. For a 100 ml gel the appropriate amount of agarose (1.3 g to 1.5 g) was added to 73 ml of DEPC-treated distilled $H_2O$. The agarose suspension was heated in a microwave oven until all of the agarose was dissolved. The agarose solution was allowed to cool to 65°C, then 10 ml of 10 x MOPS buffer (0.2 M MOPS pH 7.0, Sigma Chemical Co., 0.05 M sodium acetate, 0.01 M $Na_2EDTA$, pH 7.0) was added to it. 17 ml of formaldehyde (37 % [v/v]; Sigma Chemical Co. ) was added to this mixture, the solution was mixed by swirling and then poured into the electrophoresis apparatus. The gel was allowed to set for 30-45 min and

then it was submerged in enough 1 x MOPS running buffer to just cover the loading wells.

### 2.8.2.2 Preparation of RNA samples for denaturing electrophoresis

In preparing the RNA samples two alternative methods were used. Firstly, the RNA sample (5-20 μg) was prepared in a solution of 50 % (v/v) formamide (FLUKA Biochemicals, BDR), 1 x MOPS and 17.5 % (2.2 M) formaldehyde to give a volume less than 50 μl. The sample was heated to 65°C for 10-15 min and then chilled on ice. One-tenth the volume of RNA loading buffer (50 % glycerol, 1 mM EDTA pH 8.0, 0.25 % [w/v] bromophenol blue, 0.25 % [w/v] xylene cyanol FF) was added to the sample prior to loading. The sample was loaded into the wells of the gel with an automatic pipette and the gel was run at 50-100 mA until the bromophenol blue had migrated two-thirds of the distance down the gel. The gel was stained with 0.5-1.0 μg/ml EtBr (stock 10 mg/ml) and washed a few times in DEPC-treated distilled $H_2O$ to remove any excess stain. A UV transilluminator was used to visualise the RNA after this process of washing. Alternatively the appropriate volume of 10 mg/ml EtBr was added directly to the sample before loading.

In the second approach the RNA samples (5-20 μg per sample) were made up to 10 μl or 20 μl total volume by using DEPC-treated distilled $H_2O$. Then 7.5 μl per 10 μl sample volume of SB buffer (12 μl EtBr [10 mg/ml], 300 μl 10 x MOPS buffer pH 8.0, 80 μl formaldehyde, 900 μl formamide) was added to each sample. The sample was denatured by heating it to 65°C for 5 min and it was then chilled on ice. Prior to loading, one-tenth volume of RNA loading buffer was added to the sample and the gel was set up as described above. After the bromophenol

blue had migrated two-thirds the distance of the gel, the gel was washed in DEPC-treated distilled $H_2O$ for 1-2 hours. The gel was visualised using a UV transilluminator.

### 2.8.3 Alkaline agarose gel electrophoresis

This method was adapted from Maniatis *et al.* (1989).

Agarose was prepared in 72 ml of distilled $H_2O$ and heated in a microwave oven until all of the agarose was dissolved. The agarose solution was allowed to cool to 60°C and 8 ml of 10 x alkaline agarose buffer (300 mM NaOH, 20 mM EDTA) was added to it. The solution was mixed and poured into the electrophoresis apparatus. The gel was left to set for 30-60 min. The samples were prepared in an equal volume of 2 x loading buffer (20 % (v/v) glycerol, 25 mM NaOH, 4.6 % (v/v) saturated bromophenol blue). When set, the gel was soaked in 1 x alkaline agarose buffer for 30 min. The prepared samples were loaded into the wells of the gel. The gel was submerged in 1 x alkaline buffer and run either at 100 mA or at a reduced amperage if left overnight. As with other agarose gels the bromophenol blue migration was two-thirds the distance of the gel.

## 2.9 Digestion of DNA with restriction endonucleases

DNA to be digested was prepared in a solution of 1 x the appropriate buffer and 1-20 U/µg DNA of restriction endonuclease. The digestion reactions were incubated at 37°C for 1-12 hours. In certain cases 0.25 mg/ml RNAase A was added to the digestion mixture and for the digestion of plant genomic DNA, spermidine was added to a final

concentration of 1 mg/ml. Table 2.1 shows the combinations of restriction enzymes and buffers used in this study.

## 2.10 Isolation of DNA fragments

### 2.10.1 GeneClean® approach

GeneClean® kit was supplied by BIO 101 Inc. (La Jolla, USA) and was used according to the manufacturer's instructions.

A 1 % LMP agarose gel was prepared and used for running DNA samples. The specific DNA band was located by illumination of the gel under long-wave UV light. The band was excised with a scalpel blade and placed in an Eppendorf tube. 2.5 volumes of 6 M NaI (supplied with kit) was added to the sample. The excised agarose was dissolved by incubating this mixture at 55°C for 10 min. The required amount of glassmilk® (5 µl for up to 5 µg DNA) was added, the contents of the Eppendorf mixed and then it was left standing at room temperature. The sample was microfuged for 30 seconds to spin down the glassmilk® and the supernatant was removed. The remaining pellet was washed three times with 0.5 ml 70 % EtOH/30 % TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) solution and then resuspended in 5-30 µl of TE. The DNA was eluted by leaving the sample at 55°C for 3 min. The supernatant was removed to a fresh Eppendorf tube and the glassmilk® pellet was resuspended/extracted as above. This procedure yielded 50-80 % recovery of the isolated DNA.

**Table 2.1 Combinations of restriction enzymes and buffers**

The table below shows the different combinations of restriction enzymes and restriction enzyme buffers (REB) used in the digestion of DNA;

| Enzyme | *Eco*RI | *Bam*HI | *Hin*dIII | *Xba*I | *KpN* I | *Bgl*II |
|---|---|---|---|---|---|---|
| REB | 3 | 3 | 2 | 2 | 4 | 3 |

### 2.10.2 Tris-EDTA approach

This was done as given in Sambrook *et al.* (1989).

A 1 % LMP agarose gel was used. The desired DNA band was located by illumination of the gel under UV light and excised with a scalpel blade. The gel slice was placed in an Eppendorf tube and 300 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to it. This mixture was heated to 65°C for 10 min and then placed in a waterbath to cool to 37°C. Three phenol (saturated with Tris-EDTA buffer) extractions were carried out. After each extraction the supernatant was removed to a fresh Eppendorf tube. These extractions were followed by three chloroform extractions. After the final extraction the aqueous supernatant layer was removed to a fresh Eppendorf and 4 M sodium acetate pH 7.5 was added to a final concentration of 0.25 M. 2 volumes of ethanol (EtOH) were added to this mixture and then it was left standing at -20°C for 30-60 min. The DNA was pelleted, dried 'in vacuo' and resuspended in 30-50 µl TE buffer.

### 2.10.3 SPIN-X™ centrifuge filter unit

Gel electrophoresis of the DNA samples was carried out with a 1 % Tris-acetate (0.04 M Tris-acetate, 1 mM EDTA) agarose gel. As in the other methods the DNA band required was located by UV illumination and excised with a scalpel blade. The gel slice was placed in a SPIN-X™ tube at -70°C for 20 min. When the tube was removed, the gel slice was completely thawed and then microfuged for 3 min. After this time the tube was turned through 180° and microfuged for a further 2 min. 60-100 µl of DNA solution was obtained. To this solution 0.1 volume of 1

M NaCl and 2 volumes of EtOH were added and then mixed. The DNA was precipitated at -70°C for 15-30 min. The resulting DNA was resuspended in 30-60 µl of DEPC-treated distilled $H_2O$ after drying 'in vacuo'.

## 2.11 Transfer of nucleic acids onto nylon filters

### 2.11.1 Northern blotting

RNA was separated on a denaturing formaldehyde gel (section 2.8.2) and photographed. The gel was placed upside down on a wick of 2 layers of Whatman 3 MM paper wet with 10 x SSC (1.5 M NaCl, 0.15 mM trisodium citrate) and any air-bubbles were removed. The wick was dipped into a reservoir of 10 x SSC. Nescofilm™ was placed around the wick to the edge of the gel to prevent a short-circuit occurring. A piece of Hybond™-N nylon filter membrane (Amersham International) was then cut to the same size and placed on top. Air-bubbles between the nylon membrane and the gel were removed. Two pieces of Whatman 3 MM paper, cut to size were soaked in 10 x SSC and placed on top of the nylon membrane. This was followed by a large quantity of paper towels. A weighted glass plate was placed on top of the paper towels. The blotting apparatus was left for 6-12 hours. After this time the apparatus was dismantled and the gel examined under UV to check the efficiency of the transfer. A pencil or ink pen were used to mark the position of the gel wells on the filter membrane. The filter membrane was then placed RNA side down on a UV transilluminator for 1-2 min. This fixed the RNA onto the filter membrane. The filter membrane was washed in distilled water for 2 min, air dried and baked at 80°C for 1-2 hours. The filter

membrane, if not used immediately, was wrapped in Whatman 3 MM paper and foil. This was then stored at room temperature.

### 2.11.2 Southern blotting

This method is adapted from Southern (1975).

Plasmid or genomic DNA was separated by electrophoresis on an agarose gel containing EtBr and photographed (section 2.8.1). The immobilised DNA was prepared using two possible methods. Firstly, the DNA was denatured by washing the gel in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min and neutralising solution (1.5 M NaCl, 1 M Tris-HCl pH 8.0) for 30 min. The gel was then rinsed in 10 x SSC and was blotted onto nylon filter membranes as described in northern blotting (2.11.1).

Alternatively the gel was depurinated by a 15 min wash in a 10 ml concentrated HCl and 460 ml distilled $H_2O$ mixture. The gel was then blotted onto Hybond$^{TM}$-N filters or Hybond$^{TM}$-N$^{+}$ filters as before except that a 0.4 M NaOH solution was used to soak the Whatman 3 MM wick and in the reservoir. The blot was left for 4 to 6 hours, after this time the DNA was transferred and fixed onto the membrane filter. The membrane filter was washed in 2 x SSC, distilled $H_2O$ and allowed to air dry. Storage and packaging were as in northern blotting (2.11.1).

### 2.11.3 Phage plaques Southern blotting

Circular Hybond$^{TM}$-N nylon membrane filters, of the correct size, were placed onto agar plates containing the phage plaques. The plates were

placed at 4°C for 1 hour before applying the membrane filters. The membrane filters were allowed to contact the plate for 2 min (4 min and 8 min for lifts 2 and 3 respectively). Orientation was achieved by pricking the membrane filters using a syringe needle with a series of one, two and three dots. The membrane filters were then placed plaque side up on a series of Whatman 3 MM paper soaked in 10 % (w/v) SDS for 3 min, followed by denaturing solution for 5 min, neutralising solution for 5 min and finally rinsing solution (0.2 M Tris-HCl pH 7.5, 2 x SSC) for 30 seconds. The membrane filters were dried in air for 10 min and then baked at 80°C in a vacuum oven for 1-2 hours.

## 2.12 The purification of plasmid DNA

### 2.12.1 Mini-preparation of plasmid DNA

A 5 ml sterile bijou was filled with 4.5 ml of TB medium (composition per litre; 12 g Bacto-tryptone, 24 g Bacto-yeast extract, 4 ml glycerol, made up to 1000 ml with distilled $H_2O$), a quantity of an appropriate antibiotic and 1 µl of the specific bacterial strain containing the relevant plasmid. This was left shaking vigorously at 37°C. After this time 1.5 ml of the sample medium was decanted off into a sterile Eppendorf tube and microfuged for 1 min. The supernatant was removed and the resulting pellet resuspended in 100 µl of ice cold solution I (1 % [w/v] glucose, 10 mM EDTA pH 8.0, 25 mM Tris-HCl pH 8.0) and left standing for 5 min. 200 µl of freshly prepared solution II (0.2 M NaOH, 1 % [w/v] SDS) was added, the solutions were mixed by inverting the Eppendorf tube and the sample was left on ice for 5 min. The sample was microfuged for 5 min and the supernatant was removed to a fresh Eppendorf tube. A phenol/chloroform (1:1) extraction was carried out

and then 2 volumes of EtOH were added to the supernatant. The solutions were mixed and placed at -20°C for 15-30 min. The sample was microfuged, the supernatant removed and the resulting pellet was washed in 1 ml of 70 % (v/v) EtOH. The washed pellet was dried 'in vacuo' and resuspended in 50 µl of TE buffer containing RNAase A (20 µg/ml). The DNA sample was stored at -20°C.

### 2.12.2 Promega Wizard™ Minipreps DNA purification system

A 3 ml overnight culture of an *E. coli* strain containing the specific plasmid was grown up. The cells were pelleted by centrifugation in a microcentrifuge for 1-2 min. The pellet was resuspended in 200 µl of cell resuspension solution (RNAase A 100 µg/ml, 10 mM EDTA, 50 mM Tris-HCl pH 7.5). The resuspended cells were transferred to a sterile 1.5 ml Eppendorf tube and 200 µl of cell lysis solution (0.2 M NaOH, 1 % [w/v] SDS) was added. The solutions were mixed by inverting the Eppendorf tube several times. 200 µl of neutralisation solution (1.32 M KOAc pH 4.8) was added, mixed by inversion and then the sample was microcentrifuged for 5 min. The supernatant was decanted to a fresh Eppendorf tube. 1 ml of Wizard™ minipreps DNA purification resin (7 M guanidine HCl) was added and mixed by inverting the Eppendorf tube. A Wizard minicolumn luer-lock extension was attached to the barrel of a 3 ml sterile disposable syringe. This assembly was then inserted into a vacuum manifold (Promega's Vac-man laboratory vacuum manifold). The resin/DNA mixture was pipetted into the syringe barrel and a vacuum was applied to pull the sample into the minicolumn. The vacuum was stopped. 2 ml of column wash solution (200 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA diluted 1:1 with 95 % [v/v] EtOH) was pipetted into the syringe barrel and the vacuum reapplied, drawing the

solution through the minicolumn. After the wash solution was pulled through the resin was dried by leaving the vacuum on for 1-2 min. The minicolumn was removed from the syringe barrel and attached to an Eppendorf tube. This apparatus was microcentrifuged for 20 seconds to remove any excess column wash solution. The minicolumn was transferred to a new sterile Eppendorf tube and 50 µl of distilled $H_2O$ or TE buffer was applied to the column. This was left for 1 min and then the DNA was eluted by microcentrifuging the column/Eppendorf assembly for 20 seconds. The minicolumn was removed and the plasmid DNA was stored in the Eppendorf tube at -20°C.

## 2.13 Radiolabelling of DNA fragments

### 2.13.1 Preparation of the DNA

The DNA insert was cut out of its host plasmid using the appropriate restriction enzyme(s). After the digestion, the insert was isolated by using one of the protocols of fragment isolation (section 2.10). The concentration of the DNA insert was either estimated by comparing it to known amounts of DNA on a gel or measuring its $A_{260}$ by spectrophotometry (section 2.7).

### 2.13.2 Random priming of dsDNA

DNA was labelled with [$\alpha$-$^{32}$P]dCTP using the Multiprime, Megaprime or Rediprime DNA labelling systems supplied by Amersham International.

In the Multiprime system between 25-100 ng of DNA was heated to 95°C for 2 min and then immediately put on ice. The following mixture was

set up in an Eppendorf tube; 25-100 ng of denatured DNA, 10 µl of reaction buffer containing dATP, dGTP and dTTP, 5 µl of primer solution containing random hexanucleotides, 50 µCi [$\alpha^{32}$P]dCTP (>3000 Ci/mmol), 2 µl of enzyme solution (2 U DNA polymerase I 'Klenow' fragment) and distilled $H_2O$ to make up a total reaction volume of 50 µl . The contents were mixed and incubated at 37°C for 1-2 hours.

In the Megaprime system 25-100 ng of denatured DNA was mixed with 5 µl primer solution (nona-nucleotide primers) and the appropriate amount of distilled $H_2O$ to give a total volume of 50 µl in the final Megaprime reaction. The solutions were mixed in a screw top Eppendorf and then heated to 95°C for 5 min. The sample was then put on ice. 10 µl of Megaprime reaction buffer (dATP, dGTP, dTTP), 50 µCi [$\alpha^{32}$P]dCTP (>3000 Ci/mmol) and 2 µl of enzyme solution (2 U of DNA polymerase I 'Klenow' fragment) were added. The reaction was incubated at 37°C for 45-60 min.

In the Rediprime system 25-50 ng of DNA was diluted with sterile distilled $H_2O$ to make up a total volume of 45 µl. The DNA was denatured by heating the DNA sample to 95°C, in a boiling water bath, for 5 min, and was then briefly microcentrifuged. The denatured DNA was added to the labelling mix (dATP, dGTP, dTTP, exonuclease free 'Klenow' enzyme and random nonamer primers) and the mixture was reconstituted by gently flicking the Eppendorf tube until the blue colour of the labelling mix evenly distributed. A brief microcentrifugation was carried out and 50 µCi of [$\alpha^{32}$P]dCTP was added to the reaction mixture. This mixture was incubated at 37°C for 10-15 min.

After the incubation period of each labelling system had proceeded the reaction was stopped by adding 5 µl of 0.2 M EDTA and 45 µl of distilled $H_2O$. For use in hybridisation analysis the DNA was denatured by heating to 95-100°C for 5 min and chilling the sample on ice. This was carried out after assessing the level of incorporation and removal of unincorporated nucleotides (section 2.13.4).

### 2.13.3 Nick translation system to label DNA fragments

DNA was labelled using GIBCO-BRL Nick translation system.

The following reagents were mixed in a 1.5 ml Eppendorf tube; 5 µl solution A2 (contains nucleotides dATP, dGTP, dTTP), 1 µg DNA, 156 pmole radioactive dCTP per 50 µl reaction and distilled $H_2O$, to make up the total reaction volume to 45 µl. 5 µl of solution C (DNA polymerase I / DNAase I) was then gently mixed in by a 5 second microcentrifugation. The reaction was incubated at 15°C for 60 min. After this time 5 µl of solution D (stop buffer; 300 mM $Na_2EDTA$ pH 8.0) was added. The amount of incorporation was determined and the unincorporated nucleotides removed (section 2.13.4).

### 2.13.4 Estimation of incorporated radioactivity

#### 2.13.4.1 Adsorption to DE81 paper

A 2 µl aliquot of the radiolabelled DNA reaction mixture was removed and spotted onto a Whatman DE81 chromatography paper disc. This was repeated once more. The DE81 discs were dried under a table lamp. One DE81 disc was washed six for 5 min in 0.5 M $Na_2HPO_4$, two

times for 1 min in distilled $H_2O$ and two times for 1 min in 95 % (v/v) EtOH. All these washes were carried out at room temperature. The washed DE81 disc was then dried under a table lamp. The DE81 discs were placed in separate scintillation vials with about 5 ml of scintillation fluid (Ecoscint A, National diagnostics) and counted in an LKB Wallac 1209 RACKBETA liquid scintillation counter. The level of incorporated radioactivity was determined by comparing the radioactive counts of the two DE81 discs (before and after washing). The specific activity of the DNA probe was then determined.

Alternatively a 2 µl aliquot of the radiolabelled DNA reaction mixture was spotted onto a strip of Whatman DE81 paper. The strip was placed in a beaker containing 0.3 M ammonium formate pH 8.0. The radioactive spot was at least 1 cm above the level of the ammonium formate. This was left for 15-30 min to allow chromatographic separation to occur. The DE81 strip of paper was dried under the heat of a table lamp, wrapped in clingfilm and then laid down at room temperature for 5 min with a piece of Fuji X-ray film. The film was developed and the level of incorporation assessed visually.

### 2.13.4.2 Removal of unincorporated radioactive nucleotides by using Sephadex™ G-50 spin columns

Sephadex™ G-50 (Pharmacia, Milton Keynes) was equilibrated in TE buffer at 65°C for 1-2 hours. A 1.0 ml sterile syringe was plugged with a piece of siliconized glass wool and filled with the equilibrated Sephadex™ G-50. The syringe was placed in a 15 ml Corex® tube, containing a decapped 1.5 ml Eppendorf tube, and centrifuged at 2000 rpm for 5 min (section 2.3.7). Any liquid was removed from the Eppendorf tube, the

syringe was refilled with Sephadex™ G-50 and centrifuged as before. This process was continued until the column was completely packed with Sephadex™ G-50. 100 µl of TE buffer was then added to the top of the column and centrifuged as above. This was repeated once. The syringe was placed in a clean 15 ml Corex® tube containing a sterile decapped 1.5 ml Eppendorf tube. All of the radiolabelled DNA sample was applied to the top of the prepared column and centrifuged as above. The purified radiolabelled DNA sample was collected in the Eppendorf tube.

In an alternative column system a 0.5 ml Eppendorf tube Sephadex column was used. A hole was made in the bottom of the 0.5 ml Eppendorf tube by using a syringe needle. This was plugged with a drop of siliconized glass beads and filled with equilibrated Sephadex™ G-50. The 0.5 ml Eppendorf tube was placed in a 1.5 ml Eppendorf tube and centrifuged as described above using a BECKMAN Model TJ-6 Centrifuge. The final purified radiolabelled DNA sample was collected in the 1.5 ml Eppendorf tube.

The level of incorporated radioactivity can be estimated using these columns. A 2 µl aliquot of the radiolabelled sample was taken before and after centrifugation through the column. These samples were then placed in separate scintillation vials and counted (section 2.13.4.1). The level of incorporation was determined by comparing the counts of the two radiolabelled aliquots before and after the centrifugation steps.

## 2.14 Hybridisation analysis of nucleic acids

### 2.14.1 Hybridisation analysis of RNA using cDNA probes

### 2.14.1.1 Denhardt's approach

A nylon filter(s) containing bound RNA was prehybridised at the appropriate temperature in a shaking water bath or hybridisation oven for 6-12 hours. Enough prehybridisation solution [5 x (v/v) Denhardt's solution (0.1 % (w/v) Ficoll 400, 0.1 % (w/v) PVP 360, 0.1 % BSA), 5 x SSC, 50 % formamide, 100-200 μg/ml denatured, sonicated salmon sperm DNA, 0.1 % (w/v) SDS] was used to cover the membrane filter(s) in a sealed bag(s), plastic box(es) or hybridisation oven container(s). The radiolabelled cDNA probe(s) was denatured by heating at 95-100°C for 5 min and then chilled on ice. The prehybridisation solution was either replaced or kept in the container and the denatured radiolabelled cDNA probe was added to the prehybridisation solution with an automatic pipette. Any air bubbles were removed from the sealed bag(s)/container(s). Hybridisation was carried out at the appropriate temperature for 16 hours. After this time, the hybridisation solution was replaced and the membrane filter(s) washed at the chosen salt concentration and temperature for the required level of stringency. The hybridised membrane filter(s) was either wrapped in clingfilm or sealed in a bag(s) after washing and then autoradiographed at -80°C (section 2.14.3).

### 2.14.1.2 BSA approach

This method is adapted from Church and Gilbert (1984).

A nylon filter(s) containing bound RNA was prehybridised, in a shaking water bath or hybridisation oven for 1-2 hours, at 55°C. Enough prehybridisation solution (0.5 M $Na_2HPO_4$ pH 7.2, 7 % (w/v) SDS, 10

mg/ml BSA) was used to cover the membrane filter(s). The cDNA probe(s) was denatured as above and added to the prehybridisation solution with an automatic pipette. Hybridisation was carried out at 55°C for 16 hours. The washing and autoradiography of the hybridised membrane filter(s) was the same as above.

### 2.14.2 Hybridisation analysis of DNA using cDNA probes

#### 2.14.2.1 Plaque hybridisation

A nylon filter(s) containing plaque DNA was prehybridised for 4-6 hours in prehybridisation solution (50 % formamide, 6 x SSC, 5 x Denhardts solution, 0.5 % (w/v) SDS, 0.1 % sodium pyrophosphate, 100 µg/ml denatured, sonicated salmon sperm DNA) in a shaking water bath at the temperature of 35-42°C. The temperature depended upon the cDNA probe to be used. 10 ml of prehybridisation solution was used for every membrane filter which was prehybridised. A plastic box(es) or sealed bag(s) was used as a container for the membrane filter(s). For the hybridisation procedure 10-20 ml of fresh prehybridisation solution was added to the membrane filter(s) in its container, followed by the denatured radiolabelled cDNA probe. Any air bubbles were removed. The hybridisation reaction was carried out at the temperature of 35-42°C for 16-20 hours. After this time the hybridised filter(s) was washed and autoradiographed (sections 2.14.1 and 2.14.3).

#### 2.14.2.2 Southern blot hybridisation

A nylon filter(s) containing bound DNA was prehybridised in 20 ml of prehybridisation solution [4 x SET (0.6 M NaCl, 0.08 M Tris-HCl pH

7.8, 4 mM EDTA), 10 x Denhardt's solution, 0.1 % (w/v) SDS, 0.1 % sodium pyrophosphate, 50 μg/ml denatured, sonicated herring sperm DNA] at 55°C for 4 hours. Hybridisation was carried out in 5 ml of prehybridisation solution per membrane filter plus the denatured radiolabelled cDNA probe in a water bath at 55°C for 12-16 hours. After this time the hybridised membrane filter(s) was washed and autoradiographed (sections 2.14.1 and 2.14.3).

### 2.14.3 Autoradiography

Autoradiography was carried out either by wrapping the hybridised membrane filters in clingfilm or sealing them in bags. The wrapped hybridised filters were exposed to Fuji X-ray film type RX in a light tight film cassette with intensifying screens at the appropriate temperature (either room temperature or -80°C), depending upon the type of radioactivity that was used. The duration of exposure varied.

### 2.14.4 Removal of bound radioactive cDNA probes and blocking agents from membrane filters

The blocking agents and bound cDNA probe were removed from a membrane filter by boiling a solution of 0.1 % (w/v) SDS and pouring it over the filter. The solution was then cooled to room temperature and the procedure was repeated a further two to three times. To check that the membrane filter contained no residual probe it was again wrapped in clingfilm or sealed in a bag and autoradiographed overnight.

### 2.14.5 Methylene blue staining of nylon membrane filter-bound RNA

This method was taken from Herrin and Schmidt (1988).

The membrane filter bound with RNA was dipped into 0.04 % (w/v) methylene blue, 0.5 M sodium acetate pH 5.2 for 10 min. The methylene blue was poured off and the membrane filter washed with DEPC-treated distilled $H_2O$ until the blue bands of RNA appeared. 20 % (v/v) acetic acid was used to remove the blue stain from the membrane filter.

### 2.14.6 Autoradiograph scanning

An LKB 2202 Ultrascan laser densitometer was used to measure the absorbance of specific bands on X-ray film representing mRNA transcripts. The absorbances of the tracks of film were measured relative to a reference absorbance. The reference absorbance was represented by an area of film exposed to the filter which did not contain any hybridising bands. The amount of hybridisation was represented from the scanner as a peak, the area of which was comparable to the density of the band.

## 2.15 Construction of a cDNA library

### 2.15.1 cDNA synthesis

cDNA was synthesised using the Stratagene ZAP-cDNA™ synthesis kit.

To set up the first strand cDNA synthesis reaction the following was added, to an RNAase free Eppendorf tube, in order; 5.0 µl 10 x first strand buffer, 5.0 µl 0.1 M DTT, 3.0 µl 10 mM first strand nucleotide

mixture (10 mM dATP, dGTP, dTTP, 5 mM Me-dCTP), 2.0 µl linker-primer (1.4 µg/µl). This mixture was vortexed. 7.5 µl (5-7.5 µg) of poly(A)$^+$ mRNA was then added and the mixture was left standing at room temperature for 10 min. This allowed the template and primer to anneal. After this time 2.5 µl M-MuLV reverse transcriptase was pipetted on to the side of the Eppendorf tube, gently mixed in and then microcentrifuged briefly. 5.0 µl of the reaction mixture was removed to a separate Eppendorf tube that contained 0.5 µl of [$\alpha^{32}$P]dATP (800 Ci/mmole). This 5.0 µl radioactive control sample was used in the analysis of the quality and quantity of first strand synthesis. Both of these first strand reaction mixtures were incubated in a water bath at 37°C for 60 min. After 60 min the first strand synthesis reactions were removed from the 37°C water bath. The radioactive first strand control was stored at -20°C for later analysis and the non-radioactive first strand reaction placed on ice. To set up the second strand cDNA synthesis reaction the following was added to the 45 µl of non-radioactive first strand reaction, in order: 40.0 µl 10 x second strand buffer, 15.0 µl 0.1 M DTT, 6.0 µl 10 mM second strand nucleotide mixture (10 mM dATP, dGTP, dTTP, 26 mM dCTP), 280.6 µl sterile distilled $H_2O$ and 2.0 µl [$\alpha^{32}$P]dATP (800 Ci/mmole). The second strand reaction mixture was briefly vortexed and microcentrifuged. This was followed by the addition of 4.5 µl RNAase H (1 U/µl) and 6.9 µl DNA polymerase (14.5 U/µl). The second strand reaction mixture was again briefly vortexed and microcentrifuged. The second strand reaction mixture was incubated in a water bath at 16°C for 150 min. After this time period the second strand reaction mixture was immediately placed on ice and 400 µl of phenol/chloroform (1:1) added to it. The reaction mixture was vortexed and microcentrifuged for 2 min at room temperature. The upper aqueous layer was then transferred to a clean Eppendorf tube and a chloroform extraction was carried out, as

above. Again the upper aqueous layer was transferred to a clean Eppendorf tube and the following added to it: 33.3 µl 3 M sodium acetate and 867.0 µl 100 % EtOH. The contents were mixed by inverting the tube several times and it was then placed at -20°C, to precipitate the cDNA, overnight. The cDNA sample was microcentrifuged at room temperature for 60 min. The radioactive supernatant was carefully removed and the resulting white pellet gently washed with 1 ml 80 % (v/v) EtOH. After a 2 min microcentrifugation, the EtOH wash solution was removed and the cDNA pellet lyophilised until dry. When dry, the pellet was resuspended in 43.5 µl of sterile $H_2O$. A resuspension period of 15-30 min was allowed. For analysis of the second strand synthesis quality, 4.5 µl of the resuspended cDNA sample was removed and stored at -20°C.

The control samples of the first and second strand reactions were run on an alkaline agarose gel (section 2.8.3) with λ *Hin*dIII markers. The markers were labelled by nick translation (section 2.13.3). The gel was Southern blotted and resulting blot autoradiographed (sections 2.11.2 and 2.14.3). After developing and analysing the Fuji X-ray film, the size range of the synthesised cDNA was determined.

### 2.15.2 Blunting the cDNA termini

T4 DNA polymerase buffer, 2.5 mM dNTP mix and T4 DNA polymerase were briefly vortexed, microcentrifuged and placed on ice. The following was added to the 39 µl of synthesised cDNA; 5.0 µl 10 x T4 DNA polymerase buffer, 2.5 µl 2.5 mM dNTP mix and 3.0 µl T4 DNA polymerase (3.2 U/µl). This reaction mixture was incubated, in a water bath, at 37°C for 30 min. The total volume was brought up to 100 µl

with the addition of 50 µl of distilled $H_2O$. A phenol/chloroform (1:1) extraction was carried out. The resulting supernatant was transferred to a new Eppendorf tube and precipitated by adding 7.0 µl 3 M sodium acetate and 226 µl 100 % EtOH. The sample was left on ice for 30 min and then microcentrifuged for 60 min at room temperature. The supernatant was carefully removed to another Eppendorf tube and the resulting pellet identified using a hand held radioactivity monitor (mini-monitor type 900E). The pellet was washed with 300 µl of 80 % (v/v) EtOH. The wash solution was removed and the pellet dried 'in vacuo'.

### 2.15.3 Adaptation of cDNA termini for ligation

#### 2.15.3.1 Ligating *Eco*RI adaptors

The dried cDNA pellet was resuspended in 7.0 µl *Eco*RI adaptors. The sample was briefly mixed and microcentrifuged. The following was then added to the sample; 1.0 µl 10 x Ligase buffer, 1.0 µl 10 mM rATP and 1.0 µl T4 DNA ligase (4 Weiss U/µl). The contents were microcentrifuged and then incubated at 4-8°C for 16-48 hours. After this incubation period the ligase was heat inactivated by placing the sample in a 70°C water bath for 30 min. The sample was microcentrifuged for 5 seconds and left for 5 min to cool down to room temperature.

#### 2.15.3.2 Kinasing the *Eco*RI ends

To kinase the adaptor ends, the following components were separately vortexed, microcentrifuged and added to the cooled sample; 1.0 µl 10 x Ligase buffer, 2.0 µl 10 mM rATP, 6.0 µl sterile $H_2O$ and 1.0 µl T4 polynucleotide Kinase (10.0 U/µl). The reaction mixture was incubated

in a water bath at 37°C for 30 min. The kinase was heat inactivated by placing the sample in a 70°C waterbath for 30 min. The sample was microcentrifuged for 5 seconds and left for 5 min to cool down to room temperature.

### 2.15.3.3 *Xho*I digestion

The following components were then added to the cDNA sample; 28.0 µl *Xho*I buffer supplement and 3.0 µl *Xho*I (40 U/µl). The reaction mixture was incubated at 37°C for 90 min. The sample was cooled to room temperature and 5.0 µl of 10 x SSTE was added. The cDNA was size fractionated by running the sample through a Sephacryl S-400 spin column.

The column was prepared by plugging a sterile 1 ml plastic syringe with cotton. The syringe was then filled with Sephacryl S-400 and placed in a 15 ml Corex® tube. The column was centrifuged in an MSE Mistral centrifuge for 2 min at 600 rpm (section 2.3.7). The column was topped up with sephacryl S-400 and centrifuged as above. The column was rinsed twice with 300 µl 1 x STE. The solution collected at the bottom of the Corex® tube was removed and a 1.5 ml Eppendorf tube placed into it. The tip of the prepared column was placed into the Eppendorf tube.

The cDNA sample was added to the prepared column using an automatic pipette. The column was centrifuged at 600 rpm as above for 2 min. The column was taken out of the 15 ml Corex® tube and the eluent (1st fraction), which collected in the Eppendorf, carefully removed to a clean Eppendorf using an automatic pipette. The column was replaced into the Corex® tube, with the syringe tip placed into the Eppendorf tube, and

600 µl of 1 x STE loaded. The column was centrifuged as above. The process was repeated several more times and 8 fractions of eluent collected. A scintillation counter was used to count the individual Eppendorfs (section 2.13.4). The fractions likely to contain the large cDNA fragments were pooled together, and those fractions likely to contain the smaller cDNA fragments/free nucleotides were pooled together. To each pooled fraction phenol/chloroform (1:1) and chloroform extractions were carried out. This removed any remaining kinase from the cDNA samples. The final aqueous layers were transferred to clean Eppendorf tubes.

### 2.15.4 cDNA precipitation

The cDNA fraction(s) was precipitated by adding twice the individual sample volume of 100 % EtOH. The sample(s) was left on ice for 60 min and then microcentrifuged for 60 min at 4°C. The supernatant(s) was removed to a fresh Eppendorf tube and the sample(s) was counted using a hand held radioactive monitor. The Eppendorf tube containing the pellet(s) had a high count. The pellet(s) was washed with 200 µl 0f 80 % (v/v) EtOH. The EtOH was removed and the sample(s) dried 'in vacuo'. The cDNA fraction(s) was resuspended in 10 µl sterile water stored at -20°C.

### 2.15.5 Quantification of cDNA

The cDNA sample(s) was quantified using an ethidium bromide plate assay quantitation technique. The EtBr plates were prepared as follows; 100 ml of 0.8 % agarose/Tris-acetate was heated in a microwave and cooled to 50°C, and then 10 µl of 10 mg/ml EtBr stock solution was

added. The mixture was swirled and 10 ml aliquots poured into 100 mm petri-dishes. The plates were allowed to harden. They were stored in the dark at 4°C. The DNA concentration was determined by spotting 0.5µl of standard DNA samples (concentrations 200, 150, 100, 75, 50, 25, and 10 ng/µl) in a line onto the surface of a prepared EtBr plate. 0.5 µl of the cDNA sample(s) was spotted adjacent to the line of standards. The spots were allowed to absorb into the plate for 10-15 min at room temperature. The plate was inverted, viewed using a UV lightbox and photographed. The concentration of the cDNA sample was determined by comparing the spotted sample of unknown concentration with the standards.

### 2.15.6 Ligation of cDNA into vector arms

The cDNA was cloned in the Uni-Zap$^{TM}$ XR vector supplied in the Zap cDNA synthesis kit. The ligation mixture was set up in a clean Eppendorf tube with the following components: 100 ng resuspended cDNA, 0.5 µl 10 mM rATP, 1.0 µl Uni-Zap$^{TM}$ XR vector (1 µg/µl), $H_2O$ for a final volume of 4.5 µl. These volumes varied depending on the cDNA sample concentrations and were modified to fit the ligation components final concentrations of 100 ng cDNA, 1 x ligation buffer, 1 mM rATP and 1 µg Uni-Zap$^{TM}$ XR vector. The ligation mixture(s) was gently mixed and 0.5 µl T4 DNA ligase (4 Weiss U/µl) added. The reaction mixture(s) was incubated overnight at 12°C, or for two days at 4°C. A test ligation was also set up and incubated with the following components: 1 µl Uni-Zap$^{TM}$ XR vector (1 µg/µl), 1.6 µl test insert (0.4 µg; pBR322 digested with *Sal*I and *Eco*RI), 0.5 µl T4 DNA ligase (4 Weiss U/µl) and 0.9 µl $H_2O$. The ligated DNA, experimental DNA and

test ligation were packaged using the Gigapack® II Gold packaging extract supplied by Stratagene.

### 2.15.7 Packaging of ligated vector

Initial packaging used the *E. coli* SURE bacterial strain. It is an mcrA$^-$, mcrB$^-$ strain and will not affect the hemi-methylated cDNA introduced into the Uni-Zap XR vector. After the cDNA library passed through the SURE cells it was no longer hemi-methylated and grew on mcrA$^+$, mcrB$^+$ *E. coli* strains (eg; XL I-Blue).

#### 2.15.7.1 Preparation of plating host cells

A SURE strain colony was isolated from an LB/tetracycline plate. From this single colony a 50 ml culture of SURE cells was grown up, overnight at 39°C, in LB (10 g NaCl, 10 g Bacto-Tryptone, 5 g Yeast extract per litre) w/0.2 % maltose/10 mM $MgSO_4$. 50 ml of VCS257 cells were also cultured. This was for plating wild type DNA for the Gigapack® II packaging extract positive control. After this period the cells were centrifuged at 1500-2000 rpm in a Beckman J2-HS centrifuge for 15 min and the pellets resuspended in half the original volume of sterile 10 mM $MgSO_4$. The cells were then diluted to an $OD_{600}$ of 0.5 with sterile 10 mM $MgSO_4$.

#### 2.15.7.2 Primary library; infection of plating cells

The Gigapack® II high efficiency packaging extract supplied by Stratagene was used. The Freeze/Thaw extract was quickly thawed between the fingers. 1-4 µl (0.1-0.5 µg) of the DNA was then

immediately added to this tube and placed on ice. 15 µl of Sonic extract was added to the Freeze/Thaw extract containing the DNA. This was mixed by stirring. The reaction mixture was then incubated at room temperature for 2 hours. After this time 500 µl of SM buffer (Phage dilution buffer; 5.8 g NaCl, 2.0 g $MgSO_4$, 50 ml 1 M Tris-HCl pH 7.5, 5 ml 2 % gelatin per litre) was added to the packaged material followed by 20 µl of chloroform. The solutions were gently mixed and briefly centrifuged to sediment any debris. The supernatant was now ready for titering, storage was at 4°C.

The positive wild type DNA control was packaged as above except when diluted in the SM buffer serial dilutions were carried out to a $10^{-4}$ dilution.

### 2.15.7.3 Primary library; plating and titering

The following were mixed; 1 µl of final packaged reaction, 200 µl of SURE cells at $OD_{600}$ of 0.5 and a 1:10 dilution of final packaged reaction with SURE cells as before. These were incubated at 37°C for 15 min. Then the following were added; 5 ml of top agar (48°C), 30 µl of 0.5 M IPTG (in water) and 100 µl of 250 mg/ml X-Gal (in DMF). This was immediately poured onto NZY plates (5 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 5 g yeast extract, 10 g NZ Amine [casein hydrosylate] pH 7.5 and 15 g Difco Agar per litre NZY broth; 80 ml/150 ml plates were used) and left to set for 10 min. The plates were placed upside down at 39°C for 6-12 hours. 10 µl of the $10^{-4}$ dilution of the positive packaged control was plated on the host strain VCS257 and incubated at 37°C for 12 hours. After the incubation period the plaques were visible and counted. The recombinant plaques appear white and should be 10 to 100 fold above the non-recombinant blue plaques. Around 400 plaques on the $10^{-4}$ dilution

positive control plate were expected. When the expected results were achieved the remainder of the experimental sample ligation was packaged and titered.

### 2.15.8 Amplification of Uni-Zap$^{TM}$ XR library

The primary libraries packaged were unstable. 50 ml of LB broth supplemented with 0.2 % maltose and 10 mM $MgSO_4$ was inoculated, in a sterile conical flask, with a single SURE strain colony. The culture was left to grow with shaking overnight at 30°C. The cells were then centrifuged in a sterile conical tube for 10 min at 2000 rpm. The media was decanted off and the cell pellet resuspended in 15 ml of 10 mM $MgSO_4$. The SURE cells were diluted in 10 mM $MgSO_4$ to an $OD_{600}$ of 0.5. Then aliquots containing ~50,000 plaque forming units (pfu) of the packaged library suspension were mixed with 600 µl of the prepared SURE host cells in 2059 Falcon tubes. The tubes were incubated at 37°C for 15 min. 8 ml of top agar was mixed with each aliquot of infected bacteria (SURE cells) and spread evenly onto a freshly poured 150 mm plate of bottom agar. The plates were incubated at 39°C for 6-12 hours. When the plaques grew to 1-2 mm in size the plates were overlayed with 10 ml of SM buffer and stored at 4°C overnight, with gentle shaking. The bacteriophage suspension was recovered for each plate and pooled into a sterile polypropylene container. The plates were rinsed with an additional 2 ml of SM buffer and pooled. After adding chloroform (final concentration of 5 %) to the suspension it was left to incubate for 15 min at room temperature. The cell debris was removed by centrifugation at 2000 rpm for 10 min. The supernatant was recovered and transferred to new sterile polypropylene tubes. Chloroform was added (final concentration of 0.3 %) and the tubes were stored at 4°C. For long term

storage aliquots were stored in 7 % DMSO at -70°C. The titer of the amplified library was checked using host cells (eg; XL-I Blue) and with serial dilutions of the library.

## 2.16 Analysis of a cDNA library

### 2.16.1 DNA screening; primary, secondary and tertiary

The cDNA library was titered to determine the concentration. Aliquots of the amplified library mixture containing ~50,000 pfu were mixed with 600 µl of host cells (XL-I Blue) with an $OD_{600}$ of 0.5 and 8 ml of top agar (48°C). These were plated onto large 150 mm NZY plates. This procedure was repeated for a total of ten plates (ie; to screen 500,000 pfu). The plates were incubated at 37°C for 8-12 hours. The plates were then refrigerated at 4°C for 2 hours. Phage plaque Southern blotting procedure (section 2.11.3) was followed. Duplicate blots of each plate were made. The agar stock plates of the transfers were stored at 4°C for use after screening. The nylon membrane blots were hybridized employing plaque hybridisation techniques (section 2.14.2.1). After washing, the excess liquid was removed from the nylon membranes by blotting them on Whatman 3 MM paper. They were then wrapped in plastic sheets and autoradiographed at -70°C (section 2.14.3). The orientation of the filters was achieved by lining up the film and marking the regions where the 'dots' of the needle poked through. The strongest putative clones were assessed and marked up on the film. A square centimeter window(s) of the top agar was cut, from the stock plate(s) where a putative clone lined up with the film spot, using a scalpel and pasteur pipette. The square centimeter window(s) was put into 1 ml of SM buffer and 20 µl of chloroform in a sterile Eppendorf tube. This was

vortexed. The putative clones were diluted and titered with host cells (XL-I Blue) on small 100 mm NZY plates, so that one plate had ~50 pfu and the second plate had ~ 450 pfu. These were incubated at 37°C overnight. Then phage plaque Southern blotting and hybridization were carried out as before. Positive plaques were picked from a secondary screen by coring with a pasteur pipette and retitered as above. A tertiary screening procedure was performed to obtain isolates. After tertiary screening the isolate(s) or cored putative(s) was placed in 1 ml of SM buffer (containing ~100 pfu) with 20 µl of chloroform and stored at 4°C. The pBluescript® phagemid was removed from the Uni-Zap™ XR vector containing the insert DNA using automatic excision and so allowed analysis of the insert(s) in a plasmid system.

### 2.16.2 In vivo excision of pBluescript® from Uni-Zap™ XR vector by using the ExAssist/SOLR system

Stratagene's Zap-cDNA™ synthesis kit uses the Uni-Zap™ XR vector system. This is designed to allow in vivo excision and recircularisation of any cloned insert that is contained within the lambda vector and forms a phagemid containing the cloned insert.

The following was added to a 50 ml conical tube; 200 µl of XL-I Blue cells with an $OD_{600}$ of 1.0, 100 µl of phage stock (cored putative suspension[s]) and 1 µl of ExAssist helper phage ($1 \times 10^{10}$ pfu/ul). This mixture was incubated at 37°C for 15 min. 3 ml of 2 x YT media (10 g NaCl, 10 g yeast extract and 16 g Bacto-Tryptone per litre) was added and the mixture was incubated for 120-150 min at 37°C with shaking. After this incubation period the tube was heated at 70°C for 20 min and then centrifuged for 15 min at 4000 rpm. The supernatant was decanted

into a sterile tube, and now contained the plasmid packaged as a filamentous phage particle. Storage was at 4°C. The rescued phagemid was plated using the following procedure; 200 µl of SOLR cells ($OD_{600}$ of 1.0) were added to two 1.5 ml Eppendorf tubes. To one 1 µl of phage stock and to the other 50 µl of phage stock, obtained above, was added. The Eppendorf tubes were incubated at 37°C for 15 min. 100 µl from each tube was plated on LB/ampicillin (50 µg/ml) plates and incubated overnight at 37°C. Colonies appearing on the plates were streaked onto new LB/ampicillin plates and a glycerol stock was prepared. Storage was at -20°C. The pBluescript® double-stranded phagemid, which contained the cloned DNA insert, was isolated using Promega Wizard™ minipreps system (section 2.12.2) transformed into XL-I Blue, analysed by restriction digests and the DNA insert(s) sequenced.

### 2.16.3 Transformation of *E. coli* competent cells with pBluescript® containing cloned insert DNA

#### 2.16.3.1 Preparation of competent cells

This method was adapted from Nishimura *et al.* (1990).

A single colony of *E. coli* (XL-I Blue) was inoculated into 100 ml of ψ broth (2 % Tryptone, 0.5 % yeast extract, 0.4 % $MgSO_4$ and 10 mM KCl) with 5 ng/µl tetracycline and incubated at 37°C, with shaking, until an $OD_{550}$ of 0.35 was reached. 70 ml was transferred to two 35 ml Falcon tubes and chilled on ice for 15 min. The tubes were centrifuged at 2000 rpm for 5 min at 4°C to pellet the cells. The supernatant was removed and the cells were resuspended in 10.5 ml of ice cold TFB I solution (100 mM RbCl, 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$

and 15 % glycerol pH 5.8 with acetic acid). This suspension was incubated on ice for 90 min and the cells pelleted as before. The resulting pellet was resuspended in 2.8 ml of ice cold TFB 2 solution (10 mM morpholinosulphonic acids [MOPS], 10 mM RbCl, 75 mM $CaCl_2$ and 15 % glycerol pH 7.0). Aliquots of 200 µl were transferred to 1.5 ml Eppendorf tubes. The solution was snap frozen by placing the Eppendorf tubes in liquid nitrogen. Storage was at -80°C.

### 2.16.3.2 Transformation

200 µl aliquots of the competent *E. coli* cells (XL1-Blue) were thawed between thumb and forefinger. Once thawed they were placed on ice. The pBluescript® phagemid, which contained the cloned insert DNA (10-30 ng of vector), was added to the cells and gently mixed. This was left to incubate on ice for 20 min. The mixture was heat shocked by placing the Eppendorf tube(s) in a water bath at 37°C for 60 seconds and then returning the tube(s) to ice for 2 min. 800 µl of ψ broth was added and the cell mixture was incubated with constant shaking at 37°C for 50 min. The Eppendorf tube(s) was microcentrifuged for 30 seconds to pellet the cells. The resulting pellet was resuspended in 100 µl of ψ broth. The cells were serial diluted and plated onto selective plates. In this case the plates were LB/ampicillin plates. The cells were left to grow overnight at 37°C. Individual colonies were then streaked onto LB/ampicillin plates and LB/ampicillin cultures were grown up overnight at 37°C. Glycerol stocks (stored at -70°C) and plasmid preparations were set up using the overnight cultures.

### 2.16.4 DNA sequencing of cloned insert DNA

DNA sequencing was carried out using Stratagene's protocol for preparing a sequencing gel and the Sequenase® version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio, USA). Double stranded template DNA was prepared as in section 2.12.2.

#### 2.16.4.1 Preparation of a sequencing gel

Two glass sequencing plates were cleaned, to remove any dust, and siliconized. The gel spacers were placed on the sides of one plate and the other glass plate placed on top to sandwich the spacers between them. The bottom and sides of the assembled plates were then taped with electrical tape. This set up was placed on a 45-60° angle. 100 ml of 6 % gel solution (30:2 acrylamide/bisacrylamide) was prepared by diluting acrylamide stock (20 % acrylamide [96.5 g acrylamide, 3.35 g bis-acrylamide], 7 M urea and 1 x TBE in 500 ml batches, filtered and de-gassed) with the appropriate quantity of urea mix (7 M urea, 1 x TBE in 500 ml batches, filtered). 500 µl of 10 % ammonium persulphate was added to this 100 ml 6 % acrylamide gel mix and swirled. 50 µl of TEMED was added and the mixture gently swirled. The acrylamide mix was slowly poured down the sides of the siliconized gel plates using a 50 ml syringe. Any air bubbles that formed were removed. When the acrylamide mix reached the top of the plates a comb was inserted to form wells. The plates were clamped together and the gel was laid down at a slight angle on the benchtop for 1-2 hours, until the gel set.

### 2.16.4.2 Preparation of samples

The pBluescript® plasmid containing the insert DNA was denatured. This was achieved by mixing 20 µl of a Promega Wizard™ miniprep plasmid purification (section 2.12.2) with 2 µl of 2 M NaOH in an Eppendorf tube and incubating the mixture at 37°C for 30 min. The denatured double-stranded template was precipitated by the addition of 2 µl of 3 M sodium acetate and 60 µl EtOH, then placing the mixture at -70°C for 15-30 min. After this refrigeration, the denatured template DNA was microcentrifuged for 10 min and then resuspended in 6 µl of sterile $H_2O$.

### 2.16.4.3 DNA sequencing reactions

#### 2.16.4.3.1 Annealing of template and primer

The following was added to the 6 µl of resuspended denatured DNA in an Eppendorf tube; 2 µl sequencing reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and 2 µl Primer. Known primers that anneal to the pBluescript® vector and designed oligonucleotide primers (Figure 2.1) were used. The reaction mixture was placed at 65°C for 2 min and then allowed to slowly cool to 35°C over 15-30 min. This allowed the annealing reaction to occur. The reaction mixture was then briefly centrifuged and chilled on ice.

#### 2.16.4.3.2 Labelling and termination reactions

While the annealing reaction mixture was cooling from 65°C to 35°C 2.5 µl of each Termination mixture (note for dGTP reactions: 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 50 mM NaCl and one of the

**Figure 2.1** **Internal sequence specific primers designed to complete sequencing analysis of *Bryophyllum fedtschenkoi CAB* cDNA**

Six 18 bp primers were designed and synthesised. These were used to complete the sequencing analysis of both strands of insert DNA of the putative *CAB* clone. The primers are labelled and are shown below with base configurations with 5' and 3' ends.

| Primer | 18 bp sequence |
|---|---|
| 1691 | 5' GAA CCG CGA GCT TGA GGT 3' |
| 1729 | 5' GGT CGG CGA GGT GGT CGG 3' |
| 1730 | 5' TCG ACT ACT TGG GCA ACC 3' |
| 1748 | 5' AGC CCA GAT GGC CAA GAT 3' |
| 1749 | 5' TCC ATG TTT GGA TTC TTC 3' |
| 1773 | 5' TGG CCC ATC TGC TGT GGA 3' |

following 8 μM ddGTP, 8 μM ddATP, 8 μM ddTTP or 8 μM ddCTP) was placed in a labelled, capped Eppendorf tube. The labelling mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP) was diluted 5-fold and then the four termination Eppendorf tubes were pre-warmed in a 37°C water bath.

The labelling reaction was set up by mixing the following with the ice cold annealed DNA mixture; 1 μl 0.1 M DTT, 2 μl diluted labelling mix, 0.5 μl $^{35}$S dATP (1000-1500 Ci/mmol) and 2 μl of diluted Sequenase® version 2.0 T7 DNA polymerase (13 U/μl, 1:8 with enzyme dilution buffer: 10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA). The solutions were mixed and incubated for 2-5 min at room temperature. 3.5 μl of the labelling reaction was transferred to each of the termination tubes (ie; G, C, A, T), mixed and incubated at 37°C for 5 min. The reactions were stopped with the addition of 4 μl of Stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF) and stored on ice or at -20°C, until ready for loading onto the sequencing gel.

### 2.16.4.4 Sequencing gel electrophoresis

The sequencing gel was run in 1 x TBE buffer (10 x TBE for 1 litre: 109 g Tris, 7.3 g EDTA, 55 g boric acid or until pH 8.3 achieved). When the wells were fully covered the sequencing comb was removed and the gel pre-run at 1200 V, 25 W and ~50 mA for 30 min. Prior to loading, the wells were rinsed with 1 x TBE to remove any excess urea and the samples were heated to 75°C for 2 min. The samples were then immediately loaded into the wells (2-3 μl in each lane). Two metal plates were clamped either side of the glass plates to allow even distribution of heat during electrophoresis. The above electrophoresis conditions were

set up. The gel was left to run for 3-6 hours depending upon the region of sequence to be analysed.

After the desired period of electrophoresis the gel and glass plate sandwich was placed in a 10 % methanol/10 % acetic acid solution wash, for 30-60 min, to fix the gel. The top glass plate was carefully pulled away from the sequencing gel. The remaining plate and gel was removed from the washing solution, drained and carefully transferred onto a piece of Whatman 3 MM paper. The gel and paper combination was placed in a gel dryer for 1-2 hours at 80°C or until dry. Once dry, the gel was placed in a light tight cassette with a piece of Fuji-XR film and left overnight at room temperature (section 2.14.3). When the film was developed, the identified region of DNA sequence was determined. Both strands of the DNA insert were sequenced.

Certain oligonucleotide primers used in the sequencing reactions were designed to complete the sequence analysis of both strands of the DNA inserted into the pBluescript®. Figure 2.1 shows these designed primers. They were synthesised by using an automatic sequencing machine.

## 2.17 The measurement of carbon dioxide rhythms in detached leaves of *Bryophyllum fedtschenkoi*

Experiments were set up using the following procedure. Leaves were detached from parent *Bryophyllum fedtschenkoi* plants between 1530 h and 1600 h or at the end of the photoperiod. The leaves were weighed and placed in air-tight brass chambers (internal diameter of 6 cm). The leaf petioles or cut ends were placed in a petri-dish containing distilled water. Different numbers of leaves were placed into each chamber,

ranging from one to eight, depending on the experimental study and the leaves' size. The leaf chambers were surrounded by a water jacket to maintain a constant temperature (eg; 5°C, 15°C, 25°C or 35°C). The temperature of the water jacket was controlled by a water bath which was set up at the appropriate temperature for each individual study. A stream of normal or $CO_2$ free air was passed over the leaves at a constant rate, either 3.0 l/h or 6.0 l/h. The leaf chambers were securely closed and the $CO_2$ content of the emergent gas was determined by using an infra-red gas analyser or IRGA (ADC 225 mark 3, Analytical development company CTD, Hodderson, Herts, UK). The system was controlled by a BBC micro-computer (Model B, Commscot, Glasgow, UK). "Correction factors" for each leaf sample were entered into the computer, these were obtained by dividing the calibration value of the IRGA by the fresh weight of the leaves. The data was printed out as μg $CO_2$/hour/g fresh weight of tissue on a Radioshack TRS-80 colorgraphic printer (Tandy corp, Glasgow, UK). The leaves were either maintained in continuous darkness or light. Continuous illumination was achieved from two 20 W Philips warm white fluorescent tubes placed about 20 cm above the leaf chambers. These gave an average photon fluence rate (400-700 nm) of 15 μmol/$m^2$/s. Continuous darkness was achieved by covering the perspex top of the sealing chamber with a brass cap and covering this with a number of layers of black cloth.

## 2.18 Time course analytical experiments

A number of different time course experiments were set up. Two possible experimental methods were used. Whole plants were removed from their normal growth room at the end of the photoperiod and placed in growth rooms with continuous light or continuous dark conditions at a

constant temperature. At specific time points leaf samples were taken and snap-frozen in liquid nitrogen. Alternatively, leaf samples were taken (refer to section 2.17) and sealed in air tight chambers. Constant conditions were established (eg; constant dark, $CO_2$ free air, 15°C), and leaf samples were again taken at specific time points and snap-frozen in liquid nitrogen. The capacity of the leaf chambers was limited, so time course experiments were carried out in several stages over a few days. Leaf samples set up in continuous darkness were removed under a green safe light and snap frozen in liquid nitrogen. In all the time course studies the samples, once frozen in liquid nitrogen, were stored at -80°C for further analysis.

## Chapter 3. The comparison of *CAB* gene expression and carbon dioxide metabolism in *Bryophyllum fedtschenkoi*

### 3.1 Introduction

Morse *et al.* (1990) state that, "The biochemical mechanism of the biological clock, which is believed to be of fundamental importance in the control of diverse processes, has not been elucidated in any system." The circadian rhythms of $CO_2$ metabolism in *Bryophyllum fedtschenkoi* are well characterised in physiological and biochemical terms, and further biochemical studies of these rhythms and *CAB* gene expression should lead to a greater understanding of the biological clock.

The analysis of *CAB* gene expression should lead to identification of a possible molecular mechanism underlying transcriptional control. For example, studies carried out by Fejes *et al.* (1990) and Millar *et al.* (1991) have shown that the circadian rhythm of *CAB* gene expression is regulated at a transcriptional level by particular regions of DNA sequence.

Work by Wilkins (1960, 1962, 1983, 1989), and Anderson and Wilkins (1989b, 1989c) shows the effects of brief exposure to light, dark, extreme temperature variations or 5% $CO_2$ on the period and phase of the circadian rhythm of $CO_2$ metabolism in detached leaves of *Bryophyllum fedtschenkoi*. If *Bryophyllum fedtschenkoi CAB* gene expression follows a circadian rhythm studies of the effects of treatments that reset the $CO_2$ rhythm would be very revealing. It would then be possible to determine if a physiological link exists between the circadian rhythm of *CAB* gene expression and $CO_2$ metabolism of *Bryophyllum fedtschenkoi*.

The aim of these initial experiments was to determine the validity of studying *CAB* gene expression in *Bryophyllum fedtschenkoi*. These experiments were in relation to a circadian rhythm analysis; as to whether a link exists between *CAB* gene expression and the rhythm of carbon dioxide metabolism shown in detached leaves of *Bryophyllum fedtschenkoi*.

## 3.2 Initial assessment of *CAB* transcript levels in *Bryophyllum fedtschenkoi*

### 3.2.1 Isolation of a good quantity and quality of RNA from *Bryophyllum fedtschenkoi* leaf tissue

High quality RNA could be extracted from small leaves (size 2.0 cm by 1.0 cm) of *Bryophyllum fedtschenkoi* (Figure 3.1) by using the phenol/Kirby extraction procedure. In general the $A_{260}$ / $A_{280}$ ratios were 1.6 to 2.0 and this indicated that the RNA extracted from these small leaves was very pure. However, in some cases a lower yield and poorer ratio was obtained. In addition, the quantity and quality of the RNA obtained from leaf samples of a larger size was much less (see Figure 3.1 and Table 3.1). This was due to certain experimental difficulties. The major problem was the precipitation of a red-coloured product. It was likely that this substance was chemically linking with the RNA and preventing it from dissolving into solution. A number of different washes and RNA isolation procedures were applied in specific experiments to try to remove this impurity.

Figure 3.2 shows a formaldehyde denaturing gel of an RNA sample isolated using the phenol/Kirby method. The band patterns of the rRNA

and tRNA were not clearly visible. This was the result of a higher level of impurities in the extracted RNA sample. These factors affected the ability of the RNA to run through the formaldehyde gel during electrophoresis.

A method of RNA extraction described by Chang *et al.* (1993), section 2.4.2, was used with different sizes of *Bryophyllum fedtschenkoi* leaf material. The extraction buffer included a number of different reagents that removed high concentrations of polysaccharides and phenolics. Reducing agents were present to prevent the RNA binding irreversibly to these compounds. Figure 3.5a shows a formaldehyde denaturing northern gel of samples of extracted RNA. With this method RNA was extracted from *Bryophyllum fedtschenkoi* leaf material of all sizes. The band patterns of RNA were distinctive (Figure 3.5a).

### 3.2.2 Identification of a *CAB* transcript in *Bryophyllum fedtschenkoi* by the use of a heterologous *Arabidopsis CAB* genomic DNA fragment and studies of diurnal and circadian regulation

An *Arabidopsis thaliana CAB* genomic DNA fragment (AB165) was used as a heterologous probe to identify the presence of *CAB* transcripts in *Bryophyllum fedtschenkoi*. Isolation of the original clone pAB165 was by Leutwiler *et al.* (1986). It contains a 1.65 kbp *Eco*RI genomic fragment containing LHCP coding sequences. Figure 3.3 shows the presence of putative *CAB* transcripts identified by this probe. Moreover, it shows that the *CAB* message was present during the middle of the light period and was undetectable during the middle of the dark period. The size of the *CAB* transcript was 0.8 to 1.1 kb in length. I estimated from scans of the autoradiographs (Figure 3.3) that there was a fifty-fold

difference between the *CAB* transcript level in the middle of the light and dark periods.

Figure 3.4a identifies a peak, after 20 hours of constant darkness, in *CAB* gene expression of *Bryophyllum fedtschenkoi*. Plants placed into constant darkness, normal air and at a temperature of 15°C had leaf samples removed at specific time points. These time points were the equivalents of the middle of the light or dark periods of the normal photoperiod. The hybridisation analyses of the northern blot of these RNA samples showed that the *CAB* transcript accumulated in the subjective light treatment. Figure 3.4b identifies control transcripts hybridising to a β-tubulin probe from *C. reinhardtii* (Youngbloom *et al.*, 1985). The complementary transcripts have a constant expression in the conditions of constant darkness. The results indicate that *CAB* gene expression in *Bryophyllum fedtschenkoi* was not under diurnal regulation. The method of control was by a self-regulating mechanism that does not require light. The size of *CAB* and β-tubulin transcripts identified in these hybridisation analyses were 0.8 kb to 1.1 kb and 1.15 kb to 1.35 kb in length.

Figure 3.5a and Figure 3.5b show the denaturing formaldehyde gel and autoradiograph of an experiment to assess *CAB* gene expression in *Bryophyllum fedtschenkoi* under conditions of continuous light, normal air and at a temperature of 15-17°C. The leaf samples were removed after 12 hours and 24 hours from plants placed in these constant conditions and frozen under liquid nitrogen. These points were equivalent to the middle of the dark and light periods of the normal photoperiod. Figure 3.5b shows the autoradiograph of the northern blot of the gel hybridised with a homologous *Bryophyllum fedtschenkoi CAB* cDNA probe (see chapter 4). Although significant non-specific hybridisation to, presumably, rRNA

transcripts is evident in this experiment, the *CAB* transcripts can be identified from their size. After 12 hours in these constant conditions a reduction of the *CAB* transcript level of accumulation occurred. Consequently, the level of expression increased after 24 hours of these constant conditions. This pattern of regulation suggested that the control of *CAB* gene expression did not depend solely on light/dark changes.

These experimental results in conditions of continuous darkness and continuous light suggest that not light, but some internal mechanism(s) periodically activated the synthesis and decay of the *Bryophyllum fedtschenkoi CAB* mRNA. The indication was that the control of *CAB* gene expression in *Bryophyllum fedtschenkoi* leaves was by an endogenous or circadian-like mechanism.

### 3.3 Initial study of ontogenetic regulation of *CAB* gene expression in *Bryophyllum fedtschenkoi*

This study was carried out to assess the largest *Bryophyllum fedtschenkoi* leaf sample that could be taken that showed *CAB* gene expression. Further experiments could then be carried out to examine to what extent the rhythms of *CAB* gene expression and $CO_2$ metabolism are linked in *Bryophyllum fedtschenkoi*. Leaf samples were taken from plants in the middle of the normal light period. The samples were taken from different positions on the stem, from which the smallest leaves were 1.0 cm to 1.5 cm in length and were located near the tip of the plant. Refer to Figure 3.6 for details of all of the leaf samples obtained. The limitation on how large a leaf sample could be taken was the phenol/Kirby RNA extraction procedure. The difficulties of this are mentioned in section 3.2.1. The RNA extracted from these leaf samples was run on a

formaldehyde denaturing agarose gel and hybridised with the heterologous probes *CAB* AB165 and α-tubulin cDNA. The α-tubulin cDNA was isolated from *C. reinhardtii* (Brunke *et al.* 1984, Silflow *et al.* 1985) and was used as a control probe. Hybridisation analysis on the northern blot in Figure 3.4 has shown the α-tubulin gene to have a constant expression with a transcript size of 1.4 kb to 1.7 kb. Figure 3.6 panel (a) is a photo of the northern denaturing gel of RNA extracted from the different leaf sizes. Figure 3.6 panel (b) and panel (c) show the results of the northern blot of the gel in Figure 3.6 panel (a) hybridised with the heterologous *CAB* AB165 and α-tubulin cDNA probes. *CAB* gene expression varied depending on leaf size whereas α-tubulin gene expression remained constant. Unfortunately slight differences in the RNA loading confuse the interpretation of how *CAB* gene expression varied with leaf size. Because of this the autoradiographs, shown in Figure 3.6 panels (b) and (c), were scanned (as in section 2.14.6). The values for the *CAB* transcripts were compared to the values for the α-tubulin transcripts and a pattern of ontogenetic regulation of *CAB* gene expression in *Bryophyllum fedtschenkoi* was identified. Figure 3.6 panel (d) is a graph of how *CAB* gene expression varies with leaf size in *Bryophyllum fedtschenkoi*. This graph showed that *Bryophyllum fedtschenkoi CAB* gene expression increased up to a maximum in 2-2.5 cm leaves and then decreased in leaves of increasing size. Leaves 4-4.5 cm have very low levels of *CAB* transcripts.

## 3.4 Linking the studies of carbon dioxide gaseous exchange in detached leaves of *Bryophyllum fedtschenkoi* with its *CAB* gene expression

### 3.4.1 Carbon dioxide gaseous exchange under conditions of continuous light, normal air and a constant temperature of 15°C

Leaves that are normally used in studies of the circadian oscillations of $CO_2$ metabolism in *Bryophyllum fedtschenkoi* are 6.0 cm by 3.5 cm in size. They are usually the sixth or seventh leaf down from the tip of the plant. The *Bryophyllum fedtschenkoi* leaves which show *CAB* gene expression are much smaller in size than these leaves (section 3.2.2). The sensitivity of the IRGA apparatus that was used to measure the rhythms of $CO_2$ metabolism was such that it would be difficult to detect rhythms of $CO_2$ metabolism in a single small leaf. Hence initial experimental studies used 4 detached leaves of size 1.5 cm by 1.0 cm. The detached leaves were placed in the air-tight chambers of the IRGA (section 2.17) under conditions of continuous light, normal air and a constant temperature of 15°C. A leaf that showed the characteristic pattern of $CO_2$ metabolism under such conditions was also placed into one of the air-tight chambers for use as a control rhythm. The pattern of gaseous exchange is shown in Figure 3.7. A circadian rhythm of gaseous exchange occurred in both the experimental and control *Bryophyllum fedtschenkoi* leaves. The smaller leaves showed a rhythm with the same phase as that given by the larger leaf but with a greatly reduced amplitude. Figure 3.8 shows the results of an experiment in which the air-tight chambers contained differing numbers of leaves (1 control leaf, 4 small leaves or 8 small leaves). The results indicated that when 8 small leaves were used no apparent rhythm in $CO_2$ metabolism

occurred. The gaseous exchange of the 8 individual small leaves must have been masking each others' effects such that no variation in the $CO_2$ levels appeared to be occurring. One notable feature of these results was that the smaller leaves gave a higher rate of photosynthesis than the larger leaves. This is shown by the larger rate of $CO_2$ uptake or negative values on the y-axis of the Figures. To investigate whether this could obscure rhythms of $CO_2$ metabolism, the rate of photosynthesis was reduced by decreasing the light intensity by means of a neutral filter. The neutral filter was placed over the air-tight chamber in a number of studies to reduce the incident light intensity. Figure 3.9 shows the results of this experiment. When the light intensity was reduced the circadian rhythms of $CO_2$ gaseous exchange of the large and small leaves appeared similar. The major difference was that the rhythm in the smaller leaves dampened out much faster than that in the larger leaves.

These data suggested that rhythms of $CO_2$ metabolism and *CAB* expression could not easily be compared in either small leaves (which gave very little $CO_2$ rhythm) or larger ones (which gave very little *CAB* expression). A leaf size had to be identified from which high quality RNA could be extracted for *CAB* gene expression studies and which also showed a clear circadian rhythm in $CO_2$ metabolism. I therefore investigated an intermediate size of leaf, 3-4 leaves (length 3.5 cm to 4.0 cm) from the top of the plant. One chamber contained 2 of these middle-sized leaves while a second contained one large leaf. The results are shown in Figure 3.10. A clear circadian rhythm of $CO_2$ metabolism was seen in these conditions with the middle-sized leaves. The amplitude was larger than those shown in Figures 3.7, 3.8 and 3.9. In these leaves the rhythm damped out more quickly and the period may have been slightly different than in the larger leaves.

### 3.4.2 Carbon dioxide gaseous exchange in *Bryophyllum fedtschenkoi* leaves of differing sizes; under conditions of continuous darkness, $CO_2$ free air and a temperature of 15°C

Initial studies examined the pattern of $CO_2$ rhythms in 4 small leaves of size 1.0 cm to 2.5 cm in length under the conditions of continuous darkness, $CO_2$ free air and at a temperature of 15°C. Figure 3.11 shows the results of this experiment. The large leaves exhibited a circadian rhythm in $CO_2$ gaseous exchange for three cycles before it faded away. The 4 smaller leaves appeared to follow this pattern but the amplitude of the rhythm dropped dramatically after the first cycle. Hence the circadian rhythm of $CO_2$ metabolism was not as defined in these smaller leaves in continuous darkness and $CO_2$ free air as it was in larger leaves.

I therefore compared 3 differently sized leaves in continuous darkness and $CO_2$ free air at a temperature of 15°C. Two slightly differing studies were carried out. In one a single leaf for the middle and large leaves was sealed in individual air-tight chambers of the IRGA analytical equipment, whereas 4 small leaves were placed into the remaining air-tight chamber. In the other study one *Bryophyllum fedtschenkoi* leaf of each size was placed into individual air-tight brass chambers and the $CO_2$ gaseous exchange analysed. Figures 3.12 and 3.13 are the IRGA analyses of the detached leaves under the conditions of continuous darkness and $CO_2$ free air. For the large and middle leaves circadian rhythms of $CO_2$ gaseous exchange both followed the same cycle and had the same period lengths. The amplitude of the rhythms decreased from one cycle to the next. The smaller leaves, whether 4 leaves or 1 leaf is contained within the air-tight sealed chamber, also show a circadian rhythm which followed that of the large and middle sized leaves. As in the study in Figure 3.11 the

amplitude of this rhythm declined rapidly, but the rhythmical pattern of gaseous exchange that occurred was clearer. In Figures 3.12 and 3.13 the flow rate of $CO_2$ free air through the air-tight chambers was 6.0 l/h whereas in Figure 3.11 the flow rate was 3.0 l/h. This difference may explain why a more defined rhythm occurred in the latter experiments with the smaller leaves.

### 3.4.3 Carbon dioxide gaseous exchange under conditions of continuous darkness, normal air and a constant temperature of 15°C

When *Bryophyllum fedtschenkoi* leaves, which are normally used in the studies of the oscillations of $CO_2$ metabolism, are placed in the constant conditions of continuous darkness and normal air at a temperature of 15°C no circadian rhythm occurs (Wilkins, 1959). In the same continuous conditions a peak in *CAB* gene expression occurred after 20 hours (Figure 3.4). This implied that the control of *CAB* gene expression was by a self-regulating mechanism that did not require light. The *CAB* gene expression was identified in the smaller leaves, size 1.0 cm to 1.5 cm, of *Bryophyllum fedtschenkoi*. Therefore, experiments were carried out to determine their pattern of $CO_2$ metabolism in the constant conditions mentioned above. The experimental principles discussed in section 3.4.1 were applied. 4 small leaves were placed in an air-tight chamber under the constant conditions. A leaf that showed the characteristic pattern of $CO_2$ metabolism under such conditions was also placed into one of the air-tight chambers for use as a control rhythm. Figure 3.14 shows the results of this experiment. Both smaller and larger leaves showed one trough in $CO_2$ output corresponding to subjective night, followed by a point of higher but declining output. In agreement with earlier work, a circadian rhythm was not detected in these conditions.

## 3.5 Discussion

### 3.5.1 Isolation of RNA from *Bryophyllum fedtschenkoi* leaf material

Mitra *et al.* (1993) state that plant tissues are probably the most difficult from which to isolate RNA. The main problems that are associated with plant nucleic acid isolations have been attributed to the presence and copurification of plant polyphenolic compounds and polysaccharides. The presence of these compounds causes the formation of RNA complexes that usually appear as a coloured insoluble precipitate. The complexed nucleic acid is found to be unsuitable for cDNA synthesis, interferes with analysis by UV absorption spectrophotometry and shows poor resolution by electrophoresis through agarose gels. The aggregated material is trapped in the wells. These complications were also discussed by Tesniere *et al.* (1991), Chang *et al.* (1993), Lewinsohn *et al.* (1994), Ainsworth (1994) and Wang *et al.* (1994) in a wide variety of plants. *Bryophyllum fedtschenkoi* is a succulent plant. Consequently the leaf material is tough and fleshy and very likely to contain a large amount of these products. So it was of no surprise that extracting high amounts of good quality RNA was a laborious task.

The phenol/Kirby method enabled me to obtain high quality undegraded RNA from small leaves of *Bryophyllum fedtschenkoi*. It relied upon phenol/chloroform extractions to remove any protein and carbohydrate fractions and employed an RNAase inhibitor in 4-aminosalicyclic acid that prevented RNA degradation. The *Bryophyllum fedtschenkoi* leaf material was ground under liquid nitrogen. When too much of this ground leaf material was used, with the extraction buffer, snap freezing often occurred. Consequently the protein and carbohydrate impurities

were not removed by the phenol/chloroform washes and low quantities of RNA were extracted. Section 3.2.1 discussed the precipitation of a red coloured product that was likely to be removing the RNA, into an insoluble complex, and caused problems with its resuspension. This coprecipitation was also found to affect the analysis of RNA by UV absorption and agarose gel electrophoresis. Some of the methods used by Tesniere *et al.* (1991) were introduced into the phenol/Kirby extraction procedure. The most successful result was with a 2.5 M sodium acetate wash. This wash was reported to remove charged oligosaccharides that coprecipitate with potato tuber RNA. An alternative view (Ainsworth, 1994) was that the low pH of 5.5 stopped phenolic oxidation and polysaccharide precipitation so the RNA would be removed from its insoluble complex. RNA was not efficiently extracted from large *Bryophyllum fedtschenkoi* leaves by using the phenol/Kirby protocol. There were also difficulties in isolating poly(A)$^+$ RNA. Wang *et al.* (1994) mentioned that the phenolic compounds were compartmentalized in the vacuoles. Therefore, homogenization disrupted the cells and the polyphenols were released from the vacuoles to react with the nucleic acids. The large *Bryophyllum fedtschenkoi* leaves were physiologically more developed than their smaller counterparts. Following up the argument of Wang *et al.* (1994), it was plausible that in the large leaves the RNA formed complexes with the increased amounts of these compounds that were contained in the bigger vacuoles. This may have resulted in the formation of the insoluble complex. In the smaller leaves there may have been less of the phenolics because of the smaller vacuoles and so more RNA was free to be resuspended in solution.

An efficient method of isolating RNA from all sizes of *Bryophyllum fedtschenkoi* had to be developed. This would eventually aid in

comparing the *CAB* gene expression and $CO_2$ rhythms of *Bryophyllum fedtschenkoi*. Wang *et al.* (1994) define what has to be done to achieve this. An effective method for RNA isolation should either be able to remove polyphenolic compounds or to prevent their binding to the RNA molecules during the extraction. By using a high buffer temperature of 65°C (De Vries *et al.*, 1988) the problem of the RNA extraction buffer snap freezing, which occurred when too much of the ground *Bryophyllum fedtschenkoi* leaf tissue was used in the buffer, was eliminated. However, when large amounts of leaf tissue, especially with RNA preparations from the larger leaves, were used very small quantities of RNA were isolated. The RNA isolated was coloured in appearance, which suggested impurities were present and that the extraction buffers were saturated by the material. A number of different techniques could be applied to counteract the effects of the polyphenolics and polysaccharide compounds. Mitra *et al.* (1993) use a high salt precipitation step to maintain the polysaccharides in solution. Lewinsohn *et al.* (1994) and Wang *et al.* (1994) effectively sequestered the interfering phenolic compounds with vinylpyrrolidone polymers.

RNA was successfully extracted from different leaf sizes of *Bryophyllum fedtschenkoi* by utilising the procedures of Chang *et al.* (1993). They prevented the oxidation of phenolic compounds, which formed insoluble complexes with RNA, by using PVP and 2-mercaptoethanol as reducing agents. A high NaCl concentration in the buffer helped remove the polysaccharides and dissolved the detergent CTAB-RNA complex. A high buffer temperature prevented difficulties of the ground leaf material snap freezing the buffer and because there were no phenol extractions damage of the poly(A)$^+$ RNA was minimized. The only problem with this protocol (section 2.4.2) was that RNA isolated from *Bryophyllum*

*fedtschenkoi* leaves had a tendency to be degraded more frequently. This was because no RNAase inhibitors were present in the extraction buffers.

### 3.5.2 Initial studies of *Bryophyllum fedtschenkoi CAB* gene expression

*CAB* gene expression, measured by the steady-state level of *CAB* transcripts, was identified in *Bryophyllum fedtschenkoi* and showed a mechanism of control that was not regulated by a light/dark cycle alone. The size of the transcript identified was 0.8 to 1.1 kb. This was similar to *CAB* transcripts identified by northern hybridisation analysis, which gave messages of approximately 1.0 kb in size, in *Lycopersicon esculentum* (Piechulla, 1988), *Arabidopsis thaliana* (Zhang *et al.*, 1991) and *Hordeum vulgare* (Brandt *et al.*, 1992).

One of the major features of *CAB* genes is their transcriptional regulation by light. In the case of higher plants several investigators (Kloppstech, 1985; Piechulla *et al.*, 1987; Otto *et al.*, 1988; Nagy *et al.*, 1988; Paulsen *et al.*, 1988; Giuliano *et al.*, 1988; Stayton *et al.*, 1989; Taylor, 1989; Meyer *et al.*, 1989; Millar *et al.*, 1991) have documented that the steady-state transcript levels of the chlorophyll a/b binding protein genes (*CAB* genes) fluctuate with diurnal light/dark cycles. In most cases, these fluctuations have been shown to be controlled by a circadian clock. Also Gagné *et al.* (1992) and Salvador *et al.* (1993) identified that variations in the levels of chloroplast gene transcripts occurred during a light/dark cycle in *Chlamydomonas eugamentos* and *reinhardtii* respectively. In the majority of these studies the apparent trend was that *CAB* mRNA levels tended to reach a maximum during the middle of the light period and fall to a minimum during the dark period. In a number of cases (Kloppstech, 1985; Stayton *et al.*, 1989) the *CAB* transcript levels

do increase before the start of the light period. This was frequently stated as an anticipation of the light and was associated with endogenous rhythms. In the case of *Bryophyllum fedtschenkoi* I estimated from scans of autoradiographs that there was a fifty-fold difference between the *CAB* transcript level in the middle of the light and dark periods. This was comparable to a one hundred-fold variation between the highest (day) and lowest (night) levels of steady-state *CAB* mRNA levels of *Nicotiana tabacum* identified by Paulsen *et al.* (1988), whereas in *Euglena gracilis*, upon exposure to light, Kishore *et al* (1992) found there was a two-fold increase in the level of a *CAB* mRNA.

When *Bryophyllum fedtschenkoi* plants were placed into conditions of continuous light or dark in normal air *CAB* gene expression showed fluctuating levels. The steady state levels of the *CAB* transcripts reduced and increased over a period of 24 hours. This suggested the existence of a circadian rhythm. However, to state definitively that *CAB* gene expression in *Bryophyllum fedtschenkoi* was controlled by an endogenous oscillator further studies have to be carried out. These would be in constant conditions and over a number of days. The *CAB* gene expression will have to show a repetitive pattern with a period length of 24 hours. For example Giuliano *et al.* (1988) showed that when *Lycopersicon esculentum* plants were transferred to conditions of continuous darkness *CAB*/II transcription oscillated over three days with a period length of approximately 24 hours.

### 3.5.3 Linking the studies of carbon dioxide gaseous exchange in detached leaves of *Bryophyllum fedtschenkoi* with its *CAB* gene expression

I found that the rhythm of $CO_2$ metabolism in detached leaves of *Bryophyllum fedtschenkoi* was dependent on the size of leaf material used and upon the conditions that were set up. With the smaller leaves the circadian rhythms were less defined. Also the level of *CAB* gene expression in *Bryophyllum fedtschenkoi* tended to decline with the larger leaves. These two factors were not unexpected but they did raise the following questions. As a plant leaf develops different oscillators may exist to control specific gene expression. The morphological development of the leaf may play a role. The smaller *Bryophyllum fedtschenkoi* leaves may not carry out CAM extensively. In the older leaves an operational photosynthetic system has been established and if the chlorophyll a/b binding proteins are not continually turned over the presence of the *CAB* transcripts may not be required. In conditions of continuous light the circadian rhythm of $CO_2$ metabolism may also be affected by the level of light intensity.

Cheng *et al.* (1991) and Jones (1975) discussed the effects of developmental and environmental factors affecting the induction of CAM. Jones (1975) found that young leaves of *Bryophyllum fedtschenkoi* do not exhibit CAM; they behave like typical mesophytes. The mature leaves were found to exhibit CAM and the intermediate leaves were intermediate in their behaviour. This fact alone may account for the variations of circadian oscillations that I identified in the different leaf sizes. It was argued that the presence of CAM in the older *Bryophyllum fedtschenkoi* leaves resulted from an increased vacuole size. Low activities of the

enzymes PEPc and the PEPc kinase (discussions with Carter, P. J.) were found in the smaller *Bryophyllum fedtschenkoi* leaves. Osmond and Holtum (1981) say that in some of the constitutive CAM plants, the capacity for acid synthesis in young leaves is poorly developed. This adds to the argument that CAM may not be operating so the $CO_2$ rhythms were less likely to be clear patterns. However, Kluge *et al.* (1992) carried out an *in situ* study of CAM metabolism in *Kalanchoë beharensis* and discovered that there was no substantial contribution of the $C_3$ pathway to the fixation of external $CO_2$ by the young leaves. But, in *Kalanchoë beharensis*, there was a difference in the rates of $CO_2$ exchange of the young leaves as compared to the mature leaves in that they were much lower.

In the plant *Lycopersicon esculentum* Piechulla (1988) found that the steady-state mRNA levels of *CAB* genes varied in leaves at different developmental stages. The highest transcript levels of *CAB* genes were in leaves 4.0 cm long. The transcript levels decreased with increasing leaf size. Busheva *et al.* (1991) showed a correlation between the diurnal cycle of the level of mRNA in the cytosol and the accumulation of the LHCII in the thylakoid membranes of *Triticum aestivum*. They found that the relative changes of chl-a/chl-b ratios showed a gradual dampening with leaf age. This was argued to be the consequence of the gradual decrease of *CAB* mRNA levels during leaf development. Meyer *et al.* (1989) studied the circadian expression of *CAB* genes in a number of plant species and carried out a detailed characterisation of the pattern of *CAB* mRNA expression in *Lycopersicon esculentum* leaves. They monitored diurnal mRNA fluctuations from different leaf sizes and concluded the typical circadian oscillation was observed at different developmental stages of the leaves. However, the amplitude of the

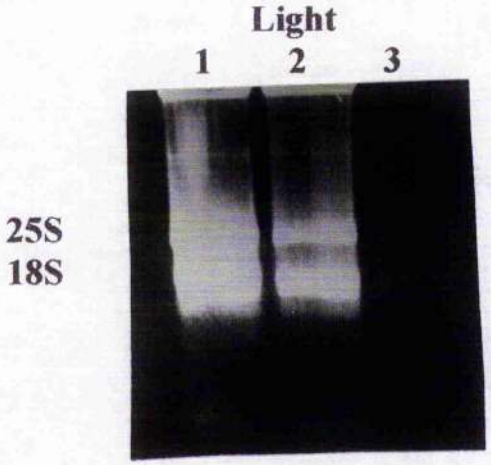
oscillation found was also dependent on the developmental stage of the leaves of *Lycopersicon esculentum*. I found that *CAB* transcript levels reached a maximum in *Bryophyllum fedtschenkoi* leaves of size 2.0 cm to 2.5 cm and decreased with increasing leaf size. These findings meant that the choice of an appropriate *Bryophyllum fedtschenkoi* leaf was important to this study. However, these findings were based on measuring transcript levels relative to a constant amount of total RNA and were achieved by comparing the scanned values of *CAB* and tubulin transcripts. The validity of this comparison was dependent on the constitutive expression of tubulin. The tubulin genes are essentially involved in microtubule formation in a cellular system. They are 'housekeeping' genes and very likely to show constitutive expression. Therefore, in *Bryophyllum fedtschenkoi*, the steady state mRNA levels of *CAB* genes was dependent on the developmental stage of the leaves.

In summary, to establish whether a circadian rhythm of *CAB* gene expression was linked to the circadian rhythm of $CO_2$ metabolism middle sized leaves (length 4.0 cm to 4.5 cm) were selected for further experiments. These leaves showed clear rhythms of $CO_2$ metabolism in various continuous conditions. Also the Chang *et al.* (1993) method of isolating RNA could be used with these leaves for use in the studies of *CAB* gene expression in *Bryophyllum fedtschenkoi*.

**Figure 3.1 RNA samples extracted from different sizes of *Bryophyllum fedtschenkoi* leaf tissue by using the phenol/Kirby extraction procedure**

RNA was extracted from *Bryophyllum fedtschenkoi* leaf sizes 1 (length 1.0 cm to 1.5 cm), 2 (length 2.0 cm to 2.5 cm) and 3 (length 3.0 cm to 3.5 cm) as described in section 2.4.1. Figure 3.1a and Figure 3.1b show the extracted RNA from these leaf samples isolated in the middle of the light and dark periods of the growth room in which the plants were located. The major rRNA bands are indicated.

a.




b.

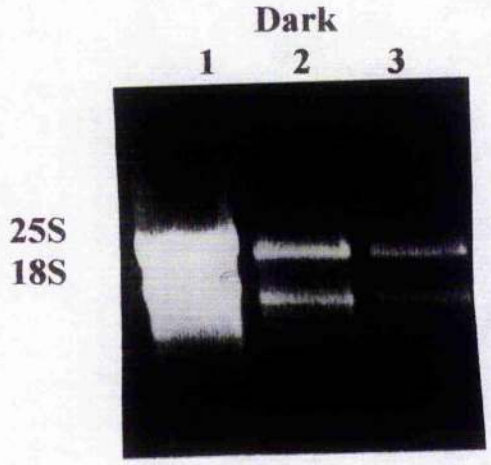


**Table 3.1 The absorbance of *Bryophyllum fedtschenkoi* total RNA, extracted from different sizes of leaf tissue, at 260 nm and 280 nm**

5 µl of each of the total RNA samples shown in Figure 3.1a and Figure 3.1b was dissolved in 995 µl of DEPC-treated distilled water and its absorbance measured at 260 nm and 280 nm against a DEPC-treated distilled water blank. These values are given along with the ratios of these absorbances ($A_{260}$ / $A_{280}$) and the calculated concentration of each RNA sample, see section 2.7.

| Leaf size | Period Isolated | Absorbance 260nm | 280nm | Ratio $A_{260}/A_{280}$ | RNA concentration (mg/ml) |
|---|---|---|---|---|---|
| 1 | Middle Light | 0.503 | 0.418 | 1.20 | 4.02 |
| 2 | Middle Light | 0.381 | 0.353 | 1.08 | 3.05 |
| 3 | Middle Light | 0.031 | 0.027 | 1.15 | 0.25 |
| 1 | Middle Dark | 0.428 | 0.261 | 1.64 | 3.42 |
| 2 | Middle Dark | 0.043 | 0.024 | 1.79 | 0.34 |
| 3 | Middle Dark | 0.028 | 0.011 | 2.55 | 0.22 |

**Figure 3.2** **Photograph of a denaturing formaldehyde gel showing four lanes of total RNA extracted from *Bryophyllum fedtschenkoi* leaf tissue using the phenol/Kirby method**

Leaf tissue, length 1.0 cm to 1.5 cm, was isolated in the middle of the light period. 10 μg of RNA extracted from this leaf material was run in each lane of this gel. The RNA sample had an $A_{260}$ / $A_{280}$ ratio of 1.6 and a concentration of 0.9 mg/ml. The samples were prepared as in section 2.4.1.2 and 2 μl of stock 10 mg/ml EtBr was added to the total RNA samples prior to loading them into the wells of the denaturing gel. The major rRNA bands are indicated.

25S

18S

**Figure 3.3 Autoradiograph showing changes in the abundance of the *Bryophyllum fedtschenkoi CAB* transcript during periods of darkness and light**

*Bryophyllum fedtschenkoi* leaf samples, length 1.0 cm to 1.5 cm, were isolated in the middle of the light (ML) and dark (MD) periods of the growth room. RNA extractions were performed, as in section 2.4.1, and 10 μg of each was run on a formaldehyde denaturing gel, as in section 2.8.2. Promega RNA markers were used in this denaturing gel. The blot was prehybridised and hybridised with Denhardts, as in section 2.14.1.1, at a temperature of 35-36°C. The *Arabidopsis* AB165 *CAB* DNA probe was prepared using Amersham's Megaprime labelling kit, section 2.13, and had a specific activity of 2.2 x $10^8$ dpm/μg. The autoradiograph shows the *CAB* transcript, size 0.8-1.1 kb. The faint upper band shown is rRNA of size 2.5-3.0 kb. These are both identified on Figure 3.3. The rRNA was identified using Methylene blue staining, section 2.14.5.

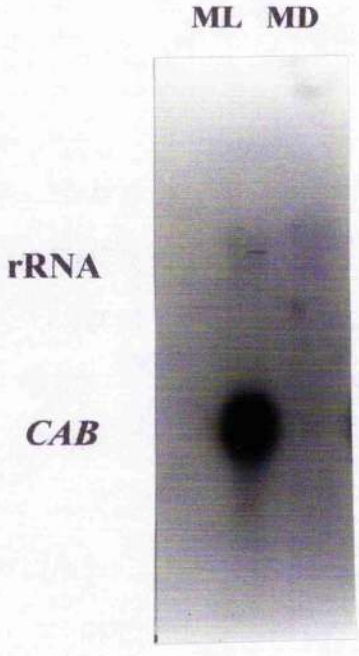
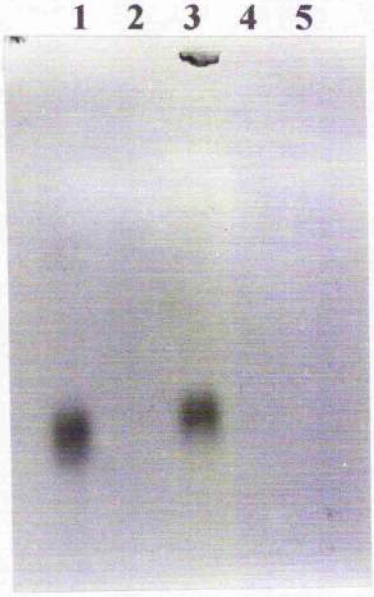

ML MD
rRNA
CAB

**Figure 3.4** ***CAB*** **and β-tubulin gene expression in** ***Bryophyllum fedtschenkoi*** **in conditions of continuous darkness, normal air and a constant temperature of 15°C**

*Bryophyllum fedtschenkoi* plants were placed into a dark room at the end of the normal light period. Leaves, length 1.0 cm to 1.5 cm, were removed at time points equivalent to the middle of the light and dark periods over two days. The samples were prepared as in Figure 3.3. Figure 3.4a shows the result of a hybridisation with the AB165 *CAB* probe (specific activity 3.6 x $10^8$ dpm/μg). The *CAB* transcript size is 0.8 - 1.1 kb. Figure 3.4b shows the result of a hybridisation with the *C. reinhardtii* β-tubulin cDNA probe (specific activity 2.2 x $10^8$ dpm/μg). Three hybridisation bands are apparent; the upper two are ribosomal RNA, sizes 1.6 - 1.8 kb and 3.0 - 3.8 kb respectively, and the lower band is presumably complementary β-tubulin, size 1.15 - 1.35 kb. The ribosomal RNA, *CAB* and β-tubulin transcripts are indicated. The different lanes are shown below (ML represents middle of light and DD represents continual darkness).

| Lane No. | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Condition | ML | DD | DD | DD | DD |
| Time point (h) | 1200 | 2400 | 1200 | 2400 | 1200 |

a.




b.

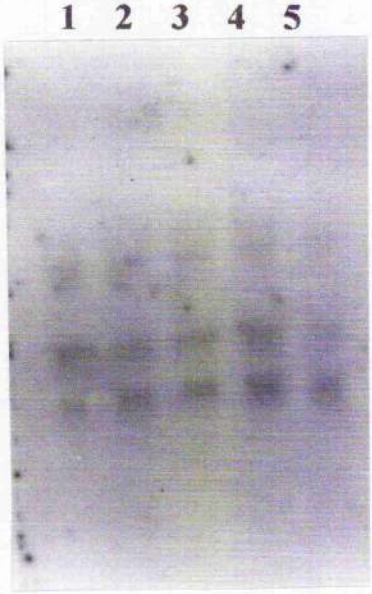
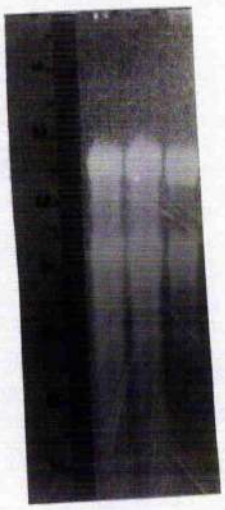


**Figure 3.5** ***Bryophyllum fedtschenkoi CAB*** **gene expression under conditions of continuous light, normal air and a constant temperature of 15 - 17°C**

*Bryophyllum fedtschenkoi* plants were removed at the end of the normal light period and placed into a growth room with continuous light and a constant temperature of 15-17°C. Leaf samples, length 1.0 cm to 1.5 cm, were isolated in the middle of the subjective dark and light periods. RNA was extracted from the leaf samples by using the method of Chang *et al* (1993), as in section 2.4.2. 10 μg of each RNA sample was run on a denaturing formaldehyde gel, as in section 2.8.2, and stained with EtBr (stock 10 mg/ml) after electrophoresis. The migration of the rRNA bands was measured to use as markers. Figure 3.5a is a photograph of this gel. The northern blot of the gel shown in Figure 3.5a was hybridised with the homologous *Bryophyllum fedtschenkoi CAB* cDNA probe (specific activity 1.3 x $10^7$ dpm/μg) at a temperature of 55°C using the BSA method (section 2.14.1.2). The filter was washed four times. The least stringent conditions were in 2 x SSC / 0.1 % (w/v) SDS and the most stringent conditions were in 0.1 x SSC / 0.1 % (w/v) SDS. All washes were of 10 minutes duration and at a temperature of 60°C. After washing, the blot was autoradiographed as discussed in section 2.14.3. Figure 3.5b shows the result of this hybridisation. Three transcripts are identified. The bottom one is the *CAB* transcript and the upper two are rRNA. The different lanes are shown below (ML represents middle of light and LL represents continual light);

| Lane No. | 1 | 2 | 3 |
|---|---|---|---|
| Condition | ML | LL | LL |
| Time point (h) | 1200 | 2400 | 1200 |

a.




b.

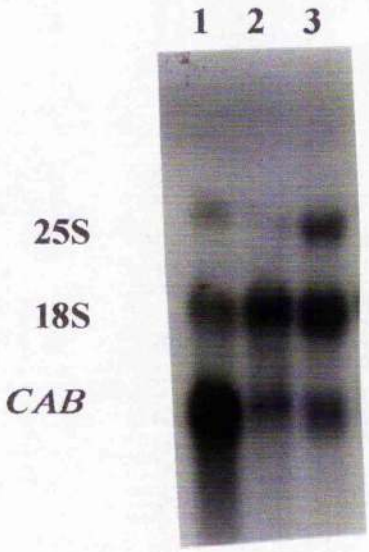
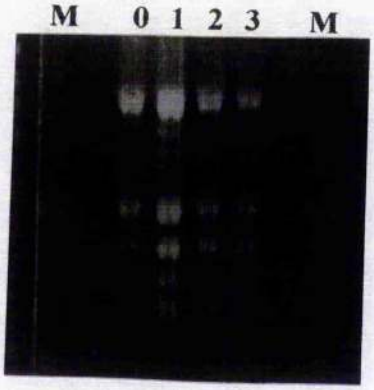


**Figure 3.6 Levels of *CAB* and α-tubulin transcripts in *Bryophyllum fedtschenkoi* leaves of different size**

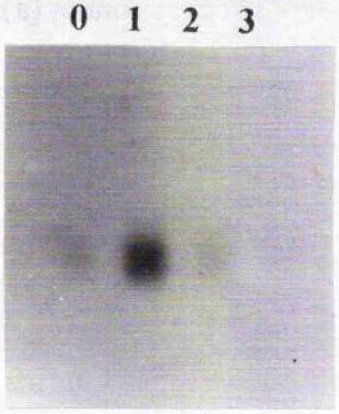
*Bryophyllum fedtschenkoi* leaf tissue of differing size was isolated in the middle of the light period of the growth room. RNA extractions were performed, as in section 2.4.1, and the RNA samples of each leaf size were pooled. Figure 3.6 panel (a) shows a photograph of the stained formaldehyde gel of these RNA samples (10 μg loaded per lane) along with the GIBCO BRL 0.16-1.77 kb RNA marker (M). The formaldehyde denaturing gel shown in panel (a) was blotted onto a nylon filter as in section 2.11.1. Figure 3.6 panels (b) and (c) show the results of hybridisation analysis with the AB165 *CAB* cDNA probe (specific activity 2.7 x $10^8$ dpm/μg) and the *C. reinhardtii* α-tubulin cDNA probe (specific activity 3.6 x $10^8$ dpm/μg). The blots were hybridised and washed as in Figure 3.3. The *CAB* and α-tubulin transcripts are identified. The *CAB* transcript was of size 0.8 - 1.1 kb. The autoradiographs of the hybridisation results shown in panel (b) and panel (c) were scanned as in section 2.14.6. The values obtained for the *CAB* / α-tubulin ratio were used in the graphical analysis shown in Figure 3.6 panel (d) that assesses how *CAB* gene expression varies with leaf size. The key to the differently sized leaf RNA samples is shown below.

Key to leaf sizes; 0 - 1.0 cm to 1.5 cm in length
1 - 2.0 cm to 2.5 cm in length
2 - 3.0 cm to 3.5 cm in length
3 - 4.0 cm to 4.5 cm in length

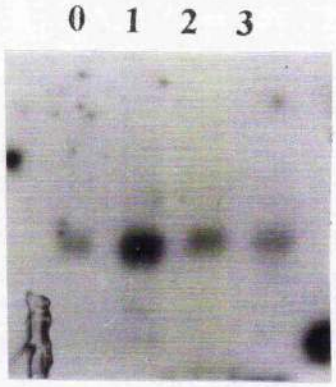
a


M 0 1 2 3 M

b


0 1 2 3
CAB

c


0 1 2 3
α–tub

d

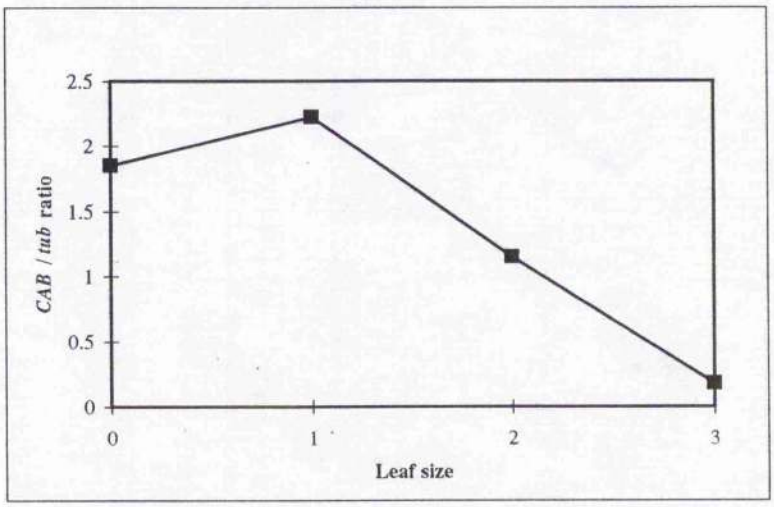

2.5
2
1.5
1
0.5
0
CAB / tub ratio
0
1
2
3
Leaf size

**Figure 3.7 Carbon dioxide gaseous exchange in detached leaves of *Bryophyllum fedtschenkoi* under conditions of continuous light, normal air and a constant temperature of 15°C**

*Bryophyllum fedtschenkoi* Leaves were detached and placed into an air-tight sealed chamber at the end of the normal light period. One large leaf and 4 small leaves were placed into separate chambers. Continuous light conditions were set up and the $CO_2$ gaseous exchange was measured, as discussed in section 2.17, by using an infra red gas analyser or IRGA. 1200 h is shown by the letter L. The table below shows the leaf size, number of leaves per chamber and the total weight of leaf tissue. The flow rate of air passing across the leaves was 3.0 l/h. Gaseous exchange was measured in μg $CO_2$ per hour per gramme of tissue.

| Leaf classification | leaf size (cm) | No. of leaves per chamber | Total weight of leaf tissue (g) |
|---|---|---|---|
| Large | 7.0 | 1 | 3.6 |
| Small | 2.5 | 4 | 1.1 |

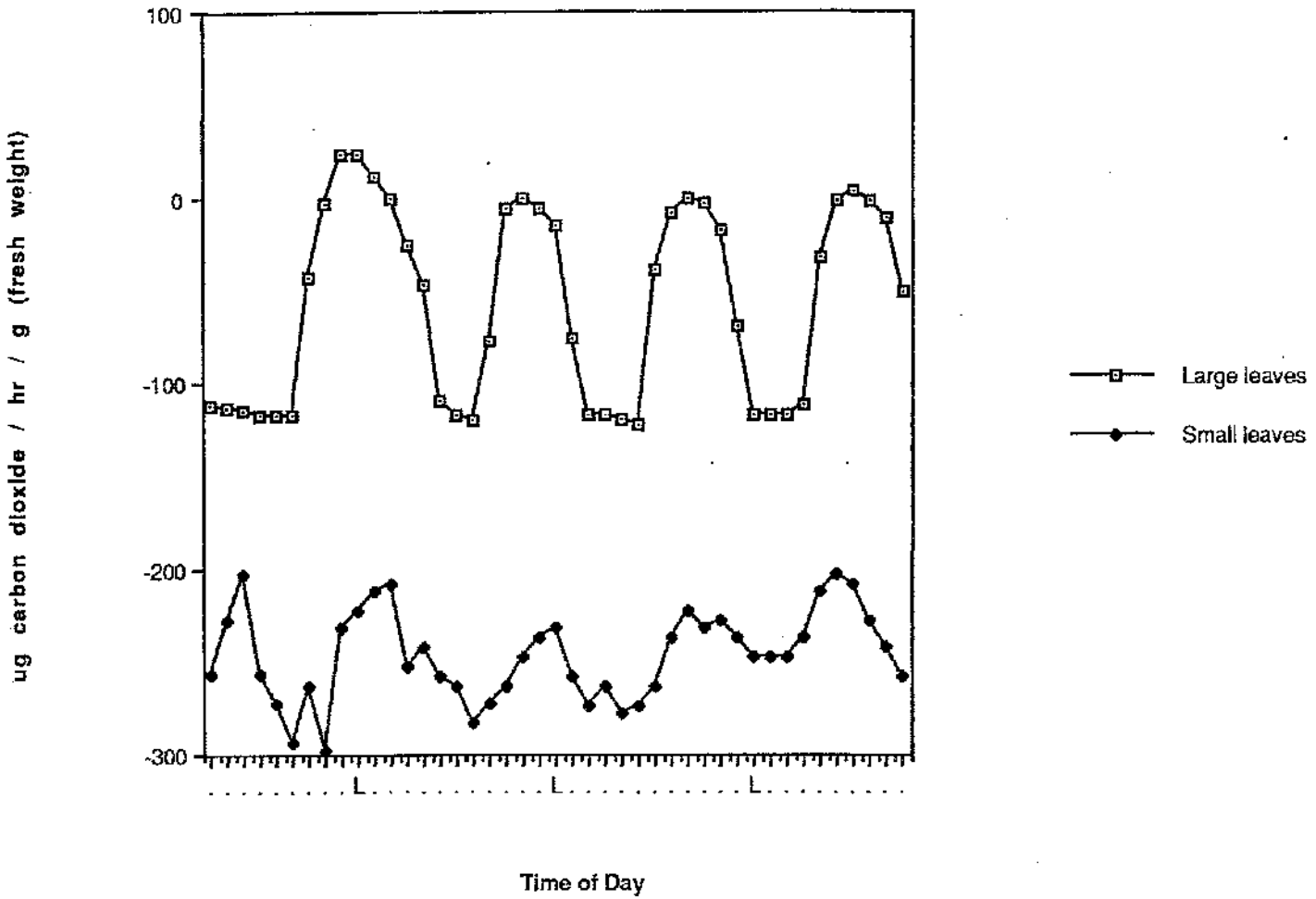
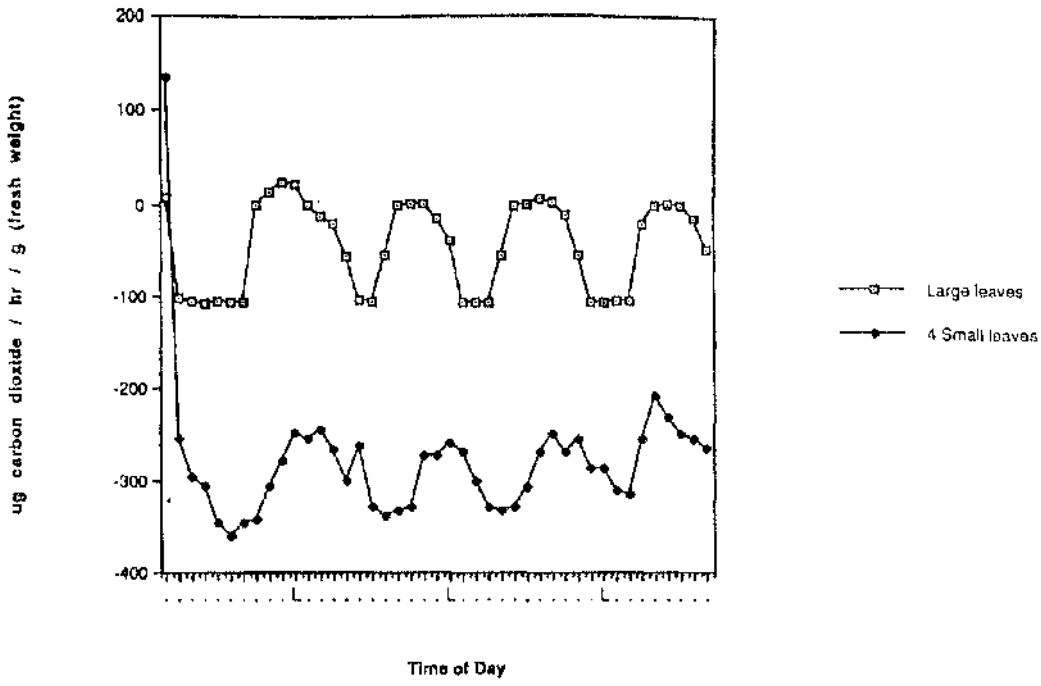

100
0
-100
-200
-300
ug carbon dioxide / hr / g (fresh weight)
L
L
L
Time of Day
Large leaves
Small leaves

**Figure 3.8** **Carbon dioxide gaseous exchange in detached leaves of *Bryophyllum fedtschenkoi* under continuous light where a variable number of small leaves is applied to a sealed chamber**

The conditions of Figure 3.7 were repeated but in this study one large leaf, 4 small leaves and 8 small leaves were placed into separate air-tight chambers and the $CO_2$ gaseous exchange measured. 1200 h is shown by the letter L. The table below shows the leaf size, number of leaves per chamber and the total weight of leaf tissue. The flow rate of air passing across the leaves was 3.0 l/h.

| Leaf classification | leaf size (cm) | No. of leaves per chamber | Total weight of leaf tissue (g) |
|---|---|---|---|
| Large | 7.5 | 1 | 4.1 |
| Small | 2.8 | 4 | 1.2 |
| Small | 2.5 | 8 | 1.6 |


ug carbon dioxide / hr / g (fresh weight)
200
100
0
-100
-200
-300
-400
L
L
L
Time of Day
Large leaves
4 Small leaves

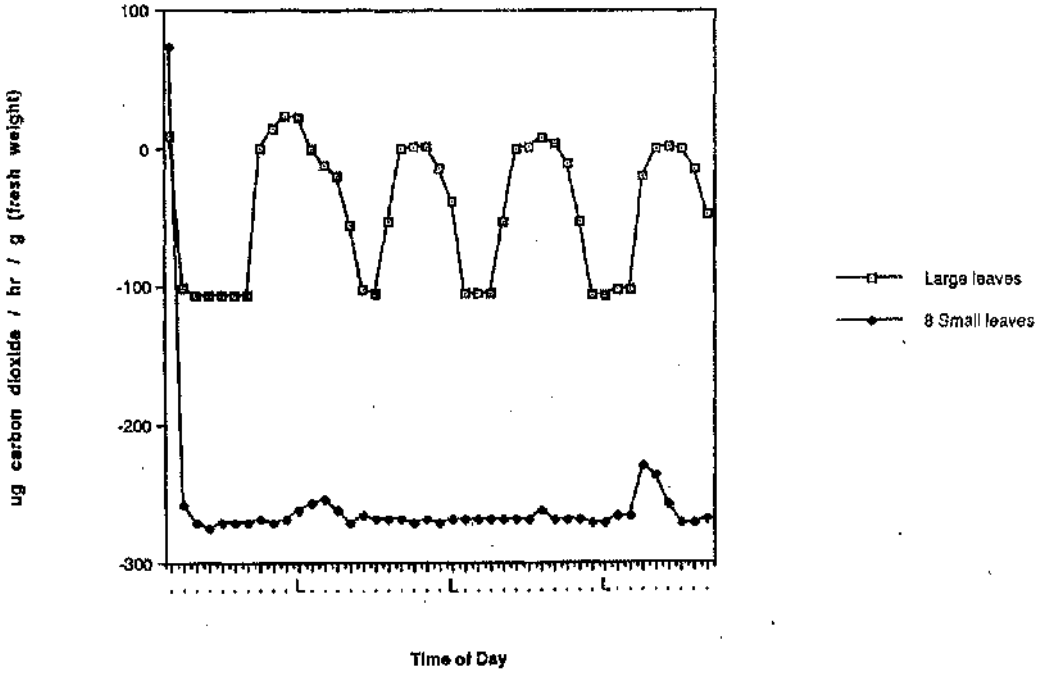

ug carbon dioxide / hr / g (fresh weight)
100
0
-100
-200
-300
L
L
L
Time of Day
Large leaves
8 Small leaves

**Figure 3.9** **The effect of reducing the light intensity on the circadian rhythm of $CO_2$ metabolism in small leaves of *Bryophyllum fedtschenkoi* under continuous light conditions**

The conditions and experimental set up discussed in Figure 3.7 were repeated in this analytical study. The only difference was that a neutral filter was placed on top of the sealed air-tight chambers to reduce the light intensity reaching the leaves. 1200 h is shown by the letter L. The table below shows the leaf size, number of leaves per chamber and the total weight of leaf tissue. The flow rate of air passing across the leaves was 3.0 l/h.

| Leaf classification | leaf size (cm) | No. of leaves per chamber | Total weight of leaf tissue (g) |
|---|---|---|---|
| Large | 6.5 | 1 | 2.3 |
| Small | 2.7 | 4 | 1.2 |

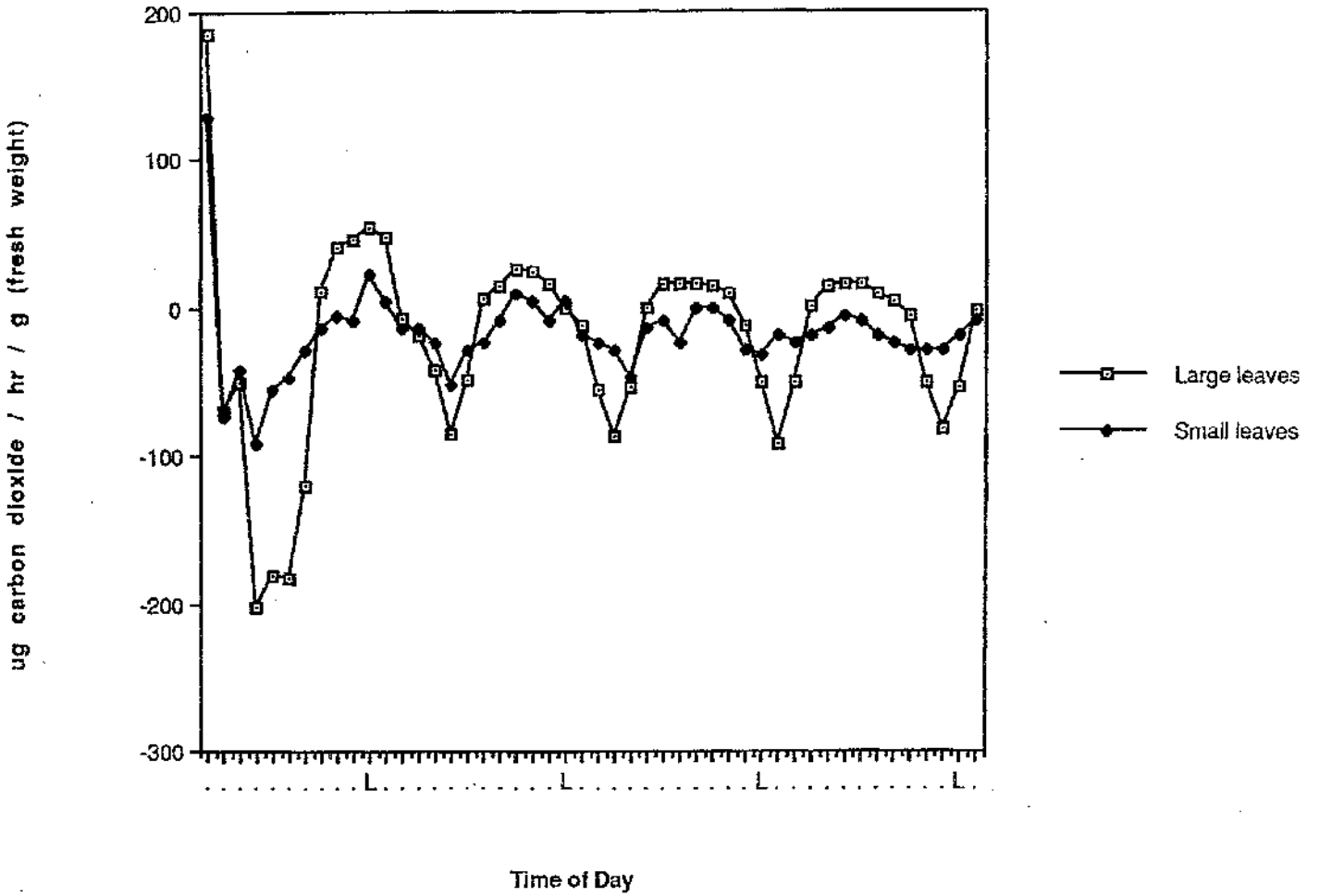

200
100
0
-100
-200
-300
ug carbon dioxide / hr / g (fresh weight)
L
L
L
L
Time of Day
Large leaves
Small leaves

**Figure 3.10 Circadian rhythm of $CO_2$ metabolism in middle leaves of *Bryophyllum fedtschenkoi* under conditions of continuous light, normal air and a constant temperature of 15°C**

*Bryophyllum fedtschenkoi* Leaves were detached and placed into an air-tight sealed chamber at the end of the normal light period. One large leaf and 2 middle leaves were placed into separate chambers. Continuous light conditions were set up and the $CO_2$ gaseous exchange was measured as discussed in section 2.17. 1200 h is shown by the letter L. The table below shows the leaf size, number of leaves per chamber and the total weight of leaf tissue. The flow rate of air passing across the leaves was 3.0 l/h.

| Leaf classification | leaf size (cm) | No. of leaves per chamber | Total weight of leaf tissue (g) |
|---|---|---|---|
| Large | 5.7 | 1 | 2.3 |
| Middle | 4.0 | 2 | 2.1 |

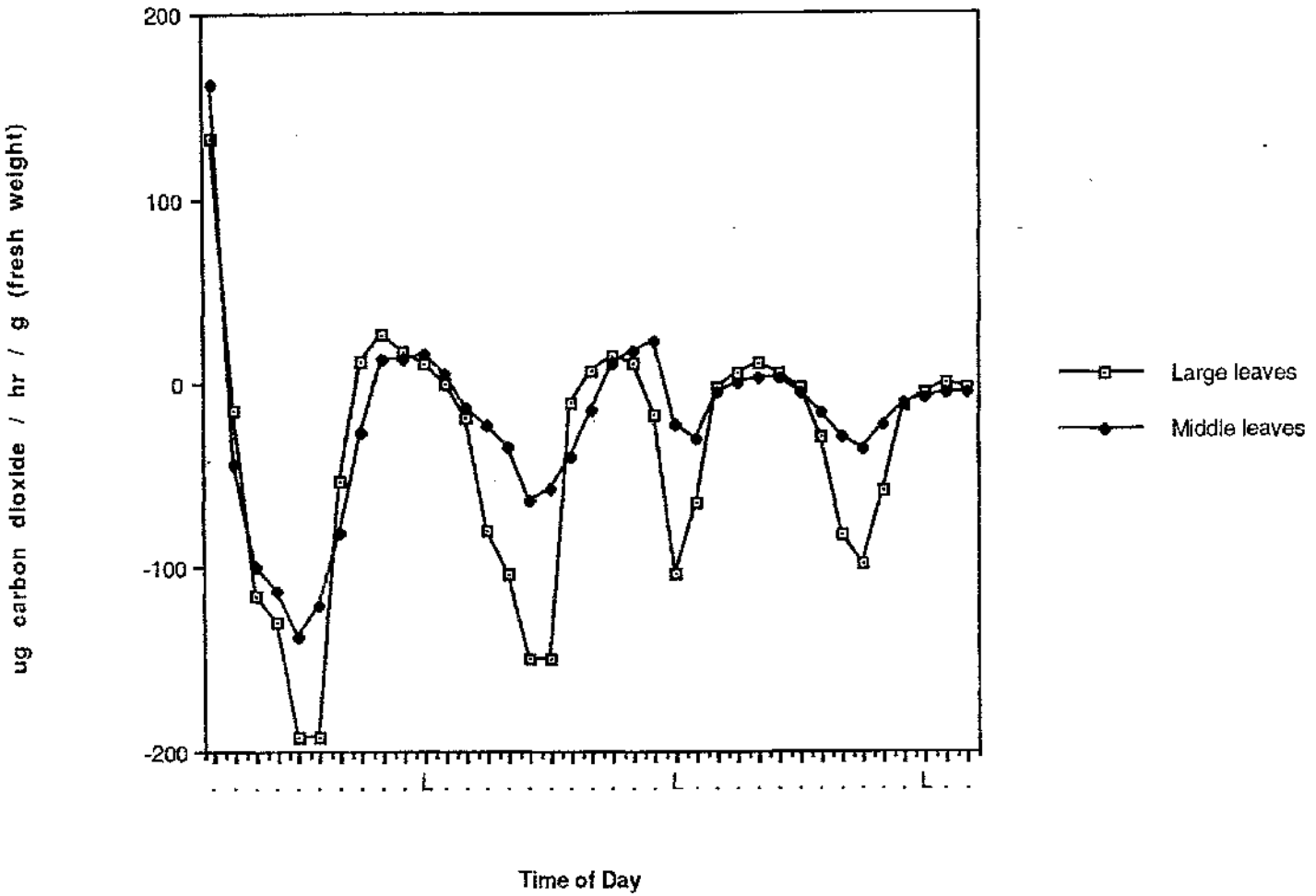

200
100
0
-100
-200
ug carbon dioxide / hr / g (fresh weight)
L
L
L
Time of Day
Large leaves
Middle leaves

**Figure 3.11 Carbon dioxide gaseous exchange in detached leaves of *Bryophyllum fedtschenkoi* under conditions of continuous darkness, $CO_2$ free air and a constant temperature of 15°C**

*Bryophyllum fedtschenkoi* leaves were detached and placed into sealed air-tight chambers at the end of the light period of the growth room. One large leaf and 4 small leaves were placed into separate chambers and the $CO_2$ gaseous exchange measured as in section 2.17. The $CO_2$ free air was achieved by passing the normal air through 20 % KOH and the $CO_2$ free air stream created was then passed across the leaves. 1200 h is shown by the letter L. The table below shows the leaf size, number of leaves per chamber and the total weight of leaf tissue. The flow rate of air passing across the leaves was 3.0 l/h.

| Leaf classification | leaf size (cm) | No. of leaves per chamber | Total weight of leaf tissue (g) |
|---|---|---|---|
| Large | 6.0 | 1 | 3.2 |
| Small | 2.2 | 4 | 1.3 |

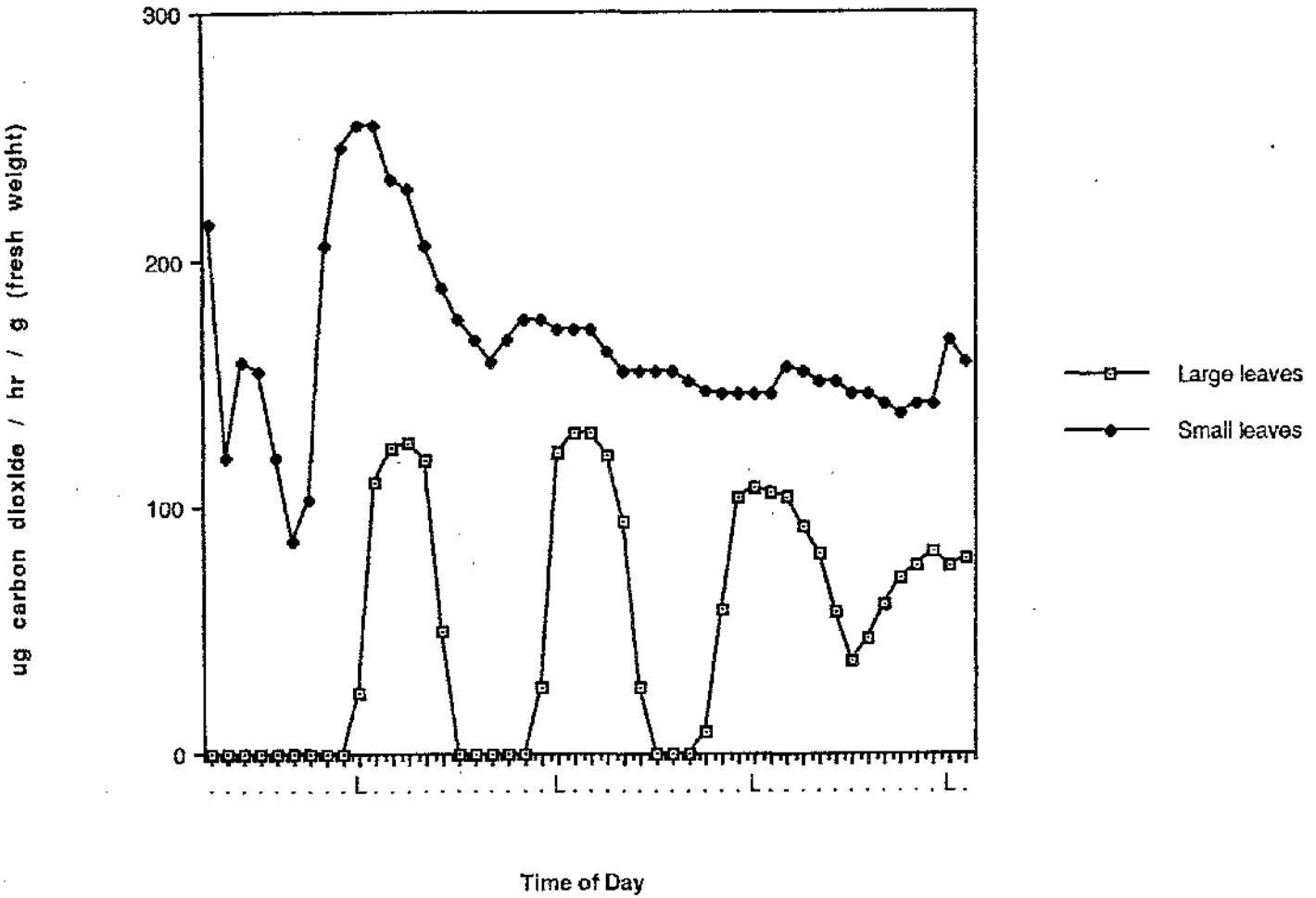

300
200
100
0
ug carbon dioxide / hr / g (fresh weight)
L
L
L
L
Time of Day
Large leaves
Small leaves

**Figure 3.12 Circadian rhythm of $CO_2$ metabolism in large, middle and small leaves of *Bryophyllum fedtschenkoi* under conditions of $CO_2$ free air, continuous darkness and a constant temperature of 15°C**

The conditions discussed in Figure 3.11 were repeated in this analytical study. One large leaf, one middle leaf and 4 small leaves of *Bryophyllum fedtschenkoi* were placed into separate air-tight chambers. The $CO_2$ gaseous exchange was recorded using the IRGA. At about the third cycle of a rhythm a light pulse was applied to test if the leaves were still photosynthetically active, which is seen as a rise in the $CO_2$ exchange level on the figure. The flow rate of the air passing across the leaves was increased from 3.0 l/h to 6.0 l/h in this study. 1200 h is shown by the letter L. The table below shows the leaf size, number of leaves per chamber and the total weight of leaf tissue.

| Leaf classification | leaf size (cm) | No. of leaves per chamber | Total weight of leaf tissue (g) |
|---|---|---|---|
| Large | 6.1 | 1 | 2.5 |
| Middle | 4.5 | 1 | 1.5 |
| Small | 2.8 | 4 | 1.4 |

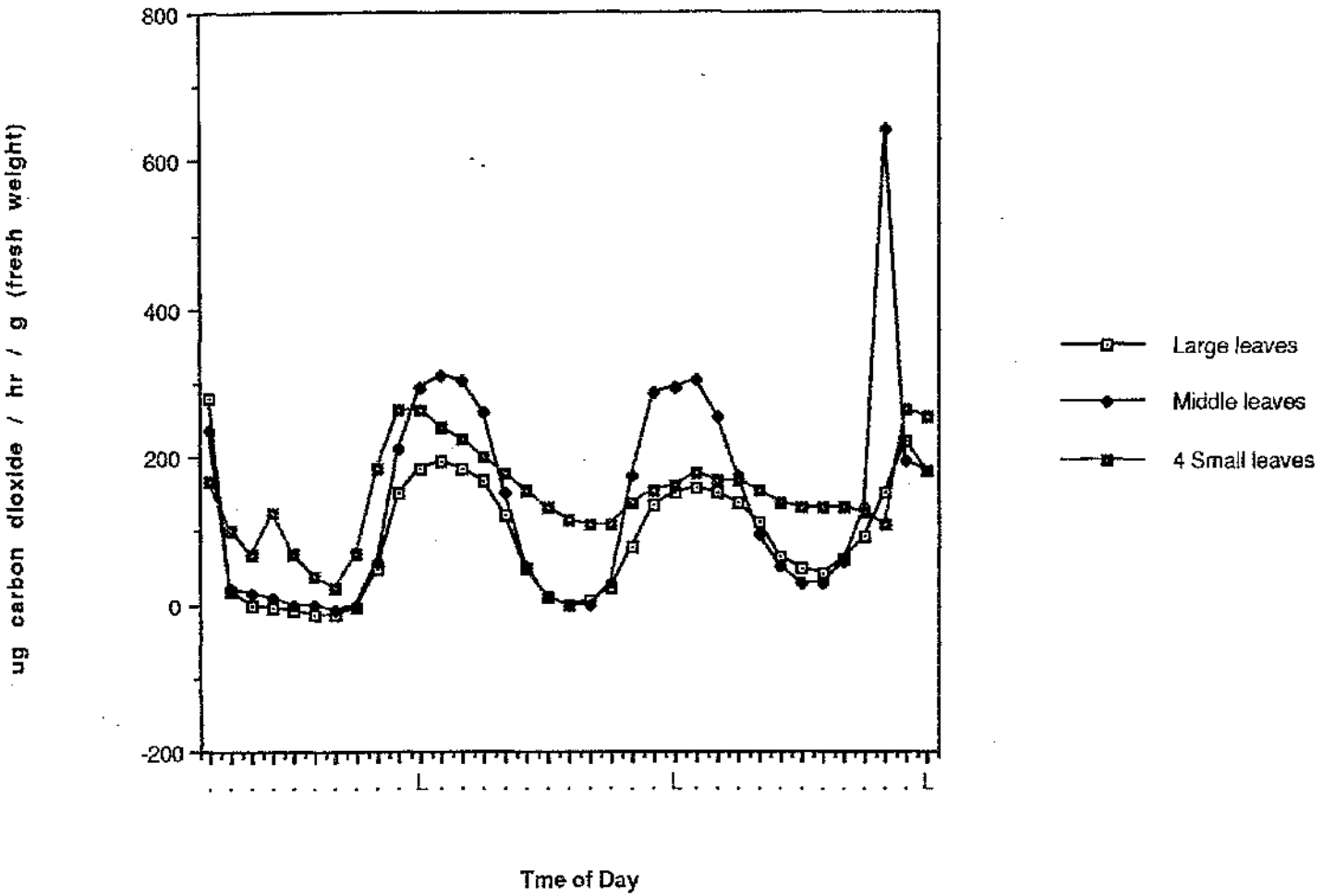

800
600
400
200
0
-200
L
L
L
ug carbon dioxide / hr / g (fresh weight)
Tme of Day
Large leaves
Middle leaves
4 Small leaves

**Figure 3.13 Circadian rhythm of $CO_2$ metabolism of a single small leaf of *Bryophyllum fedtschenkoi* under conditions of $CO_2$ free air, continuous darkness and a constant temperature of 15°C**

The conditions discussed in Figure 3.11 were repeated in this analytical study. The variation is that a single small leaf was placed into a separate air-tight chamber. Single large and middle leaves were also placed into individual chambers. The $CO_2$ gaseous exchange and test of photosynthetic activity were carried out as in Figure 3.12. The flow rate of the air was also 6.0 l/h. 1200 h is shown by the letter L. The table below shows the leaf size, number of leaves per chamber and the leaves weight.

| Leaf classification | leaf size (cm) | No. of leaves per chamber | weight of leaf (g) |
|---|---|---|---|
| Large | 5.6 | 1 | 2.5 |
| Middle | 4.7 | 1 | 1.2 |
| Small | 3.3 | 1 | 0.4 |

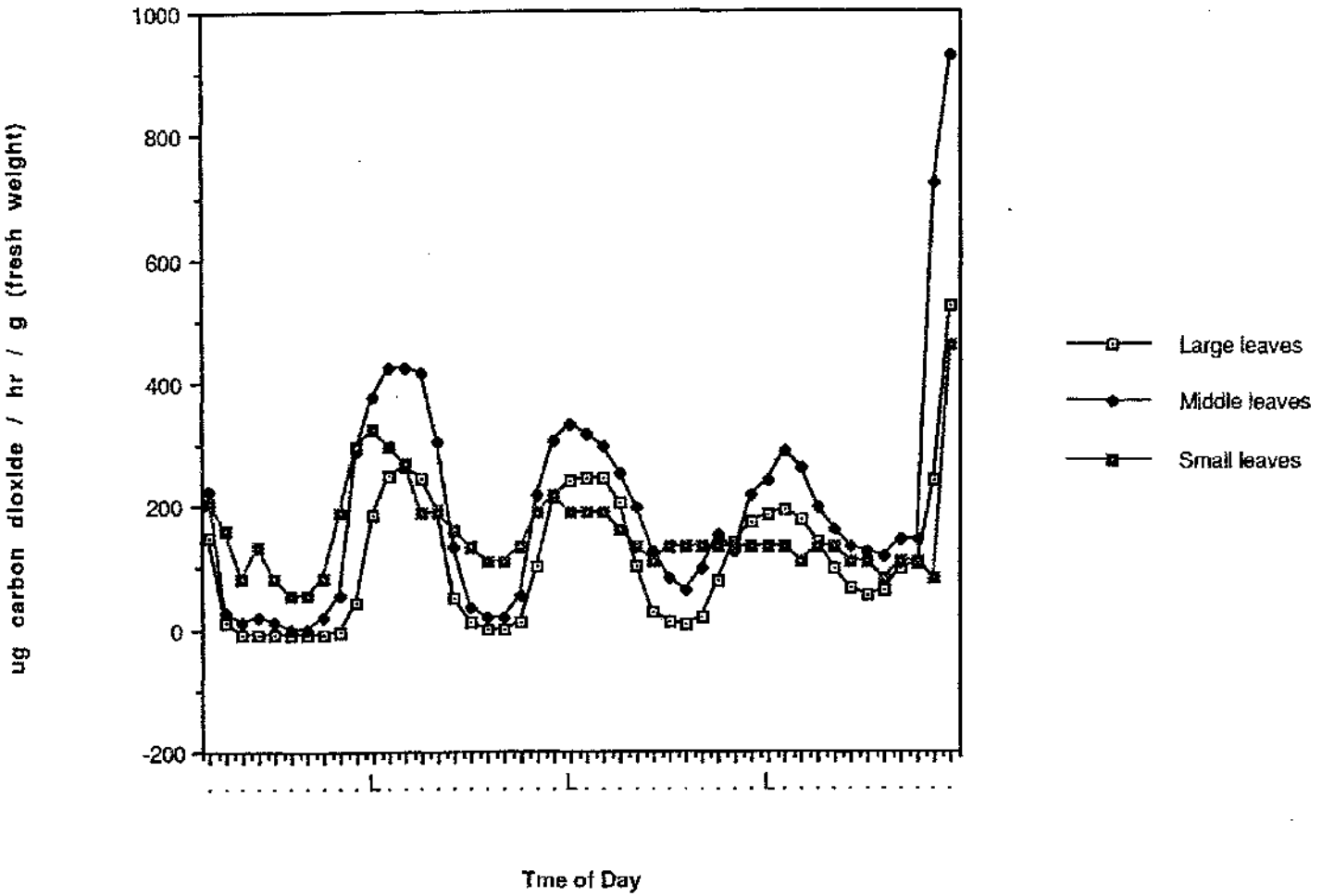
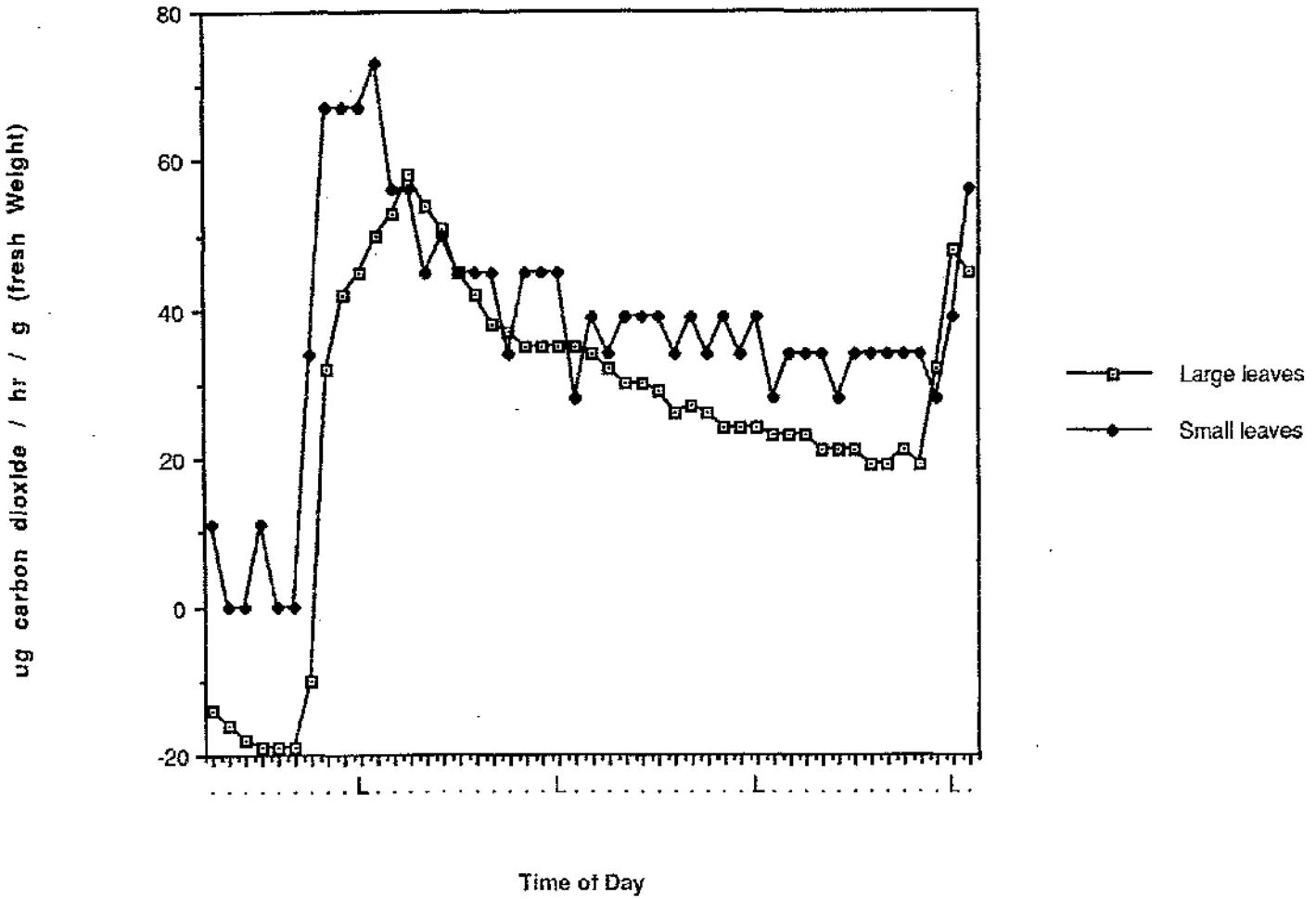

1000
800
600
400
200
0
-200
ug carbon dioxide / hr / g (fresh weight)
Tme of Day
Large leaves
Middle leaves
Small leaves

**Figure 3.14 Carbon dioxide gaseous exchange in detached leaves of *Bryophyllum fedtschenkoi* under conditions of continuous darkness, normal air and a constant temperature of 15°C**

*Bryophyllum fedtschenkoi* leaves of differing sizes were detached at the end of the normal light period and placed into sealed air-tight brass chambers under the conditions stated above. One large leaf was placed into one of these chambers and 4 small leaves were placed into another separate chamber. The $CO_2$ gaseous exchange was measured as discussed in section 2.17. The graph shows the different patterns of gaseous exchange seen in the two sets of leaves. A light pulse was applied at the end of the study to check that the leaves were photosynthetically active, which is seen as a rise in the level of $CO_2$ exchange. 1200 h is shown by the letter L. The table below shows the leaf size, number of leaves per chamber and the total weight of leaf tissue. The flow rate of air passing across the leaves was 3.0 l/h

| Leaf classification | leaf size (cm) | No. of leaves per chamber | Total weight of leaf tissue (g) |
|---|---|---|---|
| Large | 6.0 | 1 | 3.5 |
| Small | 1.5 | 4 | 1.0 |


80
60
40
20
0
-20
ug carbon dioxide / hr / g (fresh Weight)
Time of Day
Large leaves
Small leaves

# Chapter 4. Isolation of a *Bryophyllum fedtschenkoi CAB* cDNA and identification of a *Bryophyllum fedtschenkoi CAB* gene family

## 4.1 Introduction

The initial experimental work, chapter 3, carried out on the analysis of *Bryophyllum fedtschenkoi CAB* gene expression had utilised a heterologous *Arabidopsis* (AB165) genomic DNA probe in northern hybridisation analyses. This work was difficult because the hybridisation bands were often rather weak. A *Bryophyllum fedtschenkoi CAB* cDNA needed to be isolated to overcome this problem and confirm and expand the initial results of *CAB* gene expression. These experiments were carried out to isolate a *Bryophyllum fedtschenkoi CAB* cDNA which could be used as a homologous probe in *CAB* gene expression studies and in the identification of a *CAB* multigene family in *Bryophyllum fedtschenkoi*

cDNA libraries represent the information encoded in the mRNA of a particular tissue or organism. RNA is exceptionally labile and hard to amplify in its natural form. Therefore, the information encoded by the RNA is converted into stable double stranded DNA (cDNA) and is then inserted into a self-replicating vector. When the information is in the form of a cDNA library, individual segments of the original genetic information can be isolated and examined easily.

The success of library construction depends on the quality of the RNA or poly(A)$^+$ RNA isolated. These starting materials determine if a large, representative library will be produced. You also have to think about

what it is you want to use your cDNA library for. In this case it was to isolate a *Bryophyllum fedtschenkoi CAB* cDNA. When you isolate your raw material (leaf material in this instance) it should be believed to contain ample quantities of the required mRNA. As previous studies publicise the fact that *CAB* transcription was strongly regulated by light, and this was also found in my initial experiments (chapter 3), it was a good idea to isolate material in the middle of a light period.

## 4.2 Production of a *Bryophyllum fedtschenkoi* cDNA library

### 4.2.1 Extraction of poly(A)$^+$ RNA from leaves of *Bryophyllum fedtschenkoi*

Leaf samples were taken from *Bryophyllum fedtschenkoi* in the middle of the light period. Initially RNA isolations were carried out on these leaf samples using the phenol/Kirby method (2.4.1). The RNA samples were then pooled. The pooled RNA samples were first run through a cellulose column. This cleaned the RNA by removing any contaminating carbohydrates that may be associated with it. The resulting eluate was then run through an Oligo-dT cellulose column, as in section 2.5, to select the poly(A)$^+$ RNA from the isolated RNA. The resulting ribosomal and poly(A)$^+$ RNA samples collected were analysed by spectrophotometry and on a formaldehyde denaturing gel. It was apparent that the RNA was retained by the cellulose and/or Oligo-dT cellulose columns. Hence poly(A)$^+$ RNA was not obtained. This protocol was used with other plant species and gave positive results (ie, *CAB* transcripts were identified in the selected poly(A)$^+$ RNA). The problem with the *Bryophyllum fedtschenkoi* RNA samples was probably

the level of impurities (noted in chapter 3). So a different method was employed.

A new method of RNA extraction was applied (section 2.4.2) and to reduce the chances of losing any poly(A)$^+$ RNA in the isolation procedure a cellulose column was no longer used. Leaf samples were again isolated in the middle of the light period. The RNA was extracted and the samples were pooled. The procedure of isolating poly(A)$^+$ RNA from these samples followed the protocol of section 2.5. Figure 4.1 shows a photograph of the isolated ribosomal RNA and poly(A)$^+$ RNA after the total RNA sample was run through an Oligo-dT cellulose column. Table 4.1 shows the quantities of poly(A)$^+$ RNA isolated from the total RNA sample after the washes and precipitation stages were carried out. This analysis showed that some RNA was lost in the process of isolating poly(A)$^+$ RNA. The amount of poly(A)$^-$ RNA obtained was 43 % of the total RNA that was originally applied to the column. However sufficient poly(A)$^+$ RNA was isolated (approximately 30 μg) to use in cDNA synthesis and the production of a cDNA library.

### 4.2.2 Synthesis of first and second cDNA strands

7 μg of poly(A)$^+$ RNA was used in the first strand reaction mixture. The procedure in section 2.15.1 was followed. Figure 4.2 is the autoradiograph of a Southern blot from an alkaline agarose gel (section 2.8.3) showing aliquots of the synthesised first and second strand cDNA. No secondary structure was apparent and the size range of the double stranded cDNA produced was 3.5 kbp to 0.4 kbp.

The cDNA sample was size fractionated by running it through a Sephacryl S-400 spin column (section 2.15.3.3). The larger cDNAs were collected and pooled. These large cDNAs were estimated to have a concentration of 10 ng/μl.

The cDNA samples were ligated, along with the test ligation insert supplied in Stratagene's Zap-cDNA™ synthesis kit, into the Uni-Zap™ XR vector.

### 4.2.3 Packaging and screening of cDNA library

Table 4.2 shows the test results of packaging of the ligated cDNA fractions by using the Gigapack® II Gold packaging extract, as in section 2.15.7. The plaque growth on the IPTG/X-Gal plates showed the formation of blue non-recombinant and white recombinant plaques. In the Uni-Zap™ XR vector insertion of a cDNA clone disrupts a *LacZ* gene and no functional β-galactosidase is produced so white plaques develop as the X-Gal was not utilised. A 10 to 100 fold difference was expected between the non-recombinant and recombinant plaques. The cDNA isolated showed the number of recombinant plaques to be 20 fold higher than non-recombinant plaques. All controls gave expected results. The remaining quantity of the ligated cDNA fraction was packaged and the amplification of the Uni-Zap™ XR library was carried out, as in section 2.15.8. The resulting titer of the amplified library was $2.0 \times 10^9$ pfu/ml.

The *Bryophyllum fedtschenkoi* cDNA library had to be screened in order to isolate a *CAB* cDNA for use in analytical expression studies. Knight *et al.* (1992) and Chang *et al.* (1992) stated that in *Zea mays* and *Glycine max* a *CAB* cDNA has been isolated which may represent up to 2 % of the

mRNA in light-grown leaves. Therefore, as the *CAB* gene(s) in *Bryophyllum fedtschenkoi* was (were) likely to be highly represented in a cDNA library from light-grown leaf tissue a massive number of plaque forming units would not have to be screened. The screening procedure in section 2.16.1 was followed. A primary screen of about 30,000 pfu was carried out, where the set of duplicate nylon filter plaque lifts produced was hybridised with the *Arabidopsis* AB165 *CAB* probe and *C. reinhardtii* β-tubulin cDNA probe. A set of the duplicate lifts was used with each screening procedure to remove the possibility of selecting any false positives. 12 to 15 putative positive plaques appeared for the β-tubulin blots. The result of this primary screening procedure with the *Arabidopsis* AB165 *CAB* probe identified 10 putative *CAB* clones (labelled 1 to 10).

The 10 putative *CAB* clones were selected (section 2.16.1) and a secondary screening procedure was carried out. A new set of duplicate plaque lifts was produced from the primary positives. After hybridisation analysis, with the *Arabidopsis* AB165 *CAB* probe, of this new set of duplicate plaque lifts 11 putative *CAB* clones were identified (labelled A to K).

The 11 putative *CAB* clones identified after secondary screening were selected and a tertiary screening procedure was carried out as above. After hybridisation analysis with the heterologous *CAB* probe the resulting tertiary analysis showed a number of autoradiographs appearing as a black mat of plaques. This implied that these clones selected after the secondary screening were in fact putative *CAB* isolates.

This was not the case in all of the putative positives selected, a further six putative *CAB* clones (labelled i to vi) were picked from the less distinct tertiary screening results. Figure 4.3 shows the results of primary, secondary and tertiary screening of one of these putative *CAB* clones selected.

The final result of the primary, secondary and tertiary screening procedures was that there were a total of 11 putative *CAB* isolates to be analysed each of which was plaque-purified.

### 4.2.4 Isolation of putative *CAB* clones using in vivo excision

In vivo excision was carried out with the putative *CAB* isolates (E to I and i to vi) as described in section 2.16.2. The "packaged" pBluescript® DNA of the putative isolates was mixed with fresh *E. coli* SOLR bacterial cells and spread onto the selective LB/ampicillin plates to produce colonies. Colonies appeared on all of the plates for each isolate. Individual colonies were selected and cultured at 37°C in 3 ml of LB/ampicillin broth with overnight shaking. The result of this rescreening procedure was that only the isolates E, F and ii showed any signs of cell growth. A glycerol stock of E, F and ii was prepared with 750 µl of the culture and with a further 750 µl of each culture Promega Wizard™ Minipreps were carried out as in section 2.12.2. The remaining cultures were discarded. The above procedure was repeated with all of the putative isolates and the same difficulties occurred with false positive colonies growing on all of the selective plates and not in a selective LB/ampicillin broth.

The plasmid preparations of E, F and ii were analysed in a spectrophotometer. Only isolate E showed the presence of any significant amount of plasmid DNA. When the plasmid DNA of the isolates was analysed on a mini-agarose gel, plasmid DNA was present for both isolates E and F but not for ii. This was not unexpected. The putative *CAB* isolates E and F were selected from the same original plaque isolation during the cDNA library screening procedure. Figure 4.4 shows a photograph of the plasmid preparation E cut with *Xho*I and *Eco*RI restriction enzymes. This digestion cuts out the DNA insert from the plasmid as it has been designed to have these restriction enzyme sites at its 5' and 3' ends. The markers used were lambda DNA digested with the restriction enzymes *Hin*dIII and *Eco*RI. Figure 4.4 shows three bands appearing for the cut plasmid isolate E. The upper one is the pBluescript® vector (size 2921 kbp) and the lower two are the DNA insert. Therefore the DNA insert must have an internal *Eco*RI or *Xho*I restriction enzyme site. The larger of these two insert DNA fragments had a size of 580 kbp to 620 kbp. This was too small to be a complete *CAB* cDNA but was long enough to use as a homologous *CAB* cDNA probe.

The stocks of the putative *CAB* isolates E and F were small and there were difficulties in growing up the original cell stocks that contained the plasmids. Therefore the plasmids of the isolates were transformed, as in section 2.16.3, into fresh competent *E. coli* cells (XL-I Blue). Then a large scale plasmid preparation was carried out with these cells to obtain sufficient amounts for use in DNA sequence analysis and in isolating the DNA insert for use as a homologous probe for *CAB* cDNA expression studies. The DNA insert had to be analysed to confirm if it was a *Bryophyllum fedtschenkoi CAB* cDNA.

### 4.2.5 Sequence analysis of putative *CAB* clones

Both strands of the DNA insert sequence had to be determined in order to identify the exact reading frame of the putative *CAB* cDNA. The sequencing reactions and preparation were carried out as described in section 2.16.4. Initial sequencing studies used the designed primers M13 -20, T7, T3 and the SK primer which would anneal to one of the DNA strands of the pBluescript® phagemid. These initial sequencing studies proved that the DNA inserts of the plasmids of the putative *CAB* isolates E and F were of exactly the same origin and had an identical sequence. The use of the M13 -20 and T7 primers identified the poly(A)$^+$ tail, whereas the T3 and SK primers located the *Eco*RI adaptor as well as an internal *Eco*RI site on the DNA insert sequence. The size of the smaller DNA fragment, shown in Figure 4.4, was 110 kbp. This implied that the DNA insert sequence had an estimated size of 690 kbp to 730 kbp. When the DNA insert was completely sequenced the exact size would be known.

It was not possible to complete the internal sequence regions of the strands of the DNA insert with the above primers alone. The sequence was too long. Figure 2.1 shows six internal primers that were designed as the sequence of the DNA insert was identified. Both strands of the DNA inserts were completely sequenced and the two sequences were compared.

The data showed that the DNA insert sequence was 802 kbp in length with an open reading frame of 230 amino acids and a 19 bp poly(A)$^+$ tract. Figure 4.5 shows the complete sequence of the DNA insert with the open reading frame and internal *Eco*RI site identified.

The screening of the databases E.M.B.L and GenBank with the derived peptide sequence and nucleic acid sequence of the *Bryophyllum fedtschenkoi CAB* cDNA identified the twenty best scores. The comparisons identified were all other known *CAB* genes from a range of plants such as *Lycopersicon esculentum*, *Nicotiana tabacum*, *Nicotiana plumbaginifolia*, *Petunia* (Mitchell), *Pisum sativum*, *Lemna gibba*, *Zea mays*, *Glycine max*, *Arabidopsis thaliana* and *Hordeum vulgare*. The nucleic acid sequence (FASTA program used) gave a minimum of 82 % homology over (at least) a 680 bp region and the peptide sequence (TFASTA program used) gave a minimum of 93 % homology over (at least) a 225 amino acid sequence with the other known *CAB* sequences.

Figure 4.6 shows the best comparison of the derived *Bryophyllum fedtschenkoi CAB* peptide sequence to the *Lemna gibba* AB30 *CAB* gene. There is a 94.7 % identity in a 228 amino acid region between these two sequences. These findings identified the DNA insert as an incomplete *Bryophyllum fedtschenkoi CAB* cDNA which could be used as a homologous probe in *CAB* gene expression studies.

## 4.3 Analysis of genomic DNA from leaves of *Bryophyllum fedtschenkoi*

The availability of the cDNA enabled me to examine the size of the *Bryophyllum fedtschenkoi CAB* gene family. If only one gene was present there would be no scope for differential expression which would have been possible between members of a small gene family.

### 4.3.1 Isolation of DNA from leaves of *Bryophyllum fedtschenkoi*

*Bryophyllum fedtschenkoi* leaf samples of size 4.0 cm to 5.5 cm (4 to 5 from top) and size 2.0 cm to 3.5 cm (1 to 2 from top), were isolated in the middle of the light period of the growth room and snap frozen in liquid nitrogen. DNA was isolated from these leaf samples by using the method described in section 2.6. The extracted DNA was analysed using a spectrophotometer (section 2.7) and on an agarose gel (section 2.8.1). Figure 4.7 are photographs of agarose gels showing the isolated DNA samples from the differently sized *Bryophyllum fedtschenkoi* leaf samples. Heavy contamination with RNA was evident. Figure 4.7b is a photograph of DNA isolated from the larger leaves before and after treatment with an RNAase A enzyme. This treatment removes the contaminating RNA from the isolated DNA samples.

### 4.3.2 Restriction digestion of genomic DNA

A number of attempts were made to digest DNA from large leaves with various restriction endonucleases but repeated problems were encountered in digesting the DNA, probably because of the contaminating impurities which hampered experiments with RNA. DNA was isolated from the smaller leaf samples to compensate for the problems of the restriction endonucleases cutting the DNA isolated from the larger leaf samples. Individual quantities of the isolated DNA from the small leaf samples was digested with one of the following restriction enzymes; *Eco*RI, *Hin*dIII, *Bgl*II, *Bam*HI, *Xba*I and *KpN* I (section 2.9). Uncut DNA, treated with RNAase A, and the digested DNA samples were run on a 0.8 % to 1.0 % agarose gel (section 2.8.1). Duplicate restriction digestion reactions were carried out with different DNA preparations. Figure 4.8 is a photograph

of an agarose gel with duplicate sets of the digested DNA samples from the smaller leaves. The DNA was cut effectively by the restriction endonucleases as shown by the smear on the gel.

### 4.3.3 Southern analysis of genomic DNA from leaves of *Bryophyllum fedtschenkoi* using a homologous *CAB* cDNA

The agarose gels which had been run with the uncut and digested DNA samples from the small *Bryophyllum fedtschenkoi* leaf samples were Southern blotted as described in section 2.11.2. The resulting filters were hybridised with the homologous *Bryophyllum fedtschenkoi CAB* cDNA. Figure 4.9 shows the autoradiograph of the hybridisation. Several hybridising bands can be seen in the different lanes. DNA digested with the restriction endonucleases *Xba*I and *KpN* I appeared to have the least number of hybridising DNA bands. This result implied that the *Bryophyllum fedtschenkoi CAB* gene family was likely to consist of several related members.

## 4.4 Discussion

### 4.4.1 Sequence analysis of the *Bryophyllum fedtschenkoi CAB* cDNA

The screening of the databases E.M.B.L and GenBank identified that the isolated *Bryophyllum fedtschenkoi* cDNA was representative of a *CAB* gene. Jansson *et al.* (1992) proposed a nomenclature for the genes encoding the chlorophyll a/b binding proteins of higher plants. The twenty best scores that were identified through the databases were all from Type I LHCII CAB proteins. Therefore, the mature *Bryophyllum fedtschenkoi* CAB protein could be classified as a Type I LHC II gene as

it contained no introns and appeared to belong to the same LHC II lineage of the other sequenced *CAB* genes.

Coruzzi *et al.* (1983) first sequenced a cDNA (AB96) that encoded the major chlorophyll a/b binding thylakoid protein, in this case from *Pisum sativum*. This cDNA contained an 833 nucleotide sequence and a deduced 228 amino acid polypeptide. The sequence of this cDNA provided almost the entire amino acid sequence of the mature CAB protein, but none of the precursor transit polypeptide was obtained. The isolated *Bryophyllum fedtschenkoi CAB* cDNA could be compared to this clone as it consisted of a 802 nucleotide sequence and a deduced 230 amino acid sequence. Therefore, the *Bryophyllum fedtschenkoi CAB* cDNA contained an incomplete sequence of the CAB protein. Other known *CAB* sequences showed pre-CAB polypeptide sequences consisting of between 262-270 amino acid residues including a transit peptide of 30-35 amino acids (Cashmore, 1984; Walling *et al.*, 1988; Knight *et al.*, 1992; Brandt *et al.*, 1992 and McGrath *et al.*, 1992). This implied that the deduced amino acid sequence of the *Bryophyllum fedtschenkoi* CAB protein identified was 30-40 amino acids incomplete.

Figure 4.10 shows a comparison of the deduced amino acid sequence of the *Bryophyllum fedtschenkoi CAB* cDNA with four other known *CAB* sequences. These deduced amino acid sequences were from *Lemna gibba* AB30, *Petunia* (Mitchell) 22R, *Arabidopsis thaliana* AB165 (Leutwiler *et al.*, 1986) and *Zea mays* M7 (Becker *et al.*, 1992) *CAB* genes. The first two sequences were identified in the twenty best scores identified by the screening of the databases and gave 95 % and 94 % sequence homologies. The AB165 *CAB* DNA was used as a heterologous probe in the initial *Bryophyllum fedtschenkoi CAB* gene

expression studies (92 % homology) and the *Zea mays* CAB-M7 was used as an alternative to the best fit *Lemna gibba* for sequence comparison (91 % homology). All of the sequences used for comparison encoded Type I LHC II polypeptides. Notable variations in the different sequences came within the first ten amino acids and then at positions 11, 84, 100, 128, 131, 141, 150, 179 and 222. Demmin *et al.* (1989) compared a vast range of CAB amino acid sequences from different phylogenetic taxa including monocot and dicot species. All of the variations above appeared in the various CAB sequences compared. However, they noted that there were 16 amino acid residues within the mature CAB polypeptide sequence that differentiated CAB Type I and Type II proteins. Examination of these residues supports the hypothesis that the *Bryophyllum fedtschenkoi CAB* cDNA encodes a Type I LHC II polypetide. The deduced amino acid sequence did not show the Type II LHC II features. On comparing the different sequences in Figure 4.10, the amino acid residues that vary at the positions 84, 128, 131, 141, 179 and 222 (as mentioned above) within the mature CAB peptide have aided in the classification of the various species into different Type I subgroups (Demmin *et al.*, 1989). These nonsynonymous substitutions suggested that the *Fabaceae* (*Pisum sativum* and *Glycine max*) and *Brassicaceae* (*Arabidopsis thaliana*) were more closely related to each other than the other dicot species examined in the study. These nonsynonymous substitutions appear in the *Bryophyllum fedtschenkoi* CAB sequence and this connection meant that the *Crassulaceae*, as exemplified by *Bryophyllum fedtschenkoi*, could also be more closely linked to these families of plants. The fact that the *Arabidopsis thaliana* AB165 DNA probe was effective as a heterologous probe in the initial *CAB* gene expression studies, discussed in chapter 3, was likely to be linked to this factor. An interesting point was that the AB165 *CAB* DNA nucleotide

sequence was identified in the best twenty scores on comparison to the nucleotide sequence of the *Bryophyllum fedtschenkoi CAB* cDNA (82 % homology) but was not apparent in the best twenty of the deduced amino acid sequence.

Comparison of the *Bryophyllum fedtschenkoi CAB* cDNA amino acid sequence, shown in Figure 4.5, to other *CAB* genes suggests that the mature protein sequence is probably three to five amino acids short. In the majority of Type I LHC II *CAB* proteins the sequence Met-Arg-Lys-Thr-Ala/Val (MRKTV/A) occurs at the amino terminal end of the mature polypeptide. It is probable that part or all of the above sequence may be present on the complete *Bryophyllum fedtschenkoi CAB* cDNA. Demmin *et al.* (1989) discussed an algorithm of Kyte and Doolittle that predicted when regions of a polypetide will span a hydrophobic, bilayered membrane. The amino acid sequences of three potential membrane-spanning domains that occurred in *Glycine max* CAB-4 and CAB-5 precursor protein sequences were identical to three polypeptide regions in the deduced *Bryophyllum fedtschenkoi* CAB amino acid sequence. Therefore in the *Bryophyllum fedtschenkoi CAB* cDNA the potential membrane-spanning regions were encoded by the nucleotides 197-247, 353-421 and 554-604 and are shown on Figure 4.5 as the amino acid regions in bold. This was consistent with the proposed model of Karlin-Neuman *et al.* (1985) that described the association of a *Lemna gibba* CAB apoprotein with the thylakoid membrane where three regions of the polypeptide chain cross the membrane.

The *Bryophyllum fedtschenkoi CAB* cDNA untranslated region was flanked on the 3' end by 88 nucleotides preceding the poly(A)$^+$ tail. Analysis of this flanking sequence showed that no regulatory sequences

appeared in the 3' noncoding nucleotide region of the *Bryophyllum fedtschenkoi CAB* cDNA. The hexanucleotide A-A-T-A-A-A found in many eukaryotic mRNAs has been proposed as the conventional polyadenylation signal. It is usually located 10-30 bases upstream from the polyadenylation site. It was not apparent in the *Bryophyllum fedtschenkoi CAB* cDNA 3' flanking sequence. Although this consensus sequence is a common feature of the 3' untranslated region of animal and viral mRNAs, and its absence could have profound effects on the viability of a message, it is apparently not always required. A number of plant genes do not carry this consensus sequence (Coruzzi *et al.*, 1983 and Walling *et al.*, 1988). In fact, many plant genes do not strictly conform to established eukaryotic consensus sequences.

### 4.4.2 The *Bryophyllum fedtschenkoi CAB* gene family

Green *et al.* (1991) discussed the fact that the chlorophyll a/b binding polypeptides (CAB) were encoded by an extended family of nuclear genes. In fact Demmin *et al.* (1989) concluded that the plant gene family that encoded the photosystem II chlorophyll a/b binding protein genes may be useful in establishing taxonomic relatedness among angiosperms. Southern blot analysis indicates that a *Bryophyllum fedtschenkoi CAB* gene family exists and consists of several members. This belief is further supported by the fact that, out of the six restriction endonucleases used the digestion of genomic DNA (Figure 4.8), the *Bryophyllum fedtschenkoi CAB* cDNA (Figure 4.5) only has sites for *Eco*RI (Figure 4.5, nucleotide position 110) and *Bgl* II (Figure 4.5, nucleotide position 303). *CAB* genes have been isolated and characterised in several plant species. These studies have indicated that *CAB* gene families range from 3 to 19 members. Therefore, the *Bryophyllum fedtschenkoi CAB* gene

family follows this trend. In *Lycopersicon esculentum* Green *et al.* (1992) have related the members of the *CAB* gene family encoding the LHC II polypeptides to their respective polypeptides. The Type I LHC II gene of *Lycopersicon esculentum* was located in the major antenna PS II, had a chlorophyll a/b ratio of 1.2, had no introns, encoded two major polypeptides and had 8 gene copies. The *Bryophyllum fedtschenkoi* CAB protein may show some of the above features.

It would be interesting to isolate the different members of the *Bryophyllum fedtschenkoi CAB* gene family. The transcript levels of the individual members could be measured under various developmental and environmental situations. Kellman *et al.* (1993) and White *et al.* (1992) have carried out analyses on how the transcript levels of individual members of the *CAB* multigene families of *Lycopersicon esculentum* and *Pisum sativum* vary in specific conditions. Millar and Kay (1991) identified that the *Arabidopsis thaliana CAB* 1 gene showed no diurnal cycling of the mRNA level in contrast to other *Arabidopsis thaliana CAB* genes. Therefore, a comprehensive analysis of the circadian oscillation of *CAB* gene expression in *Bryophyllum fedtschenkoi* will have to look at the expression patterns of the genes encoding for the different members of the *CAB* multigene family. This will identify if the same or different patterns of expression, as Millar and Kay (1991) found, are occurring.

**Figure 4.1** **Photograph showing ribosomal and poly(A)$^+$ RNA which has been isolated from *Bryophyllum fedtschenkoi***

RNA was extracted from *Bryophyllum fedtschenkoi* leaf samples, which were isolated in the middle of the light period, by using the method of Chang *et al.* (1993), as in section 2.4.2. The only variation was that after the precipitation step the RNA sample was resuspended in 0.5 % (w/v) SDS (section 2.5) and not the usual SSTE. The poly(A)$^+$ RNA was then extracted as described in section 2.5. Figure 4.1 is a photograph of an agarose gel (section 2.8.1) showing the rRNA isolated, after washing it out of the column using the 1 x binding buffer, and the poly(A)$^+$ RNA isolated after washing the Oligo-dT cellulose column with elution buffer. Both appear as smears. The ribosomal RNA probably smeared because of the amount loaded whereas the poly(A)$^+$ RNA was expected to be heterogeneous anyway. The gel lanes are labelled; rRNA is ribosomal RNA; p(A)$^+$ is poly(A)$^+$ RNA.

rRNA p(A)$^+$

**Table 4.1 Table showing the efficiency of isolating poly(A)$^+$ RNA from *Bryophyllum fedtschenkoi* RNA samples**

The total RNA extracted using the method of Chang *et al.* (1993), section 2.4.2, was assessed at each step of the isolation of poly(A)$^+$ RNA (section 2.5). The samples A and B were pooled together before they were run through the Oligo-dT cellulose column. The table below shows the following; $A_{260}$; $A_{280}$; amount of RNA, poly(A)$^+$ RNA or poly(A)$^-$ RNA; the recovered poly(A)$^+$ RNA and poly(A)$^-$ RNA as a percentage (%) of the total RNA originally applied to the Oligo-dT cellulose column. The analysis of these different samples followed the procedure described in section 2.7.

| RNA sample | Total volume (µl) | Absorbance 260nm | 280nm | RNA or p(A)$^{+/-}$ RNA (µg) | % of total RNA |
|---|---|---|---|---|---|
| A | 500 | 0.147 | 0.089 | 588 | |
| B | 500 | 0.125 | 0.064 | 500 | |
| p(A)$^-$ | 100 | 0.581 | 0.276 | 465 | 43 |
| p(A)$^+$ | 35 | 0.044 | 0.030 | 31 | 3 |

**Figure 4.2 First and second cDNA strands synthesised from *Bryophyllum fedtschenkoi* poly(A)$^+$ RNA**

Control samples collected for the first and second strand cDNA synthesis reactions, refer to section 2.15.1, were run on an alkaline agarose gel (section 2.8.3) with λ *Hin*d III markers, which had been labelled by nick translation (section 2.13.3). The gel was Southern blotted, as in section 2.11.2, and the resulting filter membrane was autoradiographed, as in section 2.14.3. Figure 4.2 shows the result of this autoradiograph. The lanes are identified as follows; M is the λ *Hin*d III markers (fragment sizes shown), 1 is the first cDNA synthesised strand and 2 is cDNA after the second strand has been synthesised.

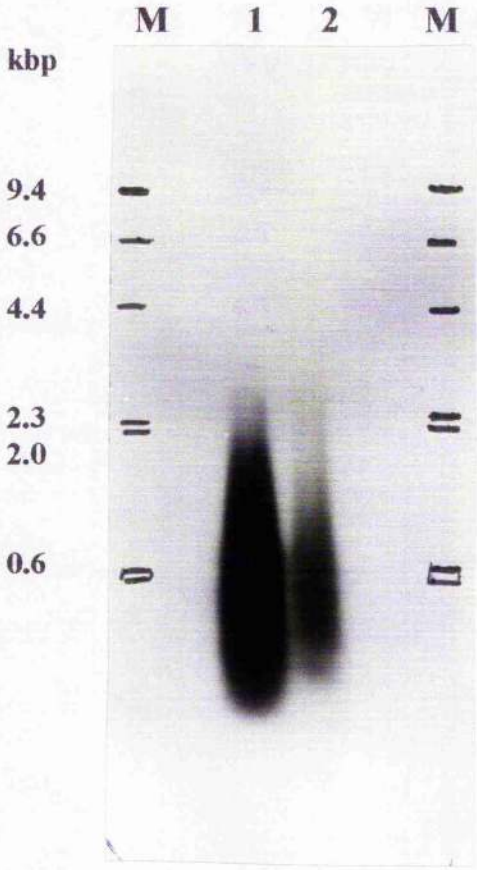

M
1
2
M
kbp
9.4
6.6
4.4
2.3
2.0
0.6

**Table 4.2 Analysis of the packaged primary library through the use of colour selection by IPTG/X-Gal**

The packaged Uni-Zap™ XR vector containing insert DNA was analysed by infecting *E. coli* SURE cells and allowing them to grow on top agar, in the presence of IPTG and X-Gal, that was poured onto NZY plates at a temperature of 39°C (refer to section 2.15.7.3). The results of this principal infection of host SURE cells are shown in the table below, along with the control tests for checking the ligation and the positive control for the Gigapack® II packaging extract;

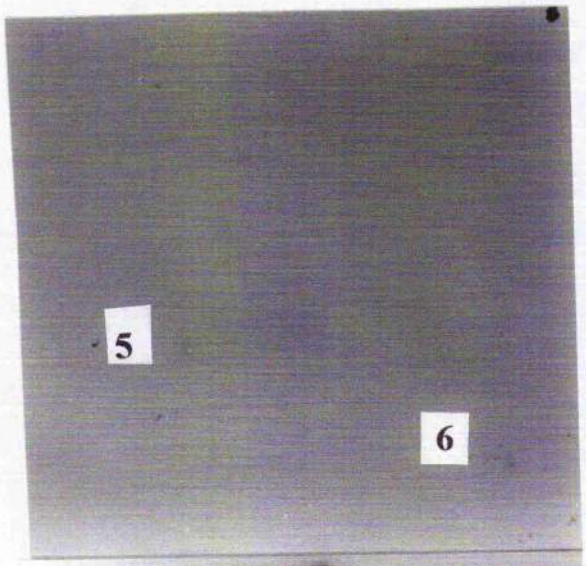
| Bacterial cell | No. of pfu | | Dilution | Ratio of white/blue pfu |
|---|---|---|---|---|
| | White | Blue | | |
| SURE | 491 | 24 | $10^{0}$ | ~20 |
| | 124 | 8 | $10^{-1}$ | ~15 |

| Bacterial cell | No. of pfu | Dilution |
|---|---|---|
| VCS257 (packaging control) | 300 | $10^{-4}$ |
| SURE (packaged test ligation) | 956 | $10^{0}$ |
| | 184 | $10^{-1}$ |

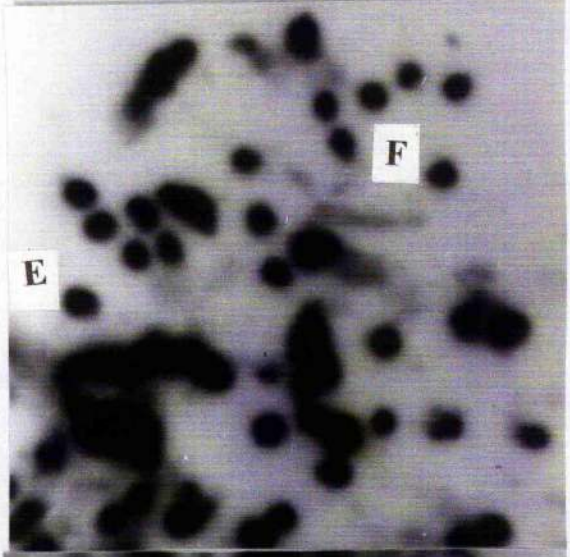
**Figure 4.3** **Nylon filters showing the results of primary, secondary and tertiary screening of a putative *Bryophyllum fedtschenkoi CAB* clone from the constructed cDNA library**

30,000 pfu of the *Bryophyllum fedtschenkoi* amplified cDNA library were initially screened with the heterologous AB165 *CAB* probe as described in section 2.16.1. 10 putative *CAB* clones were identified. Figure 4.3a shows a photograph of two of these, labelled 5 and 6, after primary screening. A subsequent secondary screening procedure was carried out with these primary positives, again using the AB165 *CAB* probe and 11 putative clones were selected. Figure 4.3b shows a photograph of the secondary screening result with the putative *CAB* clone 5, putative *CAB* clones E and F were selected from this plate. Figure 4.3c shows the result of tertiary screening with the putative *CAB* clone F. The fact that the filter lift appears black implies that isolated clone F is very likely to be a *CAB* clone.

a. 1°




b. 2°




c. 3°

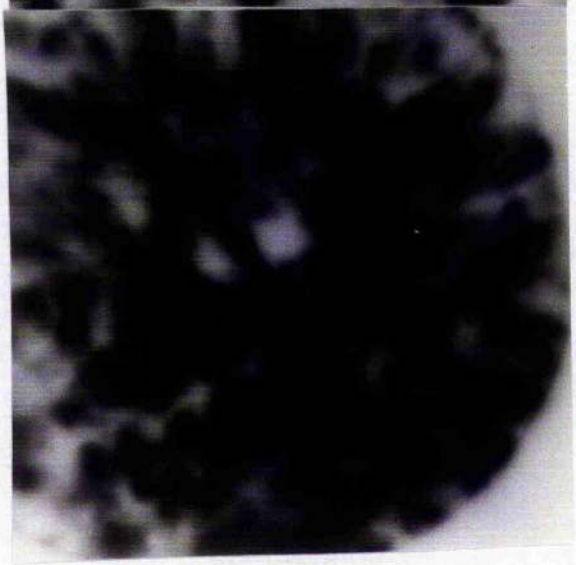


**Figure 4.4** **Restriction digest of the isolated plasmid containing the putative *Bryophyllum fedtschenkoi CAB* insert cDNA with the restriction endonucleases *Xho*I and *Eco*RI**

10 µl of the plasmid preparation E was mixed with 6 µl $dH_2O$, 2 µl REACT 4 buffer (GIBCO BRL), 1 µl *Xho*I and 1 µl *Eco*RI. The reaction mixture was left in a water bath at 37°C for 2 hours. After the reaction time had proceeded 2 µl of gel loading buffer (section 2.8.1) was added and the digested sample was run on a 1 % agarose gel (section 2.8.1). Figure 4.4 is a photograph of this restriction digest reaction. The markers used were λ DNA cut with *Eco*RI and *Hin*dIII. The different fragments seen are identified. The lanes are labelled M for the marker and E for the plasmid.

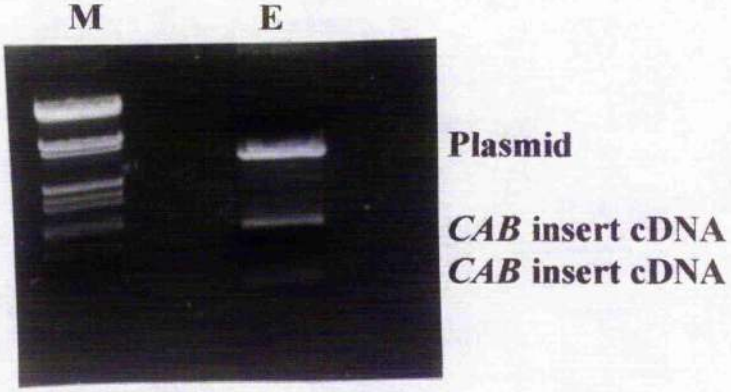

M
E
Plasmid
*CAB* insert cDNA
*CAB* insert cDNA

**Figure 4.5 Sequence of isolated *Bryophyllum fedtschenkoi CAB* cDNA insert**

The completed DNA sequence analysis of the cDNA insert is shown. The 230 amino acid open reading frame is identified under the nucleotide sequence and begins at the start of the sequence. The 19 kbp poly(A)$^+$ tract begins at nucleotide 784 and ends at nucleotide 802. The internal *Eco*RI site is underlined. The three amino acid regions shown in bold are representative of the proposed transmembrane domains of the mature *CAB* peptide.

GGCACGAGCTAAGCCGAAGCCAGCGGCATCATCCAGCCCATGGTACGGTCCTGACCGTGT 60
A R A K P K P A A S S S P W Y G P D R V 20

CAAGTACTTGGGACCGTTCTCGGGTGAGGCTCCATCGTACCTGACCGGTGAATTCCCAGG 120
K Y L G P F S G E A P S Y L T G E F P G 40

TGACTACGGCTGGGACACCGCTGGACTCTCTGCTGACCCCGAGACATTCGCTAAGAACCG 180
D Y G W D T A G L S A D P E T F A K N R 60

CGAGCTTGAGGTGATCCACAGCAGATGGGCCATGCTCGGCGCTCTCGGATGCGTCTTCCC 240
E L E V I H S R W A M L G A L G C V F P 80

CGAGCTCTTGTCTCGCAATGGTGTCAAGTTCGGCGAGGCTGTCTGGTTCAAGGCTGGCGC 300
E L L S R N G V K F G E A V W F K A G A 100

CCAGATCTTCAGCGAGGGAGGACTCGACTACTTGGGCAACCCGAGCTTGGTCCACGCCCA 360
Q I F S E G G L D Y L G N P S L V H A Q 120

AAGCATCTTGGCCATCTGGGCTACCCAGGTTATCCTGATGGGAGCCGTCGAGGGCTACCG 420
S I L A I W A T Q V I L M G A V E G Y R 140

CATTGCTGGAGGGCCACTCGGCGAGGTCACCGACCCGTTGTACCCCGGTGGCAGCTTCGA 480
I A G G P L G E V T D P L Y P G G S F D 160

CCCGTTAGGCCTTGCTGACGACCCAGAGGCTTTCGCTGAGCTTAAAGTGAAGGAGATCAA 540
P L G L A D D P E A F A E L K V K E I K 180

GAATGGAAGACTGGCCATGTTCTCCATGTTTGGATTCTTCGTCCAGGCCATCGTCACCGG 600
N G R L A M F S M F G F F V Q A I V T G 200

AAAGGGACCCTTGGAGAACCTCGCCGACCACCTCGCCGACCCAGTCAACAACAATGCCTG 660
K G P L E N L A D H L A D P V N N N A W 220

GGCTTATGCCACCAACTTTGTCCCCGGAAAGTGAGAACTGAGAATTTCAAATTTCACCAA 720
A Y A T N F V P G K * 230

AAACTGTGAGACTGTTGTAATGTAAATCATATGCACATTATTAAAGAAGTTTAATTTCAA 780

ACAAAAAAAAAAAAAAAAAAAC 802

**Figure 4.6** **Comparison between the derived peptide sequence of the *Bryophyllum fedtschenkoi CAB* cDNA and a *L. gibba* light harvesting chlorophyll a/b binding protein gene AB30**

This figure shows a comparison between the derived peptide sequence of the *Bryophyllum fedtschenkoi CAB* cDNA (labelled F1.Pep) and a *Lemna gibba* light harvesting chlorophyll a/b binding protein gene AB30 (labelled Lgilhc). A 95 % identity exists between the two sequences in a 228 amino acid region.

```
SCORES      Frame: (2) Initl: 1142 Initn: 1142 Opt: 1143
             94.7% identity in 228 aa overlap

                                                    10        20        30
Fl.Pep                                          ARAKPKPAASSSPWYGPDRVKYLGPFSGEAPS
                                                  :||||::|:||||||||||||||||||||||
Lgilhc SPSLVGKAVKLAPAASEVFGEGRVSMRKTAGKPKPVSSGSPWYGPDRVKYLGPFSGEAPS
     140       150       160       170       180       190

               40        50        60        70        80        90
Fl.Pep YLTGEFPGDYGWDTAGLSADPETFAKNRELEVIHSRWAMLGALGCVFPELLSRNGVKFGE
       ||||||:||||||||||||||||||||||||||:||||||||||||||:||||||||
Lgilhc YLTGEFAGDYGWDTAGLSADPETFAKNRELEVIHARWAMLGALGCVFPELLARNGVKFGE
     200       210       220       230       240       250

              100       110       120       130       140       150
Fl.Pep AVWFKAGAQIFSEGGLDYLGNPSLVHAQSILAIWATQVILMGAVEGYRIAGGPLGEVTDP
       |||||||:||||||||||||||||||||||||||||:|||||||||:|||||||||:||
Lgilhc AVWFKAGSQIFSEGGLDYLGNPSLVHAQSILAIWATQVVLMGAVEGYRVAGGPLGEVVDP
     260       270       280       290       300       310

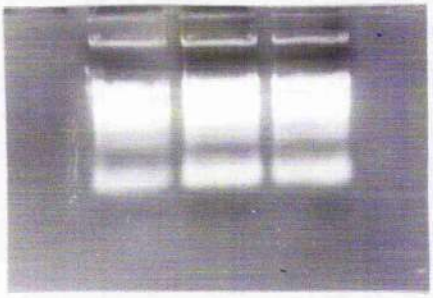
              160       170       180       190       200       210
Fl.Pep LYPGGSFDPLGLADDPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLENLADHLA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Lgilhc LYPGGSFDPLGLADDPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLENLADHLA
     320       330       340       350       360       370

              220       230
Fl.Pep DPVNNNAWAYATNFVPGK
       |||||||||:||||||||
Lgilhc DPVNNNAWAFATNFVPGKXEAPSCSRDPCPMYMRRRLCSIMYHYRSHFIHFILLPFPTLD
     380       390       400       410       420       430
```

**Figure 4.7 Photographs showing isolated DNA from *Bryophyllum fedtschenkoi* leaf tissue**

Figure 4.7a is a photograph of DNA, isolated using the procedure described in section 2.6, from the *Bryophyllum fedtschenkoi* leaves of size 1 to 2. Each lane is of an individual preparation from separate leaf samples. 8 leaves of size 1 to 2 were used in the isolation of each sample. Figure 4.7b shows isolated DNA from large leaves of *Bryophyllum fedtschenkoi* before and after treatment with the enzyme RNAase A. In lane 1 there is 20 μl of untreated DNA. In lane 2 there is 20 μl of DNA which has been treated with RNAase A. The reaction in lane 2 was carried out at 37°C for 15 minutes to 30 minutes with a 2 μl addition of RNAase A. The contaminating RNA is removed by this treatment. The marker used was a GIBCO BRL 1 kb DNA ladder. The DNA and RNA bands are labelled accordingly.

a.

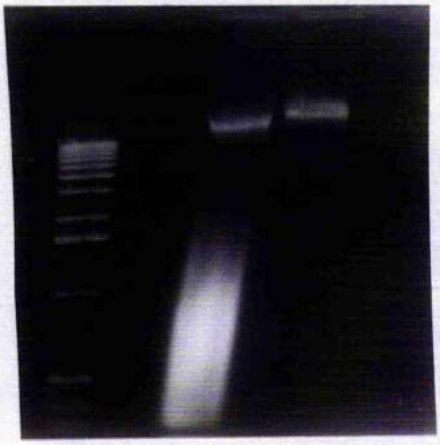


DNA

}RNA

b.

1 2



DNA

RNA

**Figure 4.8** **Photograph of an agarose gel showing the restriction digestion of DNA isolated from small leaves of *Bryophyllum fedtschenkoi* with a number of restriction endonucleases**

The restriction digestion reactions, shown in Figure 4.8, were set up as follows. 30 μg to 40 μg of DNA isolated from small leaves was incubated with 5 μl RNAase A, as described in section 2.9. The DNA was digested with restriction endonucleases (section 2.9) and precipitated on a 0.8 % agarose gel (section 2.8.1). λ DNA cut with the restriction endonucleases *Hin*d III and *Eco*R I was used as a marker. Digestions of two DNA samples, A and B, isolated from the small leaves of *Bryophyllum fedtschenkoi* are shown in Figure 4.8. The different lanes and restriction endonucleases are identified below;

| Lane No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Enzyme | Uncut | *Eco*RI | *Bam*HI | *Hin*dIII | *Xba*I | *KpN* I | *Bgl*II |

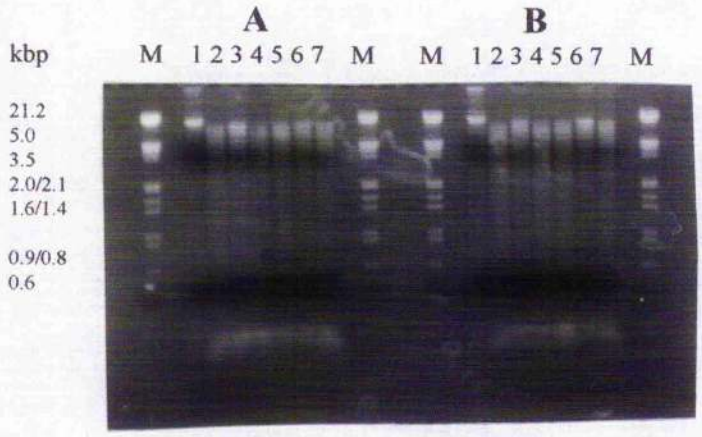

A
B
kbp
M 1 2 3 4 5 6 7 M
M 1 2 3 4 5 6 7 M
21.2
5.0
3.5
2.0/2.1
1.6/1.4
0.9/0.8
0.6

**Figure 4.9** **Autoradiograph showing the hybridisation of a homologous *Bryophyllum fedtschenkoi CAB* cDNA with restriction digested DNA isolated from small leaves of *Bryophyllum fedtschenkoi***

The Southern blot of the gel shown in Figure 4.8 was hybridised with the homologous *Bryophyllum fedtschenkoi CAB* cDNA (specific activity 8.1 x $10^8$ dpm/μg) as in section 2.14.2.2. The hybridised filter was washed in a series of stages. The least stringent wash was in 2 x SSC/0.1 % (w/v) at a temperature of 58°C to 60°C. The most stringent wash was in 0.1 x SSC/0.1 % (w/v) SDS at 65°C. The duration of each wash was for 10 minutes. The resulting autoradiograph is shown. The exposure time was 72 hours. The different samples A and B are labelled. The different lanes are shown below;

| Lane No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Enzyme | Uncut | *Eco*RI | *Bam*HI | *Hin*dIII | *Xba*I | *KpN* I | *Bgl*II |

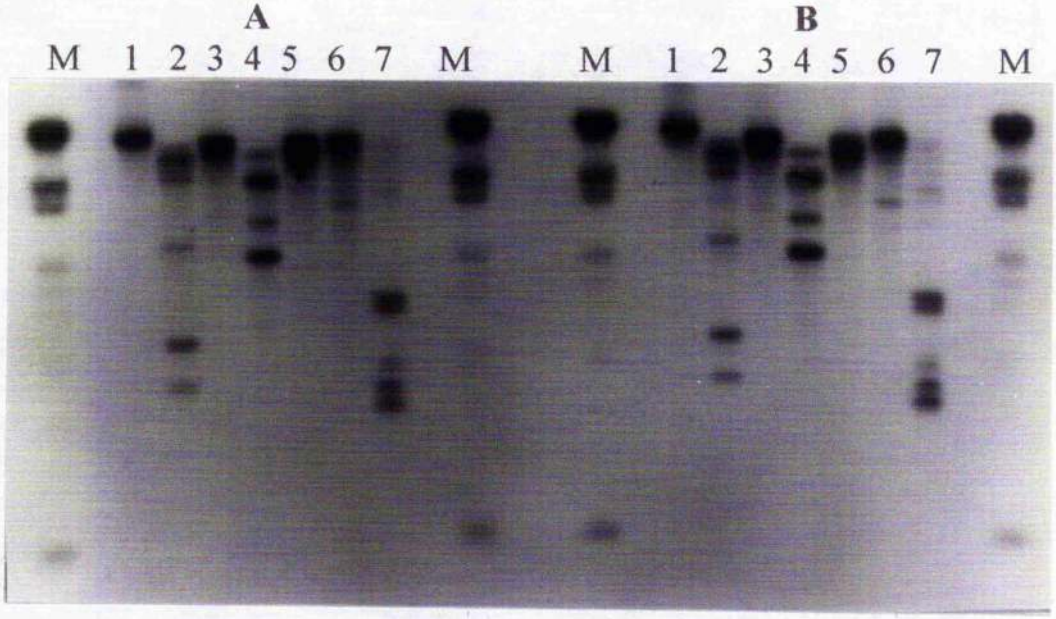

A
M 1 2 3 4 5 6 7 M
B
M 1 2 3 4 5 6 7 M

**Figure 4.10 Comparison of the deduced amino acid sequence of the *Bryophyllum fedtschenkoi CAB* cDNA with other known *CAB* genes**

The deduced amino acid sequences of the *Bryophyllum fedtschenkoi CAB* cDNA (Bf.*CAB*), *Lemna gibba* AB30 *CAB* gene (Lg.AB30), *Petunia* (Mitchell) *CAB* 22R gene (Pt.22R), *Arabidopsis thaliana* AB165 *CAB* gene (AB165) and *Zea mays CAB*-M7 gene (M.*CAB*7) are compared in Figure 4.10. The *Bryophyllum fedtschenkoi CAB* cDNA deduced amino acid sequence is shown. Regions of homology are identified by a * and any sequence variations are shown by the respective amino acid residue. The *Arabidopsis thaliana* AB165 CAB protein has been realigned to best fit the *Bryophyllum fedtschenkoi* CAB sequence; the symbol + shows a shift and the bold **N** (position 144) is not aligned to the other CAB sequences. The amino acid residues underlined at positions 67, 84, 128, 131, 141 and 222 aid in the classification of the *Bryophyllum fedtschenkoi* CAB peptide into a specific Type I LHC II subgroup (Demmin *et al.*, 1989).

```
Bf.CAB   ARAKPKPAAS  SSPWYGPDRV  KYLGPFSGEA  PSYLTGEFPG  DYGWDTAGLS  50
Lg.AB30  TAG ****VS *  G **** *****  ***** *****  ***** *A***  *****  *****
Pt.22R   TVT *A***S *  ***** *****  ***** *****  ***** ****S  *****  *****
AB165    TV ****G P+*  G **** *S***  ***** ****S  ***** *****  *****  *****
M.CAB7   *AKAKPA***  G **** *****  L**** L***P  ***** *****  *****  *****

Bf. CAB  ADPETFAKNR  ELEVIHSRWA  MLGALGCVFP  ELLSRNGVKF  GEAVWFKAGA  100
Lgilhc   ***** *****  ******A***  ***** *****  ***A* *****  *****  **** S
Pt.22R   ***** *****  ******C***  ***** *****  ***A* **I **  *****  *****
AB165    ***** **R**  **********  ***** *****  ***A* *****  *****  **** S
M.CAB7   ***** *****  **********  ***** *****  ***A* *****  *****  **** S

Bf. CAB  QIFSEGGLDY  LGNPSLVHAQ  SILAIWATQV  ILMGAVEGYR  IAG  GPLGEVT  150
Lgilhc   **********  ***** *****  **********  V**** *****  V**  ******V
Pt.22R   **********  ***** *****  *******C**  V**** *****  V**  ****** I
AB165    *********D  ***** *****  **********  ***** *****  V**N*****AE
M.CAB7   **********  ***** *I ***  *******C**  V**** *****  ***  ******V

Bf. CAB  DPLYPGGSFD  PLGLADDPEA  FAELKVKEIK  NGRLAMFSMF  GFFVQAIVTG  200
Lgilhc   ***** *****  ***** *****  ***** *****  ***** *****  ***** *****
Pt.22R   ***** *****  ***** E****  ***** *****  ***** *****  ***** *****
AB165    *L*** *****  ***** T****  ***** ***L*  ***** *****  ***** *****
M.CAB7   ***** *****  ***** *****  ***** ***L*  K **** *****  ***** *****

Bf. CAB  KGPLENLADH  LADPVNNNAW  AYATNFVPGK  230
Lgilhc   ***** *****  ***** *****  *F***  *****
Pt.22R   ***** *****  ***** *****  S **** *****
AB165    ***I * *****  ***** *****  *F*** *****
M.CAB7   ***** *****  I **** *****  ***** *****
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## Chapter 5. *CAB* gene expression in *Bryophyllum fedtschenkoi*

### 5.1 Introduction

Plants are sessile organisms and so are at the mercy of their surrounding environment. Consequently the environmental factors of light and temperature have a profound effect on the growth and development of plants. Light influences a multitude of responses in plants and affects the activity of a number of genes that are involved in developmental or metabolic processes. Light has been found to stimulate the expression of the nuclear genes encoding the major light-harvesting chlorophyll a/b binding protein of the thylakoid LHC II complex (*CAB* genes). The photoregulation of expression starts with the initial reception of light, involves a series of signal transduction processes and ends with the eventual control of gene expression. The expression of the genes can be controlled at transcriptional, post-transcriptional, translational or post translational events. Taylor (1989) stated that, "The accumulation of *CAB* mRNA appears to be regulated exclusively at the transcriptional level, with a number of factors affecting *CAB* gene transcription." The majority of my work in this chapter analyses the change in the levels of the *CAB* transcripts in *Bryophyllum fedtschenkoi* under differing light controlled conditions.

Light affects plant growth and development at virtually every stage of the life cycle. An example is during seedling emergence where light stimulates chlorophyll production, leaf development, cotyledon expansion, chloroplast biogenesis and the coordinated induction of many nuclear and chloroplast encoded genes. In addition to light regulation, *CAB* gene expression is spatially regulated in different organs and cells,

and temporally regulated during development (Brusslan *et al.*, 1992; Chang *et al.*, 1992a and 1992b; Kretsch *et al.*, 1995; Tavladoraki *et al.*, 1989). A result of this is that the analysis of *CAB* genes has involved a number of different areas of study.

These experiments use the *Bryophyllum fedtschenkoi CAB* cDNA as a homologous probe in gene expression studies. The effects of leaf age, light conditions and temperature on *Bryophyllum fedtschenkoi CAB* gene expression were analysed. A series of time course studies were carried out, in different conditions, to allow me to compare the circadian rhythms of *CAB* gene expression with those of carbon dioxide metabolism.

## 5.2 Ontogenetic control of *CAB* gene expression in *Bryophyllum fedtschenkoi*

The initial experimental work (chapter 3) concluded that to establish whether a circadian rhythm of *CAB* gene expression was linked to the circadian rhythm of $CO_2$ metabolism of *Bryophyllum fedtschenkoi*, middle sized leaves (length 4.0 cm to 4.5 cm) should be used for further experiments. The aim of this experiment was to establish if *CAB* gene expression could be identified in this size of leaves and, therefore, be used in circadian rhythm experiments.

Leaf samples were removed from *Bryophyllum fedtschenkoi* plants in the middle of the light period of the growth room. The leaf samples were taken from the tip of the plant down to the sixth pair of leaves (length 6.0 cm to 7.0 cm) on the stem. Figure 5.1 is a photograph of the different sizes of *Bryophyllum fedtschenkoi* leaves analysed. This study was more

comprehensive than the ontogenetic analysis described in section 3.3. RNA extracted as in section 2.4.2 (Chang *et al.*, 1993), was run on a denaturing formaldehyde agarose gel and northern blotted. Hybridisation analysis was carried out using the homologous *Bryophyllum fedtschenkoi CAB* cDNA as a probe. The nylon membrane was also hybridised with a *Triticum aestivum* ribosomal genomic DNA fragment of 9.0 kbp (Gerlach and Bedbrook, 1979). This was used to serve as a control for the RNA loading of the formaldehyde denaturing gel. This study was carried out three times with different leaf samples. Figure 5.2 shows the autoradiographs of one of these hybridisation analyses. In Figure 5.2 air bubbles occurred during the hybridisation analyses, and show up at size 0 leaves for the *CAB* hybridisation on Figure 5.2a and where the 18S ribosomal transcript is not present (leaf sizes 2 and 3) for the wheat ribosomal DNA hybridisation. There is no lane for the RNA isolated from the tip of the *Bryophyllum fedtschenkoi* plant as the samples were not of a high enough concentration. The autoradiographs from each of the three experimental studies were scanned as in section 2.14.6. The values obtained for the *CAB* transcripts were compared to the values for the 25S ribosomal DNA transcripts and a pattern of ontogenetic regulation of *Bryophyllum fedtschenkoi CAB* gene expression was developed. Figure 5.3a illustrates how *CAB* gene expression varies with leaf size in *Bryophyllum fedtschenkoi*. I also analysed how actual leaf area varies with leaf size. For these experiments the leaves were placed onto a piece of graph paper and the leaf area calculated. Figure 5.3b is a graph of how *Bryophyllum fedtschenkoi* leaf area varies with leaf size.

A pattern was seen (Figure 5.3a) where a peak of *CAB* gene expression occurred in size 2 leaves (3.0 cm to 3.5 cm in length), and this was followed by a subsequent general decline. The younger and older leaf

sizes showed increasing and decreasing levels of expression respectively. The variability of the data suggests that the peak at size 4 leaves (5.0 cm to 5.5 cm in length) may not be significant. These patterns were also seen in each of the three replicate experiments. The change in leaf area showed a different pattern where the leaf area increased with leaf size (Figure 5.3b) but appeared to be reaching a maximum level just after size 6 leaves (6.0 cm to 6.5 cm in length).

## 5.3 *CAB* gene expression in normal growth conditions with an 8 hour light period and 16 hour dark period

The aim of this investigation was to follow *CAB* gene expression in *Bryophyllum fedtschenkoi* through its entire diurnal growth cycle. This showed me the change in *CAB* gene expression that I expected to find in the circadian rhythms of *CAB* gene expression. Therefore, when *Bryophyllum fedtschenkoi* plants are placed into constant conditions the circadian pattern of *CAB* gene expression can be compared to its diurnal pattern.

*Bryophyllum fedtschenkoi* plants were grown in a growth room with an 8 hour light period and 16 hour dark period. Leaf samples, of length 3.0 cm to 5.0 cm, were taken at time points of 4 hour intervals to cover the beginning and end of the cycle over a time of 24 hours duration. The result was that six time points were used (see Figure 5.4). RNA extractions were carried out on these samples as described in section 2.4.2. The samples were run on a formaldehyde denaturing agarose gel (section 2.8.2), northern blotted (section 2.11.1) and analysed by hybridisation. Hybridisation analysis was carried out using the homologous *Bryophyllum fedtschenkoi CAB* cDNA probe and the

*Triticum aestivum* ribosomal genomic DNA fragment (section 5.2) as a control. Duplicate samples were collected and analysed in exactly the same way. Figure 5.4 shows the result of hybridisations with the *CAB* cDNA and ribosomal DNA probes from one of these studies. The *CAB* transcript showed a low level of accumulation at 0800 h, reached a maximum level at 1200 h and was undetectable from 1600 h to 0400 h. The autoradiographs of each study were scanned, as in section 2.14.6, and the values for the *CAB* transcripts were compared to the values for the 25S ribosomal DNA transcripts. The *CAB*/25S rRNA ratios for each time point were then averaged. The resulting diurnal pattern of *CAB* gene expression in *Bryophyllum fedtschenkoi* is shown in Figure 5.4c.

## 5.4 The circadian rhythms of *CAB* gene expression in *Bryophyllum fedtschenkoi*

### 5.4.1 *CAB* gene expression in carbon dioxide free air and continuous darkness

The circadian rhythms of $CO_2$ metabolism of detached leaves of *Bryophyllum fedtschenkoi* were found to continue for about three cycles in conditions of continuous darkness and carbon dioxide free air at a temperature of 15°C. Nimmo *et al.* (1987) and Carter *et al.* (1991) identified circadian rhythms in the phosphorylation state of phosphoenolpyruvate carboxylase (PEPc) and PEPc kinase respectively. My earlier work (chapter 3) suggested that *CAB* gene expression was under circadian control. Therefore, I compared the rhythms of $CO_2$ metabolism and *CAB* gene expression.

Pairs of *Bryophyllum fedtschenkoi* leaves, of size 4.5 cm to 5.0 cm, were detached, at the end of a normal light period, weighed and placed into an air-tight sealed brass container which was connected to the IRGA under the conditions of continuous darkness, $CO_2$ free air and a constant temperature of 15°C (section 3.4.2). The leaves were removed at specific time points, under a green safe light, and snap frozen under liquid nitrogen. The peaks and troughs of the circadian rhythm of $CO_2$ metabolism were chosen as these specific time points, because they showed the points at which the $CO_2$ rhythm was alternating. These time points are identified on Figure 5.5. RNA was extracted from the leaf samples using the Chang *et al.* (1993) procedure (section 2.4.2), run on a formaldehyde denaturing agarose gel (section 2.8.2) and northern blotted (section 2.11.1). The resulting filter was hybridised with the *Bryophyllum fedtschenkoi CAB* cDNA and with an H1 cDNA by using the Denhardts method (section 2.14.1.1). The H1 cDNA probe was used as a control since H1 transcripts are constitutively and abundantly expressed in *Phaseolus vulgaris* (Lawton and Lamb, 1987). The experiment was carried out in duplicate. Figure 5.5a and Figure 5.5b show the results of one set of hybridisations. A *CAB* transcript of size 0.8 kb to 1.1 kb was identified and an H1 transcript of size 3.7 kb was identified. The autoradiographs of both experiments were scanned as in section 2.14.6 and the values obtained for the *CAB* and H1 transcripts compared. The average value of the *CAB*/H1 ratios was calculated at each time point (Figure 5.6a). *Bryophyllum fedtschenkoi CAB* gene expression appeared to follow a circadian rhythm in the conditions of $CO_2$ free air and continuous darkness. The initial peak of expression was at 1000 h to 1200 h and the second peak was at 1200 h to 1600 h. The initial trough of expression was at 2200 h to 0200 h and the second trough was at 2400 h to 0400 h. This implied that the circadian rhythm had a period length

of 26 hours to 30 hours. The level of *CAB* gene expression and amplitude of the rhythm tended to drop after some time in the constant conditions. It was usually possible to detect three complete cycles of the rhythm in $CO_2$ output in detached leaves of *Bryophyllum fedtschenkoi* under the same conditions. It was possible that the *CAB* rhythm would also be detectable over three days but the analysis was not extended this far. Figure 5.6b shows the IRGA analysis of three pairs of the middle sized leaves used under these conditions over the same period of study.

There are a number of differences between the rhythm of *CAB* gene expression and $CO_2$ metabolism. Wilkins (1962) showed that when detached leaves of *Bryophyllum fedtschenkoi* were placed in the conditions of $CO_2$ free air, continuous darkness and at a constant temperature of 16°C the period length of the $CO_2$ metabolic rhythm was $23.8 \pm 0.3$ hours. The period length of the *CAB* rhythm appears to be longer (26-30 hours). Also the first peak of the *CAB* rhythm occurs at least 4-6 hours before that of the $CO_2$ rhythm (see Figure 5.6a and b).

### 5.4.2 *CAB* gene expression in conditions of continuous light/normal air and continuous dark/normal air at a constant temperature

The initial experimental analysis of *CAB* gene expression in continuous dark and light conditions (section 3.2.2) showed that the regulation did not solely depend on light/dark changes. The indication was that the control of *CAB* gene expression in *Bryophyllum fedtschenkoi* leaves was by a circadian mechanism. To extend this work and identify if the mechanism of control was circadian, I carried out time course analyses over the duration of a few cycles.

*Bryophyllum fedtschenkoi* plants were removed from their growth room at the end of the normal light period and placed in a growth room either in constant light (temperature 18°C) or constant darkness (temperature 15°C). The experiments were carried out in duplicate with each particular constant condition. In each experiment leaf samples, of size 3.5 cm to 5.0 cm, were collected at 4 hour time intervals and snap frozen in liquid nitrogen. In the case of the plants placed into constant darkness the samples were taken under a green safe light to ensure that there could be no effects on *CAB* gene expression by an outside light source. RNA was extracted from the samples by using the Chang *et al.* (1993) procedure (section 2.4.2) and the samples were run on a denaturing formaldehyde agarose gel (section 2.8.2). Northern blotting was carried out (section 2.11.1) and the resulting nylon membrane filters were hybridised with the homologous *Bryophyllum fedtschenkoi CAB* cDNA and with the H1 cDNA probe (discussed in section 5.4.1). Figure 5.7 and Figure 5.9 show the results from one of these hybridisation analyses with the samples collected under continuous light and continuous dark conditions.

In continuous light a number of different transcripts were seen. These were assumed to be *CAB* transcripts because of the stringency of hybridisation and washing. However, it is not clear why they were resolved in this experiment and not in other experiments (see Discussion, section 5.6). The putative different *Bryophyllum fedtschenkoi CAB* transcripts were named *CAB* 1, *CAB* 2 and *CAB* 3. The two strongest transcripts were of size 1.0 kb to 1.1 kb (*CAB* 1) and 0.6 kb to 0.7 kb (*CAB* 3). There was a weak transcript of size 0.8 kb (*CAB* 2). An H1 transcript of size 3.5 kb to 3.7 kb was identified. The *CAB* 1 transcript was present all the time but showed some oscillation in its level of expression whereas the *CAB* 3 transcript showed a cycle of accumulation

and reduction. After one day of continuous light the *CAB* 3 transcript appeared between 0400 h and 1600 h and disappeared at 1600 h to 0800 h the following day. The *CAB* 3 transcript level was reduced going into the second day but it was present until at least 2400 h. The weak *CAB* 2 transcript only clearly shows expression after one day of continuous light between 0800 h to 1200 h.

Figure 5.7b shows the result of one of the hybridisation analyses with the control H1 cDNA probe. The apparent rhythm is probably an artifact caused by loading variable amounts of RNA onto different lanes, as judged by the photograph of the gel. Therefore, to aid in the interpretation of the experiments, the autoradiographs from each study for continuous light conditions were scanned (section 2.14.6). The values for the *CAB* transcripts were compared to the values for the H1 transcripts. The average value of *CAB*/H1 ratios was calculated for each time point. Figure 5.8 shows the graphical representations of these results for the *CAB* 1 and *CAB* 3 transcripts identified. While the *CAB* 1 transcript appeared to exhibit a circadian rhythm, it was difficult to analyse because each of the second and third peaks was actually split into two. However, as judged by both peaks and troughs, expression showed a rhythm with a fairly small amplitude and a period of about 20 hours. *CAB* 1 showed 3 peaks at about 1200 h, 0800 h and 0400 h (period length 20 hours). The troughs of *CAB* 1 expression were at 2000 h, 1600 h and 1200 h (period length 20 hours). *CAB* 3 also showed a cyclical pattern in which each appeared as a doublet. However, the nature of the *CAB* 3 signal is not clear. Wilkins (1984) shows that under the conditions of continuous light, normal air and a constant temperature of 15°C, the carbon dioxide circadian rhythm in detached leaves of *Bryophyllum fedtschenkoi* was

found to continue for a number of cycles with a period length of 18.1 $\pm$ 0.1 hours.

In continuous darkness a *Bryophyllum fedtschenkoi CAB* transcript of size 1.2 kb to 1.3 kb and an H1 transcript of size 3.7 kb to 4.0 kb were identified (Figure 5.9). The *CAB* transcript was absent for the first 8 hours of darkness. It appeared 4 hours prior to the time at which a normal light period would start and reached maximum expression at the start of what would be the light period. The level of *CAB* gene expression then declined and no transcript was detectable over the next 36 hours of continuous darkness. After the plants had been left in these conditions for two days they were returned to their normal growth room and leaf samples taken at the middle of the next two light periods. This was to test if the plants were still photosynthetically active. These are shown on Figure 5.9. *CAB* gene expression returned to normal after two days. As has been discussed (see section 3.4.3) the rhythm of carbon dioxide metabolism under these conditions showed a very similar pattern in that only one period of $CO_2$ uptake was observed, followed by a steady rate of $CO_2$ output. In the conditions of continuous darkness and normal air I can not say if *CAB* gene expression was under the control of an endogenous oscillator. However, since one peak of *CAB* expression is seen in continuous darkness, it is clear that expression is not controlled simply by light/dark changes.

## 5.5 The regulation of *CAB* gene expression in continuous darkness/normal air at temperatures of 5°C, 27°C and 35°C

The aim of this investigation was to see how *Bryophyllum fedtschenkoi CAB* gene expression was affected by changing the temperature of the

surrounding environment. In *Bryophyllum fedtschenkoi* Carter *et al.* (1995a, 1995b) found that the phosphorylation state and the malate sensitivity of phosphoenolpyruvate carboxylase (PEPc) were altered by changes in the ambient temperature. This in turn affected the patterns of $CO_2$ gaseous exchange that occurred in conditions of continuous darkness and normal air. A comprehensive time course analysis of *Bryophyllum fedtschenkoi CAB* gene expression in the conditions of normal air and continuous darkness at a constant temperature of 15°C had been carried out (section 5.4.2). Three separate experiments were set up with the same conditions except for differences in temperature.

As in section 5.4.1 pairs of *Bryophyllum fedtschenkoi* leaves, of size 4.5 cm to 5.3 cm, were detached at the end of a normal light period, weighed and placed into an air-tight sealed brass container that was connected to the IRGA under the conditions of continuous darkness, normal air and a constant temperature of 5°C, 27°C or 35°C. The leaves were removed at specific time points, under a green safe light, and snap frozen under liquid nitrogen. The specific time points were chosen in relation to the results shown in section 5.4.2. In each temperature analysis the final samples collected had been illuminated for four hours at a time point that was equivalent to the beginning of a normal light period. This was to test whether the leaves were still photosynthetically active and whether *CAB* gene expression would occur. Duplicate studies were carried out in each case. As in section 5.4.1, RNA was extracted from the leaf samples using the Chang *et al.* (1993) method (section 2.4.2), run on a formaldehyde denaturing agarose gel (section 2.8.2) and northern blotted (section 2.11.1). The resulting filters were hybridised with the *Bryophyllum fedtschenkoi CAB* cDNA and with the H1 cDNA (discussed in section 5.4.1) using Denhardts method (section 2.14.1.1). Figures

5.10, 5.11 and 5.12 show the results of these hybridisations with the analyses at the temperatures of 5°C, 27°C and 35°C. In each case an H1 transcript of size 3.7 kb was observed. At a constant temperature of 27°C and 35°C a *CAB* transcript of size 0.8 kb to 1.1 kb was observed. At 5°C essentially no *CAB* transcript appeared (Figure 5.10a). However, prolonged exposure of the autoradiograph (not shown) suggested that a very low amount of the *CAB* transcript was present initially at a temperature of 5°C. The *CAB* transcript was only present for the first 8 hours in these conditions. No *CAB* gene expression was observed after illumination at the end of the period of continuous darkness.

At a temperature of 27°C (Figure 5.11) the *CAB* transcript showed a similar pattern to that seen when a *Bryophyllum fedtschenkoi* plant was left in continuous darkness and normal air at a constant temperature of 15°C. The *CAB* transcript appeared 4 hours before a normal light period would start (0400 h), reached maximum expression at the start of the subjective light period (0800 h) and disappeared rapidly from 0800 h to 1200 h. The amplitude changes in the *CAB* transcript were bigger than those in Figure 5.9. The *CAB* transcript was not apparent again until the leaves were illuminated at the end of the analysis. The leaves were still photosynthetically active.

At a temperature of 35°C (Figure 5.12) the *CAB* transcript appeared and disappeared a number of times. The *CAB* transcript was detectable but the expression showed no clear pattern. Each time the *CAB* transcript appeared the level of expression seen dropped immediately afterwards. The *CAB* transcript was only slightly apparent when the leaves were illuminated at the end of the analysis. In Figure 5.12b the H1 transcript did not show a constant appearance (see lanes 7 and 8) so any conclusions

on the patterns of expression were subjective. However, in the duplicate experiment (not shown) the *CAB* transcript was harder to detect but the pattern of expression was the same as described above, and in this case the H1 transcript shows a constant expression for the duration of the experiment.

Figures 5.10c, 5.11c and 5.12c show the IRGA analysis of three pairs of the middle sized leaves used under the conditions of continuous darkness and normal air at the temperatures of 5°C, 27°C and 35°C. At the different temperatures of analysis distinctive variations were seen in the *Bryophyllum fedtschenkoi CAB* gene expression and in the $CO_2$ metabolism. Figure 5.13 compares the patterns of $CO_2$ gaseous exchange of three separate pairs of middle sized leaves at the different temperatures. At a temperature of 27°C the same pattern of $CO_2$ gaseous exchange was seen as shown in Figure 3.14 whereas at a temperature of 35°C a partial rhythmical variation of $CO_2$ gaseous exchange occurred, but with a very short period length and which tended to fade out rapidly. But there was never any dramatic decrease in the level of $CO_2$ gaseous exchange at a temperature of 35°C. At a temperature of 5°C the pattern of $CO_2$ gaseous exchange began as that seen in Figure 3.14 and Figure 5.11c. However, the level of $CO_2$ gaseous exchange increased very slowly unlike that seen in the studies at the temperatures of 15°C and 27°C. The detached leaves in continuous darkness, normal air and at a constant temperature of 5°C, 27°C and 35°C even showed the effect of the introduced light on the IRGA analyses by the sudden drop in the $CO_2$ gaseous exchange at the end of the study. These different patterns were showing a similarity between the circadian rhythms of *Bryophyllum fedtschenkoi CAB* gene expression and $CO_2$ metabolism.

## 5.6 Discussion

### 5.6.1 Ontogenetic control and diurnal pattern of *CAB* gene expression

*Bryophyllum fedtschenkoi CAB* gene expression showed two peaks for leaves 3.0 cm to 3.5 cm and 5.0 cm to 5.5 cm in length whereas leaves 4.0 cm to 4.5 cm in length showed a trough. Leaves that were of a smaller and a larger size tended to show increasing and decreasing *CAB* transcript levels respectively. As the result shown in Figure 5.3a was only based on three separate studies it can be argued that the trough of *CAB* gene expression in size is not statistically significant. In the plant *Lycopersicon esculentum* Meyer *et al.* (1989) suggested that the extent of *CAB* mRNA synthesis was adapted to the needs of the individual leaves (developmental stage, light intensity, light quality). Therefore, the variable up/down pattern of the *Bryophyllum fedtschenkoi CAB* transcript (Figure 5.3a) levels may be a result of leaf development. In comparison to the results shown in Figure 3.6 a much higher level of *CAB* gene expression was seen in the larger leaves (3.0 cm to 4.5 cm in length). This implied that the previous analysis in section 3.3 did not give a full representation of the ontogenetic pattern of *CAB* gene expression in *Bryophyllum fedtschenkoi*. This was most probably on account of the limitations that were encountered with isolating high quality RNA, by using the phenol/Kirby method of extraction (section 2.4.1), from *Bryophyllum fedtschenkoi* leaves.

In *Bryophyllum fedtschenkoi* the general trend of *CAB* gene expression was that leaves 3.0 cm to 5.5 cm in length tended to show higher *CAB* transcript levels than the smaller and larger leaves. This was not unlike

the patterns of *CAB* transcript levels identified in *Lycopersicon esculentum* leaves and developing tomato fruits by Piechulla (1988), Meyer *et al.* (1989) and Piechulla *et al.* (1987). They found that the steady-state transcript levels of nuclear *CAB* genes increased to a maximum at a certain developmental stage and then proceeded to decline. Developing leaves of the plant *Phaseolus vulgaris* (Tavladoraki *et al.*, 1989) showed an increase in the level of the mRNA coding for the LHC II apoprotein up to leaves that were 13 days old and then this level decreased thereafter. These patterns of *CAB* gene expression may have similar mechanisms of control.

Studies on the developing seedlings of *Arabidopsis* (Brusslan *et al.*, 1992), *Glycine max* (Chang and Walling, 1992a and 1992b) and *Sinapsis alba* (Kretsch *et al.*, 1995) found changes in the amount of mRNAs of *CAB* genes to be under developmental regulation. In this latter case, reporter constructs were introduced into the plant *Nicotiana tabacum* and the same pattern of *CAB* gene regulation of the host plant was identified. It appears that the expression of the *CAB* gene(s) in *Bryophyllum fedtschenkoi* was regulated by a developmental programme that controlled the level of gene activation at specific stages of development (*ie*; vacuole size, chlorophyll content etc.). The implication of this was that *CAB* gene expression was affected by the developmental stage of the plant. Since I was analysing the circadian rhythms of *CAB* gene expression and comparing them to $CO_2$ metabolic rhythms in *Bryophyllum fedtschenkoi*, I subsequently used leaf material 3.5 cm to 5.5 cm in length that had higher levels of the *CAB* transcript appearing and yet showed clear rhythms of $CO_2$ gaseous exchange. This is also briefly discussed in section 3.5.3.

The diurnal pattern of *Bryophyllum fedtschenkoi CAB* gene expression showed that the *CAB* transcript accumulated, during the light period, to a maximum level at 1200 h. The *CAB* transcript decreased to a low level at 1600 h and was then absent for the duration of the dark period. These results indicated that the *CAB* transcript was degraded and newly synthesised over a 24 hour period, which suggested that a high turnover rate of the *CAB* message was occurring in the *Bryophyllum fedtschenkoi* leaves. However, direct measurements of *CAB* gene transcription and *CAB* transcript stability would be needed to test this hypothesis. This pattern of expression was very similar to the diurnal patterns of *CAB* transcripts in *Lycopersicon esculentum* (Piechulla, 1988; Kellman *et al.*, 1993), *Zea mays* (Taylor, 1989) and *Pisum sativum* (Adamska *et al.*, 1991; Spiller *et al.*, 1987). The expression of the *Bryophyllum fedtschenkoi CAB* gene was regulated by light. Kloppstech (1985) and Stayton *et al.* (1989) found that *CAB* transcripts of *Pisum sativum* and *Petunia* (Mitchell) were present at elevated levels 2 hours prior to illumination and did not decrease to undetectable levels. This was not the case with *Bryophyllum fedtschenkoi* as the *CAB* transcripts appear to show no anticipation of the light. It should be noted that there may be variations in expression between members of the *CAB* multigene family and different diurnal patterns of expression may occur. Without information on the sequences of the family members it is difficult to know whether the probe, at the stringency of hybridisation and washing used, detected one or several members of the gene family.

Figure 5.7 is the only example where putative different *Bryophyllum fedtschenkoi CAB* transcripts were detected. Whether I detected different members of the gene family is speculative. Since light is known to stimulate the expression of *CAB* genes (section 5.1) and conditions of

continuous light were present in the experiment (see Figure 5.7), it is possible that numerous *CAB* transcripts were identified. In Figures 5.4 and 5.7 after a period of 8 hours of light a single *CAB* transcript was identified whereas after a period of 20 hours of continuous light (Figure 5.7) three putative *CAB* transcripts were detected. The longer period of continuous light may have increased the transcription of a number of *CAB* genes to the extent that enhanced levels of the different *CAB* transcripts could be detected by hybridisation. However, in the preliminary experiment (Figure 3.5) numerous transcripts were not identified after 20 hours of constant light. In this study the RNA samples showed partial degradation (not shown) and this may explain why the different transcripts were not identified. *CAB* 1 is most likely to be the transcript that I normally detect since it is present within the first 8 hours of continuous light. Further analysis is necessary to discover if the different transcripts in Figure 5.7 are from the *Bryophyllum fedtschenkoi CAB* gene family. This might be achieved through the use of primer extension analysis (Kellman *et al.*, 1993).

Kellman *et al.* (1993) have found that the nineteen members of the *Lycopersicon esculentum CAB* gene family all show typical diurnal expression patterns. However, certain distinct members show detectable levels of *CAB* transcripts prior to the dark/light transition, which may be due to the abundant expression of these genes. Further analysis of the *Bryophyllum fedtschenkoi CAB* gene family may also identify such variations in the expression patterns of its members. Busheva *et al.* (1991) and Riesselmann *et al.* (1992) extended the study of the diurnal fluctuation of the steady-state level of *CAB* mRNA to the thylakoid membranes. They found that the synthesis of the LHC II polypeptide followed a diurnal fluctuation as well. These fluctuations were correlated

with the diurnal oscillation in the level of *CAB* mRNA accumulation. Further investigations are necessary to determine if this is the case in *Bryophyllum fedtschenkoi*.

### 5.6.2 The circadian rhythms of *CAB* gene expression

*Bryophyllum fedtschenkoi CAB* expression was under the control of a circadian oscillator. *CAB* gene expression was found to follow rhythmical patterns of expression under specific conditions (continuous darkness and $CO_2$ free air, continuous light and normal air) whereas a continuous rhythm did not occur in conditions of continuous darkness and normal air. These results show a degree of similarity to the rhythms of $CO_2$ metabolism in *Bryophyllum fedtschenkoi*. The circadian rhythms of $CO_2$ metabolism, in detached leaves of *Bryophyllum fedtschenkoi*, were found to persist in continuous darkness and $CO_2$ free air (Wilkins, 1959, 1960), and in continuous light and normal air (Wilkins, 1984; Anderson and Wilkins, 1989a). A non-rhythmic pattern of $CO_2$ uptake and output of detached leaves was found to occur in continuous darkness and normal air (Wilkins, 1959, 1984; Anderson and Wilkins, 1989b).

The period length of *Bryophyllum fedtschenkoi CAB* gene expression in continuous darkness and $CO_2$ free air, at a temperature of 15°C, appeared to be between 26 to 30 hours whereas the period length of the $CO_2$ rhythm, in similar conditions, was found to be approximately 24 hours. The peaks were between 1000 h to 1600 h and 1200 h to 1800 h, and the troughs were between 2000 h to 0400 h and 2200 h to 0800 h respectively. A certain degree of dampening occurred in the rhythm of *CAB* gene expression which also was apparent in the rhythm of $CO_2$ metabolism. A future study will have to be carried out to assess if the rhythm of

*Bryophyllum fedtschenkoi CAB* gene expression continues for another cycle as is seen in the rhythm of $CO_2$ metabolism.

The period length of *Bryophyllum fedtschenkoi CAB* gene expression in continuous light and normal air at a temperature of 18°C appeared to be 20 hours and the rhythm showed persistence for a longer time period as compared to that seen in $CO_2$ free air and continuous darkness, even though the amplitude of oscillation was rather small. Wilkins (1984) found that under the conditions of continuous light, normal air and at a temperature of 15°C the rhythm of $CO_2$ metabolism persisted for about 10 days and had a period of 18 hours. This was shorter than that seen in $CO_2$ free air and darkness. Piechulla and Riesselmann (1990) have documented that *Lycopersicon esculentum* seedlings that were grown in continuous illumination and at a constant temperature (24°C) expressed an oscillating accumulation pattern of the *CAB* mRNAs. In this case a period length of 30 to 32 hours was observed. In leaves of *Bryophyllum daigremontianum* Buchann-Bollig (1984) found that a circadian rhythm in $CO_2$ metabolism occurred in the conditions of continuous light and normal air at a temperature of 23.3°C with a period length of 24 hours. Since the period lengths of the *CAB* and $CO_2$ rhythms of *Bryophyllum fedtschenkoi* in continuous darkness and $CO_2$ free air, and continuous light and normal air were similar in duration it was possible that they were controlled by the same oscillator.

The lack of a rhythm of $CO_2$ metabolism in detached leaves of *Bryophyllum fedtschenkoi* kept in normal air and continuous darkness at 15°C seemed to correlate with the lack of a rhythm in *CAB* gene expression in the same conditions. However, under the above conditions, Wilkins (1984) found that an initial period of $CO_2$ fixation

occurred that was followed by a period of $CO_2$ output which increased to a maximum at the end of the following day's subjective light period. This output then gradually declined over the following days. Under the conditions of continuous darkness and normal air *Bryophyllum fedtschenkoi CAB* gene expression was found to be absent for the first 8 hours of darkness and then appeared 4 hours prior to the start of the subjective light period and reached maximum expression at the start of what would be the light period. The level of *CAB* gene expression then declined until no transcript was apparent. These two patterns were similar in that only one cycle occurred. In the case of the $CO_2$ metabolism maximums of $CO_2$ fixation and output occurred at 0100 h and 1600 h, whereas in *CAB* gene expression minimum and maximum levels occurred between 1600 h to 2400 h, and 0400 to 1200 h respectively. Therefore, the two patterns were similar but the actual timing of events was different. Kellman *et al.* (1993) showed that the individual *CAB* genes of the plant *Lycopersicon esculentum* revealed circadian oscillations in conditions of continuous darkness. But the majority of members of the *CAB* gene family only showed one period of expression in the subjective light period and the levels of the *CAB* mRNA decreased to almost undetectable levels in the following period of continuous darkness. This contrasts with the findings of Piechulla (1988) with total *CAB* mRNA levels of *Lycopersicon esculentum* under continuous dark conditions which show a detectable cycle occurring for a period of 5 days. Therefore, although a continual rhythm of *CAB* gene expression does not occur in *Bryophyllum fedtschenkoi* under the conditions of continuous darkness and normal air it could be on account of two factors; the lack of *CAB* gene expression was related to CAM and the fact that a $CO_2$ rhythm does not occur in these conditions in *Bryophyllum fedtschenkoi*, or the

*CAB* transcript levels were reaching undetectable levels in the second and third days of darkness.

These results suggest that there is a link between the circadian rhythms of $CO_2$ metabolism and *CAB* gene expression in *Bryophyllum fedtschenkoi*. The appearance of a rhythm in the *Bryophyllum fedtschenkoi CAB* transcript under continuous darkness and $CO_2$ free air and the subsequent lack of a rhythm when normal air was present parallels what was seen in the $CO_2$ rhythm of detached leaves. This seemed to correlate with the level of malate that accumulated in the two different conditions. Nimmo *et al.* (1987) found that leaves maintained in continuous darkness, $CO_2$ free air and at 15°C accumulated very low amounts of malate whereas in normal air there was a period of malate accumulation after which its levels slowly declined. This difference in malate levels was thought to inhibit the circadian rhythm of $CO_2$ gaseous exchange in *Bryophyllum fedtschenkoi* leaves. In continuous darkness, the fact that the *Bryophyllum fedtschenkoi CAB* rhythm was present in $CO_2$ free air and absent in normal air suggests that the levels of malate were also affecting it. In continuous light, normal air and a constant temperature the levels of malate in *Bryophyllum fedtschenkoi* leaves were found to remain low (Nimmo *et al.*, 1987), and the *Bryophyllum fedtschenkoi CAB* and $CO_2$ rhythms both occurred. Therefore, it is probable that the *Bryophyllum fedtschenkoi* rhythms of *CAB* gene expression and $CO_2$ metabolism are affected by the levels of malate and so controlled by the same oscillator.

The occurrence of rhythms in *CAB* gene expression is well documented in a number of higher plants under the conditions of continuous light and dark. Different plants show circadian rhythms in *CAB* gene expression under specific conditions. In continuous light and continuous dark in the

plants *Lycopersicon esculentum* (Giuliano *et al.*, 1988; Piechulla, 1988), *Triticum aestivum* (Nagy *et al.*, 1988) and *Phaseolus vulgaris* (Tavladoraki *et al.*, 1989), *CAB* mRNA transcripts oscillated over a number of days. In the conditions of continuous darkness the amplitude of the transcript levels tended to decrease the longer the particular plant stayed in the dark. The plants *Pisum sativum* (Kloppstech, 1985; Otto *et al.*, 1988; Spiller *et al.*, 1987) and *Nicotiana tabacum* (Paulsen and Bogorad, 1988) showed circadian oscillations in the levels of *CAB* mRNA under continuous light conditions. In the case of *Nicotiana tabacum* no circadian oscillation of *CAB* mRNA occurred under constant darkness but the rhythm was reinitiated upon illumination of the plant. In *Arabidopsis thaliana* (Millar and Kay, 1991) specific *CAB* genes, *CAB* 2 and *CAB* 3, showed circadian cycling under continuous darkness. In the case of *CAB* 1 mRNA little or no cycling was exhibited under the same conditions.

Circadian cycling of *CAB* gene expression was expected in *Bryophyllum fedtschenkoi*. The constant conditions that were set up appeared to determine the particular characteristic features of the rhythms produced. This was associated with the function of the *CAB* genes and to the plant itself. Light exerts two different effects on the expression of light-inducible (*CAB*) genes: the induction of circadian rhythmicity in the mRNA levels and a longer-lasting enhancement of transcript levels. Under illumination these two effects are not experimentally separable. In conditions of continuous light *Bryophyllum fedtschenkoi CAB* gene expression was very likely to be identified and to follow a continuous cycle over the period of a few days. This occurrence also related to the *Bryophyllum fedtschenkoi* rhythm of $CO_2$ metabolism, in the above conditions, that continued for at least 10 days with a short period length.

In comparison to continuous darkness, in which there was no trigger for *CAB* gene synthesis, the trend was that if a circadian rhythm occurred the continuous cycling was going to be hard to detect due to the rhythm dampening out. Of course, as no continuous cycling rhythm of *CAB* gene expression was identified in *Bryophyllum fedtschenkoi* this could also be related to the fact that there was no subsequent rhythm of $CO_2$ metabolism and not undetectable levels of *CAB* transcripts.

The difference between *Bryophyllum fedtschenkoi* and the various plants that have been mentioned is that it is a CAM plant. CAM plants operate with a diurnal cycle of $CO_2$ metabolism, and are defined as showing massive diurnal fluctuations of titratable acidity. This extreme change of acidity levels does not occur in the other plants discussed. In *Bryophyllum fedtschenkoi* a distinct rhythm of *CAB* gene expression occurred in $CO_2$ free air and continuous darkness, and a rhythm of $CO_2$ metabolism showed continuous cycling in these conditions, but these rhythms did not occur in normal air. The implication of these findings was that the rhythms of $CO_2$ metabolism and *CAB* gene expression might be connected. Since the levels of malate do not show a change in plants that do not use CAM this may explain why rhythms of *CAB* gene expression have been seen in other plants in conditions of constant darkness. Further experiments will have to be carried out to determine if this link is through a single oscillator or another mechanism of control. Numerous phase shifting studies have been carried out on the circadian rhythms of $CO_2$ metabolism of *Bryophyllum fedtschenkoi*. Various effects of temperature, light pulses and phytochrome have been documented. The rhythms of *CAB* gene expression in *Bryophyllum fedtschenkoi* will have to be subjected to similar studies to determine the

extent to which the rhythms of $CO_2$ metabolism and *CAB* gene expression are linked.

### 5.6.3 The effect of temperature on *CAB* gene expression

Temperature is a very useful tool with which to probe the basic oscillator to establish its characteristics and mechanism of operation at both the physiological and biochemical levels. Piechulla and Riesselmann (1990) found that temperature alterations support the setting and arrangement of the *CAB* mRNA circadian rhythm, in much the same way as a day/night transition, of growing *Lycopersicon esculentum* seedlings. The rhythm occurred in both continuous light and dark conditions with a period of 24 hours. In *Bryophyllum fedtschenkoi* under the conditions of continuous darkness and normal air, different temperatures affected the observed patterns of *CAB* gene expression and $CO_2$ metabolism.

It was reported in *Gossypium hirsutum* (Rikin, 1991; Rikin *et al.*, 1993), *Bryophyllum fedtschenkoi* (Anderson and Wilkins, 1989) and a number of other higher plants that physiological extreme temperatures inhibit circadian rhythms by driving the basic oscillator to, and holding it at, a fixed phase of the cycle. Alterations of illuminating conditions as well as alternating low and high temperature regimes also play an important role in the synchronising process of the oscillations.

In CAM plants like *Bryophyllum fedtschenkoi* temperature variations between the light and dark periods are essential to its photosynthetic functioning. Temperature and light intensity are known to influence the gas exchange and acid accumulation of several CAM plants (Osmond and Holtum, 1981). High daytime and relatively low night time (10°C)

temperatures are optimal for efficient photosynthesis to occur in CAM plants. Haag-Kerwaar *et al.* (1992) showed that increasing the difference between the day and night temperatures led to the $C_3$-CAM plant *Clusia minor* adopting a CAM cycle. Buchanan-Bollig (1984) showed that high temperatures and high irradiances interfered with the establishment of the endogenous $CO_2$ rhythms in *Kalanchoë daigremontiana*. Chardot and Wedding (1992) showed that the affinity of *Crassula argentea* PEPc, which catalyses the primary fixation of atmospheric $CO_2$ in CAM plants, for its substrate decreases with increasing temperature.

I found that at 5°C the middle leaves of *Bryophyllum fedtschenkoi* showed a single period of $CO_2$ fixation, which continued for a period of roughly 44 hours, and was followed by a constant rate of $CO_2$ output. The pattern of $CO_2$ metabolism at 27°C was very similar to leaves that were placed in the same conditions at 15°C. Again there was a single period of $CO_2$ fixation, which reached a maximum between 2400 h to 0100 h, that occurred for approximately 8 hours. However, Carter *et al.* (1995b) showed that a circadian rhythm of $CO_2$ gaseous exchange was exhibited by detached leaves of *Bryophyllum fedtschenkoi* in continuous darkness, normal air and at a constant temperature of 25-30°C. Since the $CO_2$ rhythm in continuous darkness and normal air, identified by Carter *et al.* (1995b), at 25-30°C was not huge and I used pairs of leaves (Figure 5.11c) as compared to only a single leaf (Carter *et al.*, 1995b), the rhythm was probably masked and no detectable variation in the $CO_2$ levels appeared. At 35°C no net $CO_2$ fixation occurred and an erratic rhythm in $CO_2$ gaseous exchange was exhibited.

I assessed how *CAB* gene expression was affected by temperature variations. *Bryophyllum fedtschenkoi CAB* transcripts were not detected

at 5°C. When the temperature was increased to 27°C the pattern of *CAB* gene expression showed a single period of transcript accumulation. At 35°C an erratic pattern of *CAB* gene expression, with no discernible rhythm, occurred. These patterns of expression can be compared to recent findings by Carter *et al.* (1995a) on the activity of the PEPc kinase. Carter *et al.* (1995a) showed the activity of PEPc, in *Bryophyllum fedtschenkoi*, was affected by temperature in three ways; the catalytic activity of the enzyme increases with increasing temperature, temperature can bring about a change in the phosphorylation state of the enzyme and temperature has a direct effect on the allosteric properties of the enzyme. The effects of temperature on PEPc subsequently regulate the pattern of $CO_2$ fixation in *Bryophyllum fedtschenkoi*. These findings also showed that PEPc kinase activity varied with temperature. High temperature led to a loss of PEPc kinase activity promoting the dephosphorylation of PEPc, and low temperature stabilised PEPc kinase activity maintaining PEPc in its phosphorylated form. Therefore, *Bryophyllum fedtschenkoi CAB* gene expression shows the opposite expression pattern to the PEPc kinase.

Although it has been shown that the circadian rhythm in *CAB* gene expression in *Lycopersicon esculentum* was insensitive to temperature over a wide range, at some critical temperature below 10°C the mechanism for temperature compensation fails and the circadian rhythm fails. Many plant species that are adapted to warm habitats are susceptible to injury by low temperature exposure. An important element of chill sensitive species is an inhibition of photosynthesis. Low temperature at night can cause severe reductions in $CO_2$ fixation but essentially the loss of activity is due to impairment of chloroplast function. Martino-Catt and Ort (1992) found that in *Lycopersicon esculentum* the circadian rhythms of

transcriptional activity of the *CAB* and Rubisco activase genes were also visible at the level of the newly synthesised *CAB* and Rubisco proteins. A low temperature treatment (4°C) was found to affect the normal pattern of expression of these two proteins by suspending both the progression of the timing of the circadian clock controlling gene transcription and the normal turnover of the transcripts. Any messages that were stabilised at the low temperature could no longer be translated into the protein. Therefore, at the low temperature of 5°C *Bryophyllum fedtschenkoi CAB* gene expression may be inhibited. This may explain why *CAB* gene expression was not seen even when the leaf samples were illuminated for 4 hours at the end of the experiment (Figure 5.10a).

High temperatures have also been found to affect *CAB* gene expression in a number of plants. Beator *et al.* (1992) showed that heat shock treatments during the emergence of *Hordeum vulgare* and *Pisum sativum* seedlings in the dark resulted in elevated levels of nuclear-encoded *CAB* mRNAs. In *Hordeum vulgare* heat was perceived to some extent as a substitute for a light signal, in that a circadian oscillation in *CAB* mRNA levels occurred after such heat treatments and continued for at least 3 days. This was similar to oscillations observed by Tavladoraki *et al.* (1989) after light pulse treatments. It was perceived that heat might affect the specific DNA sequence region or domain that was under control of the circadian oscillator. In my experimental analysis the high temperature of 35°C (Figure 5.12) was maintained for the duration of analysis and not as a shock treatment (Beator *et al.*, 1992). Therefore, it was unlikely that the plant perceived this temperature as a light pulse.

In *Bryophyllum fedtschenkoi* there is probably a link between the rhythms of $CO_2$ metabolism and *CAB* gene expression (see section 5.6.2). When

*Bryophyllum fedtschenkoi* leaves are placed into conditions of constant darkness, normal air and at specific temperature malate accumulates. This accumulation of malate is probably inhibiting the $CO_2$ and *CAB* rhythms of *Bryophyllum fedtschenkoi*. Carter *et al.* (1995b) found that the accumulation of malate in *Bryophyllum fedtschenkoi* leaves, which were maintained in the constant conditions described above over a period of 4 days, declined with increasing temperature. Therefore, this could account for the fact that I do not see the *Bryophyllum fedtschenkoi CAB* transcript after illumination at the end of the 5°C experiment (Figure 5.10c). Since these leaves would have a very high concentration of malate (Carter *et al.*, 1995) whereas in constant darkness at a temperature of 27°C or 35°C there is some *CAB* expression but these leaves will accumulate much less malate.

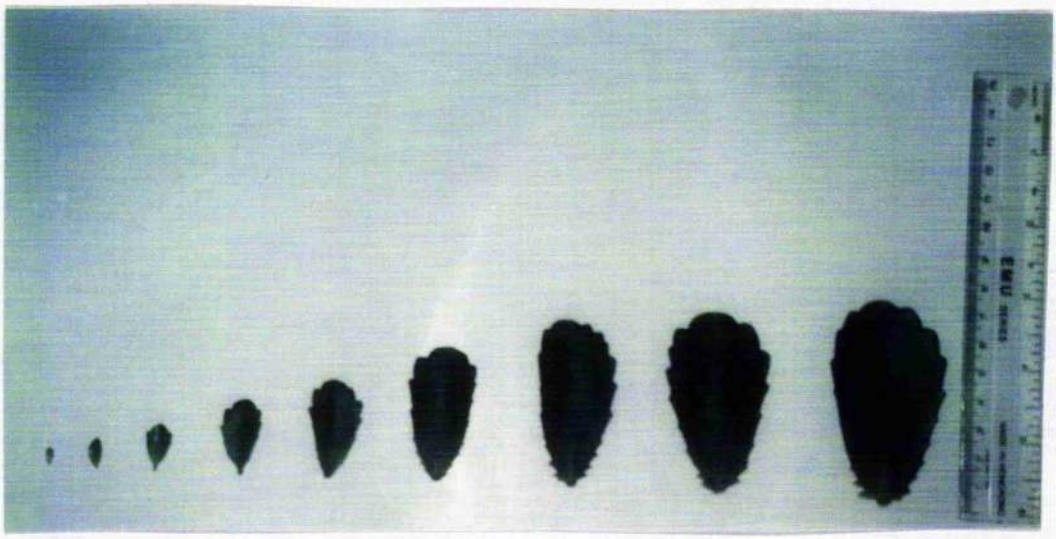
Summarising the results, *Bryophyllum fedtschenkoi* shows circadian rhythms of $CO_2$ metabolism and *CAB* gene expression in conditions of constant light and normal air, constant dark and $CO_2$ free air when both are at a constant temperature. The period lengths of each rhythm are approximately the same duration and it appears that malate accumulation prevents *CAB* expression. Therefore, the evidence suggests that the rhythms of $CO_2$ metabolism and *CAB* gene expression may be linked by the same oscillator.

**Figure 5.1 Photograph showing the different leaf sizes of *Bryophyllum fedtschenkoi***

The photograph shows the different sizes of *Bryophyllum fedtschenkoi* leaves analysed, from the tip to the sixth pair of leaves down the stem. The key to the different leaf sizes is shown below;

Key to leaf sizes:

| | |
|---|---|
| Tip | - 0.1 cm to 0.3 cm |
| o | - 0.3 cm to 1.0 cm |
| 0 | - 1.0 cm to 1.5 cm |
| 1 | - 2.0 cm to 2.5 cm |
| 2 | - 3.0 cm to 3.5 cm |
| 3 | - 4.0 cm to 4.5 cm |
| 4 | - 5.0 cm to 5.5 cm |
| 5 | - 6.0 cm to 6.5 cm |
| 6 | - 7.0 cm to 7.5 cm |



Tip o 0 1 2 3 4 5 6

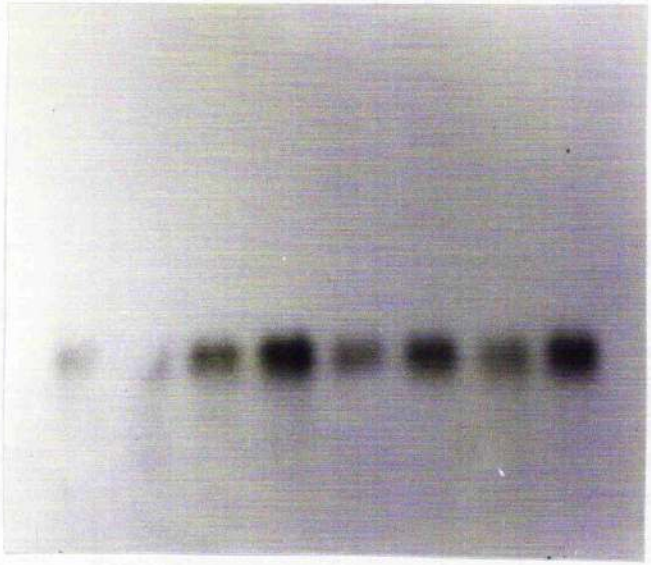
**Figure 5.2 The variation of expression of *CAB* and ribosomal RNA transcripts with leaf size in *Bryophyllum fedtschenkoi***

*Bryophyllum fedtschenkoi* leaf samples of sizes from the tip to size 6 (Figure 5.1) were taken in the middle of the light period and frozen in liquid nitrogen. RNA extraction, denaturing gel electrophoresis and northern blotting was followed as in sections 2.4.2, 2.8.2 and 2.11.1. The resulting filter was prehybridised and hybridised with Denhardts, as in section 2.14.1.1, at a temperature of 42°C. Figure 5.2a shows the result of a hybridisation with the *Bryophyllum fedtschenkoi CAB* cDNA probe (specific activity 3.1 x $10^8$ dpm/μg). The filter was washed three times for 10 minutes at a temperature of 60°C. The least stringent wash was with 2 x SSC/0.1 % (w/v) SDS and the most stringent wash was with 0.5 x SSC/0.1 % (w/v) SDS. After washing, the filter was autoradiographed as in section 2.14.3. Figure 5.2b shows the result of a hybridisation of the same filter with an isolated wheat ribosomal DNA probe (specific activity not calculated, DE81 strip used as in section 2.13.4.1). The filter was washed as above except that a temperature of 65°C was employed and one extra wash with 0.1 x SSC/0.1 % (w/v) SDS was used. Two transcripts are apparent, representing the 25S and 18S ribosomal RNAs. The Lane numbers refer to the leaf sizes in Figure 5.1. GIBCO BRL RNA ladders 0.16 - 1.77 kb and 0.24 - 9.5 kb were used as markers.

a.

o 0 1 2 3 4 5 6

*CAB*



b.

o 0 1 2 3 4 5 6

25S

18S

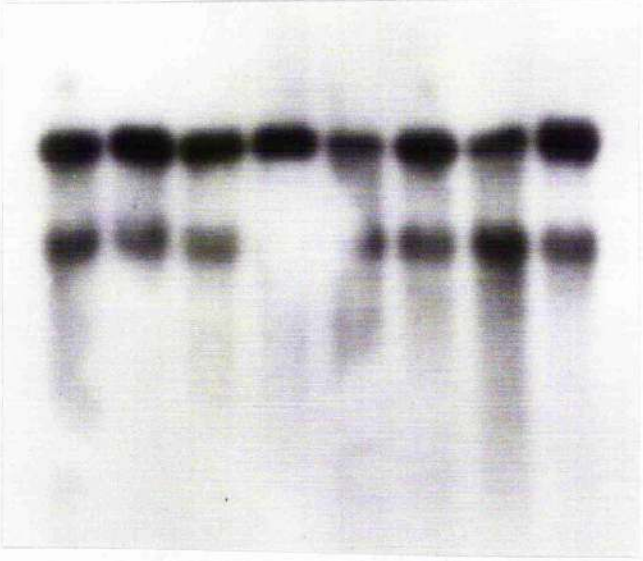
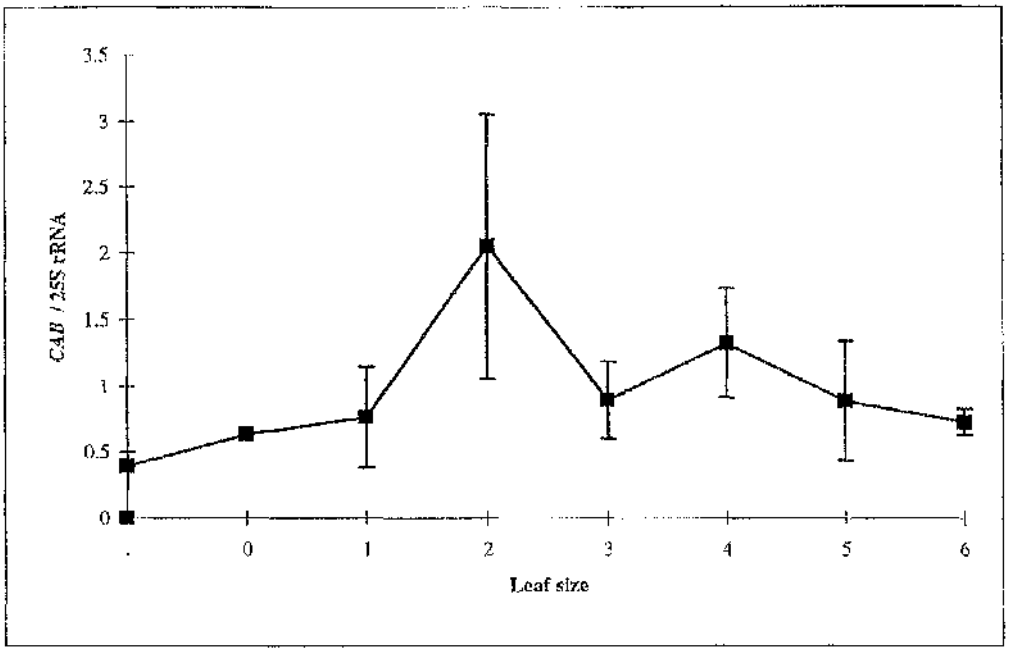


**Figure 5.3 Graphs showing the result of an extensive study of how *CAB* gene expression and leaf area varies with leaf size in *Bryophyllum fedtschenkoi***

Autoradiographs of three separate hybridisation studies, one of which is shown in Figure 5.2, were scanned (section 2.14.6). The values for the *CAB*/25S rRNA average ratios and standard deviations were used in this graphical analysis, to assess how *CAB* gene expression varies with leaf size. Figure 5.3a illustrates the result of this.

A different number of *Bryophyllum fedtschenkoi* leaves of varying sizes were collected. An outline of each leaf was drawn onto a piece of graph paper and the leaf area calculated for each leaf size. The average leaf area and standard deviation was calculated for each leaf size. Figure 5.3b shows the results of this analysis.

**a**


3.5
3
2.5
2
1.5
1
0.5
0
CAB / 25S rRNA
0
1
2
3
4
5
6
Leaf size

**b**

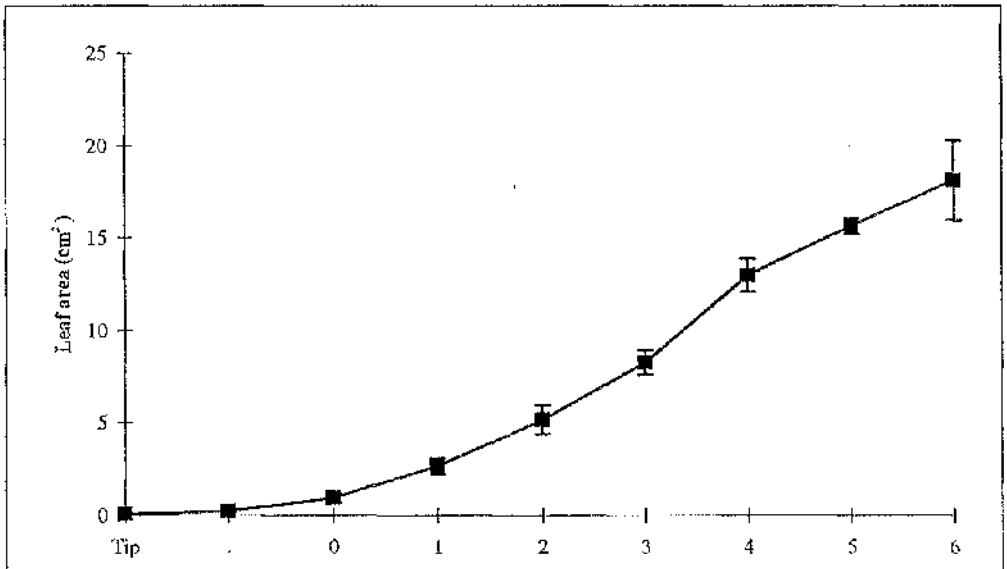
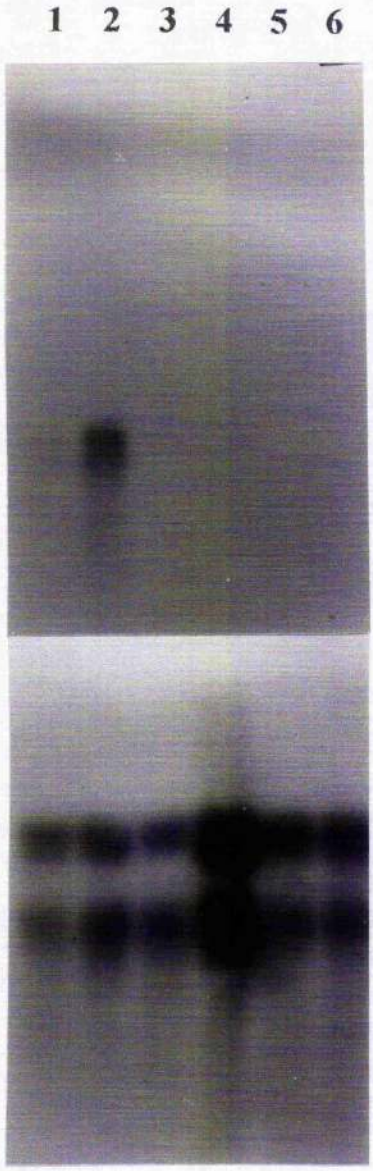

25
20
15
10
5
0
Leaf area (cm²)
Tip
0
1
2
3
4
5
6
Leaf size

**Figure 5.4 Diurnal pattern of *Bryophyllum fedtschenkoi CAB* gene expression**

*Bryophyllum fedtschenkoi* plants grown with a light period from 0800 h to 1600 h and a dark period from 1600 h to 0800 h, had leaf samples (size 3.0 cm to 5.0 cm in length) removed at the start of the light period and then at 4 hour intervals until the end of the dark period. RNA was extracted from the samples (Chang *et al.*, 1993 ), run on a denaturing formaldehyde gel and northern blotted (see Figure 5.2). Figure 5.4a and Figure 5.4b show the result of hybridisations with the homologous *Bryophyllum fedtschenkoi CAB* cDNA probe and with the control wheat ribosomal genomic DNA fragment. The hybridisation conditions and washes were as in Figure 5.2. The formaldehyde denaturing gel was run with GIBCO BRL RNA markers of size 0.16 kb to 1.77 kb and 0.24 kb to 9.5 kb. The lanes are identified below with the times of leaf isolation and whether leaves were in the light (LP) or the dark (DP). The *CAB* and ribosomal RNA bands are indicated. Figure 5.4c shows a graph of the average results for *CAB*/25S rRNA ratios (values determined by autoradiograph scanning, section 2.14.6) with time.

| Lane No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Time (h) | 0800 | 1200 | 1600 | 2000 | 2400 | 0400 |
| Period | LP | LP | LP | DP | DP | DP |

a


1 2 3 4 5 6
CAB
b
25S
18S

c

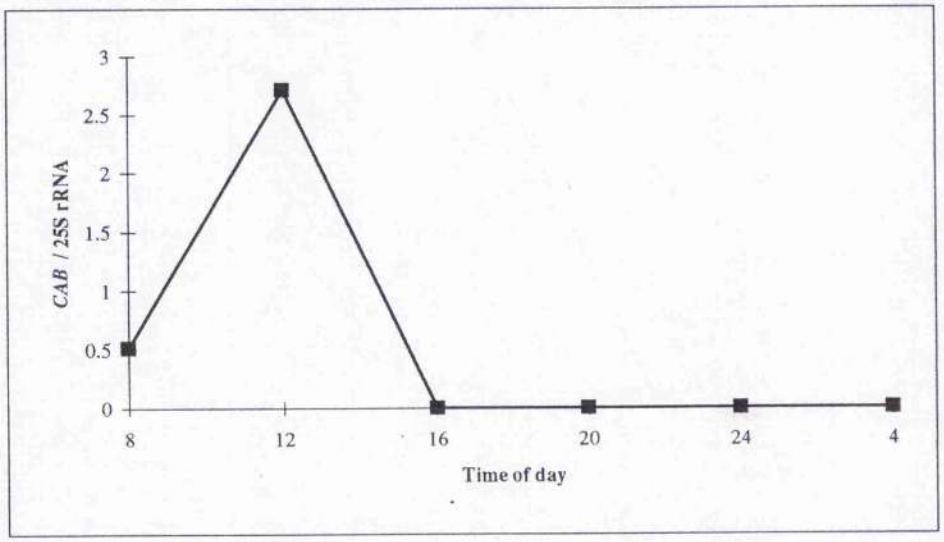

CAB / 25S rRNA
3
2.5
2
1.5
1
0.5
0
8
12
16
20
24
4
Time of day

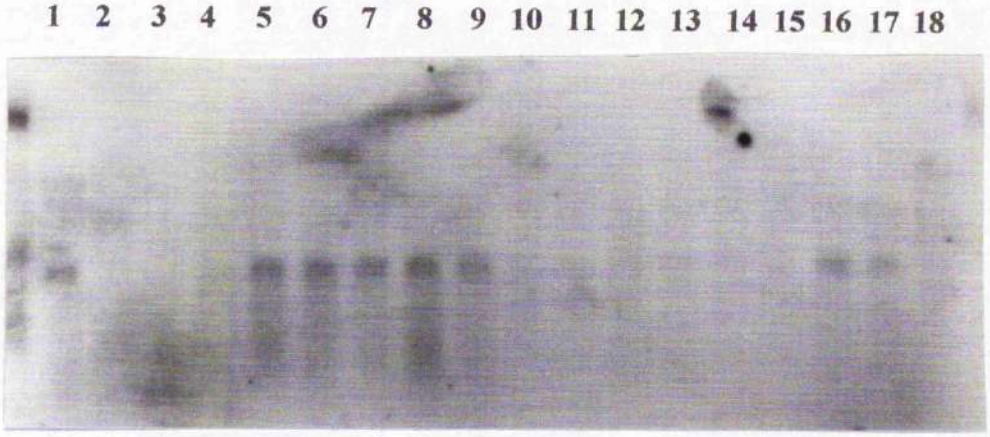
**Figure 5.5 Time course showing *Bryophyllum fedtschenkoi CAB* and H1 gene expression in conditions of $CO_2$ free air, continuous darkness at a constant temperature of 15°C**

Pairs of *Bryophyllum fedtschenkoi* leaves were detached at the end of a normal light period and placed into a sealed air-tight container in conditions of $CO_2$ free air, continuous darkness and a constant temperature of 15°C. At specific time points, identified below, leaf samples were removed from these containers, under a green safe light and snap frozen in liquid nitrogen. RNA was extracted (section 2.4.2) from the leaf tissue, run on a formaldehyde denaturing agarose gel (section 2.8.2) and northern blotted (section 2.11.1). The filter was hybridised at 42°C by using Denhardts method (section 2.14.1.1) with the homologous *Bryophyllum fedtschenkoi CAB* cDNA (specific activity $1.4 \times 10^9$ dpm/μg) and subsequently with the H1 cDNA (specific activity $8 \times 10^8$ dpm/μg). The most stringent washing step was in 1 x SSC/0.1 % (w/v) SDS at a temperature of 60°C to 65°C for 10 minutes duration. Figure 5.5a and Figure 5.5b show the results of these hybridisation analyses with the *CAB* cDNA and H1 cDNA respectively. The *CAB* and H1 transcripts identified are 0.8 kb - 1.1 kb and 3.7 kb in size. The GIBCO BRL 0.24 kb to 9.5 kb RNA ladder and migration of the ribosomal bands were used as markers. The different lanes are shown below.

| Lane No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 1600 | 2000 | 2400 | 0400 | 0600 | 0800 | 1000 | 1200 | 1600 |

| Lane No. | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 2000 | 2400 | 0200 | 0400 | 0600 | 0800 | 1200 | 1600 | 2000 |

a.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
CAB

b.

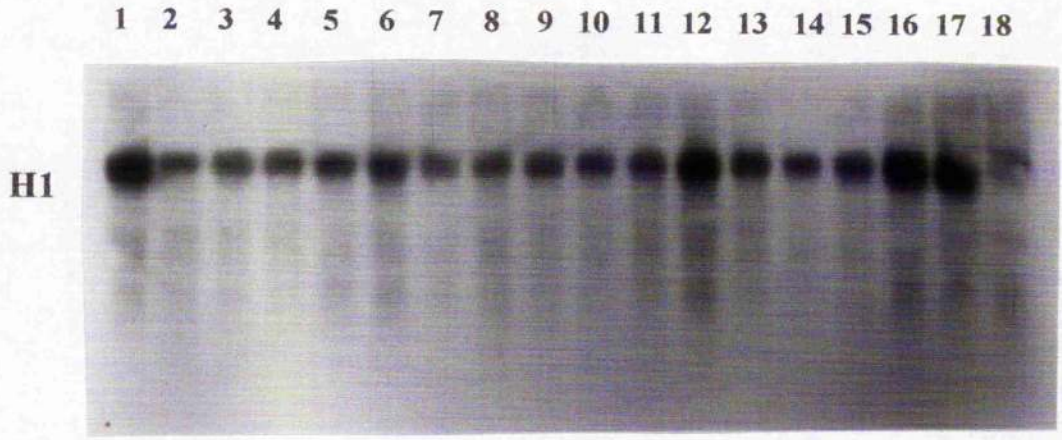
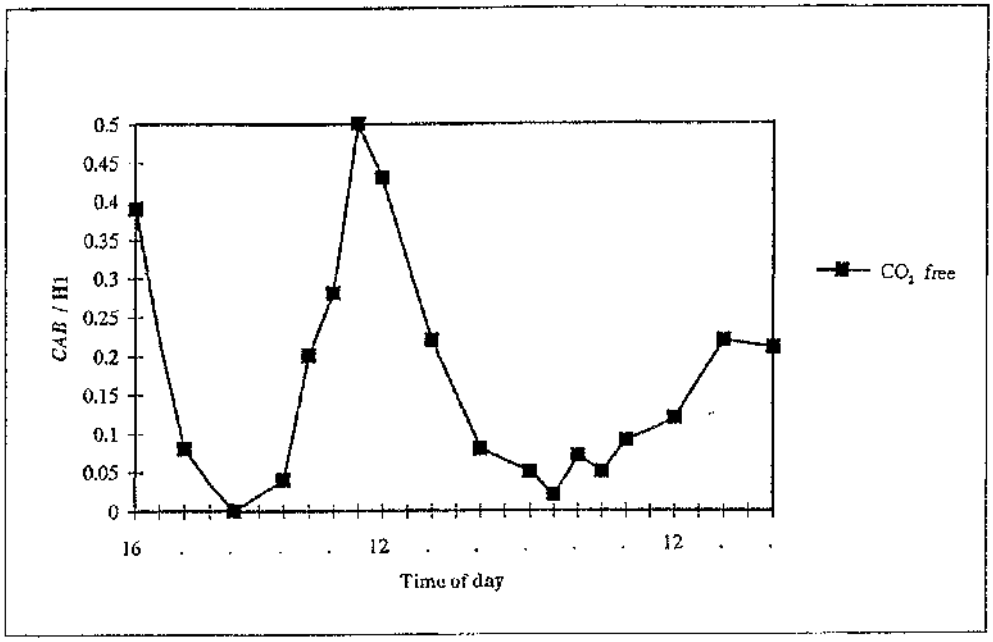

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
H1

**Figure 5.6** **Graphs showing *Bryophyllum fedtschenkoi CAB* gene expression and carbon dioxide gaseous exchange in conditions of $CO_2$ free air and continuous darkness at a temperature of 15°C**

Figure 5.6a is a graph of the average values for the *CAB*/H1 ratios (values determined by autoradiographic scanning, section 2.14.6) versus the time of day in the constant conditions described in Figure 5.5. *Bryophyllum fedtschenkoi CAB* gene expression shows a repeating cycle. Figure 5.6b shows the $CO_2$ gaseous exchange of three pairs of *Bryophyllum fedtschenkoi* leaves, in the conditions stated above, as recorded by using the IRGA over the duration of the time course. The pairs of leaves had a fresh weight of 2.8 g to 3.3 g and a length of 4.5 cm to 4.8 cm. The flow rate of the $CO_2$ free air passing across the leaves was 6 l/h. The time of day is labelled accordingly.

**a**


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
CAB / H1
16
12
12
Time of day
CO2 free

**b**

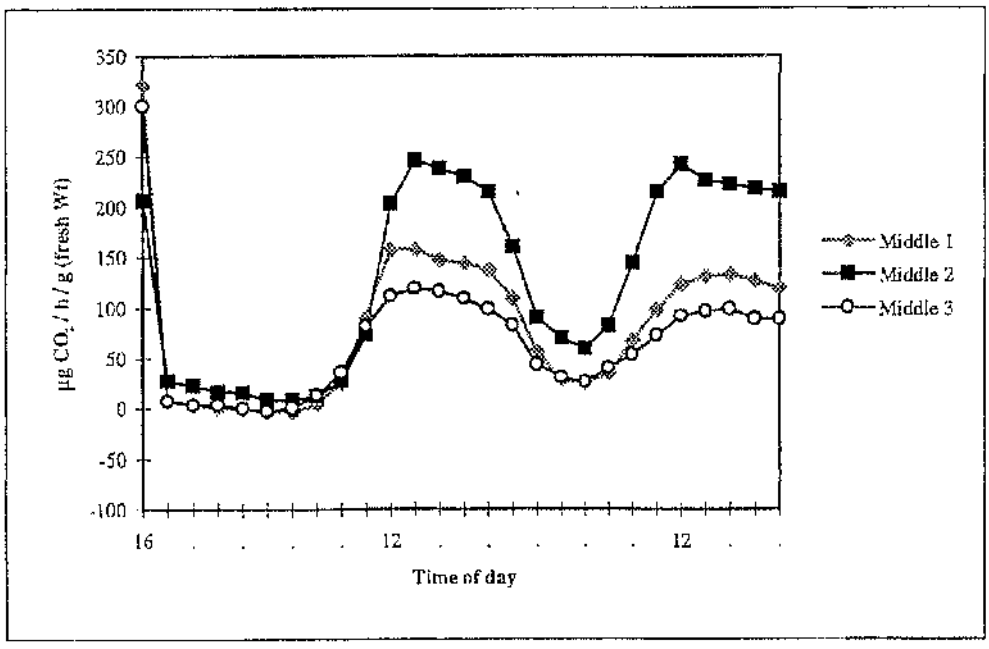

350
300
250
200
150
100
50
0
-50
-100
µg CO2 / h / g (fresh Wt)
16
12
12
Time of day
Middle 1
Middle 2
Middle 3

**Figure 5.7 Time course showing *Bryophyllum fedtschenkoi CAB* and H1 gene expression in continuous light, normal air and at a temperature of 18°C**

*Bryophyllum fedtschenkoi* plants were removed from the growth room at the end of the light period and placed into a continuous light room, at a temperature of 18°C. Leaf samples were taken every 4 hours and snap frozen in liquid nitrogen. RNA was extracted from the leaf tissue by using the Chang *et al.* procedure (section 2.4.2), run on a formaldehyde denaturing agarose gel (section 2.8.2) and northern blotted (section 2.11.1). The filter was hybridised with the *Bryophyllum fedtschenkoi CAB* cDNA (specific activity 2.5 x $10^8$ dpm/µg) and an H1 cDNA (specific activity 8 x $10^8$ dpm/µg) by using the BSA and Denhardts methods respectively (sections 2.14.1.1 and 2.14.1.2). The most stringent washing conditions applied with the *CAB* cDNA and H1 cDNA hybridisations were in 1 x SSC/0.1 % (w/v) SDS at a temperature of 55°C to 60°C and 60°C to 65°C for 10 minutes respectively. Figure 5.7a shows the result of the hybridisation analysis with the *CAB* cDNA and identifies three transcripts of size 1.0 kb to 1.1 kb, 0.8 kb and 0.6 kb - 0.7 kb (labelled *CAB* 1, *CAB* 2 and *CAB* 3). Figure 5.7b shows the result of the hybridisation analysis with the H1 cDNA and identifies a transcript of size 3.5 kb to 3.7 kb. The ribosomal bands on the original formaldehyde denaturing gel were used as markers. The different lanes are shown below;

| Lane No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 2000 | 2400 | 0400 | 0800 | 1200 | 1600 | 2000 | 2400 | 0400 |

| Lane No. | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 0800 | 1200 | 1600 | 2000 | 2400 | 0400 | 0800 | 1200 | 1600 |

| Lane No. | 19 | 20 |
|---|---|---|
| Time (h) | 2000 | 2400 |

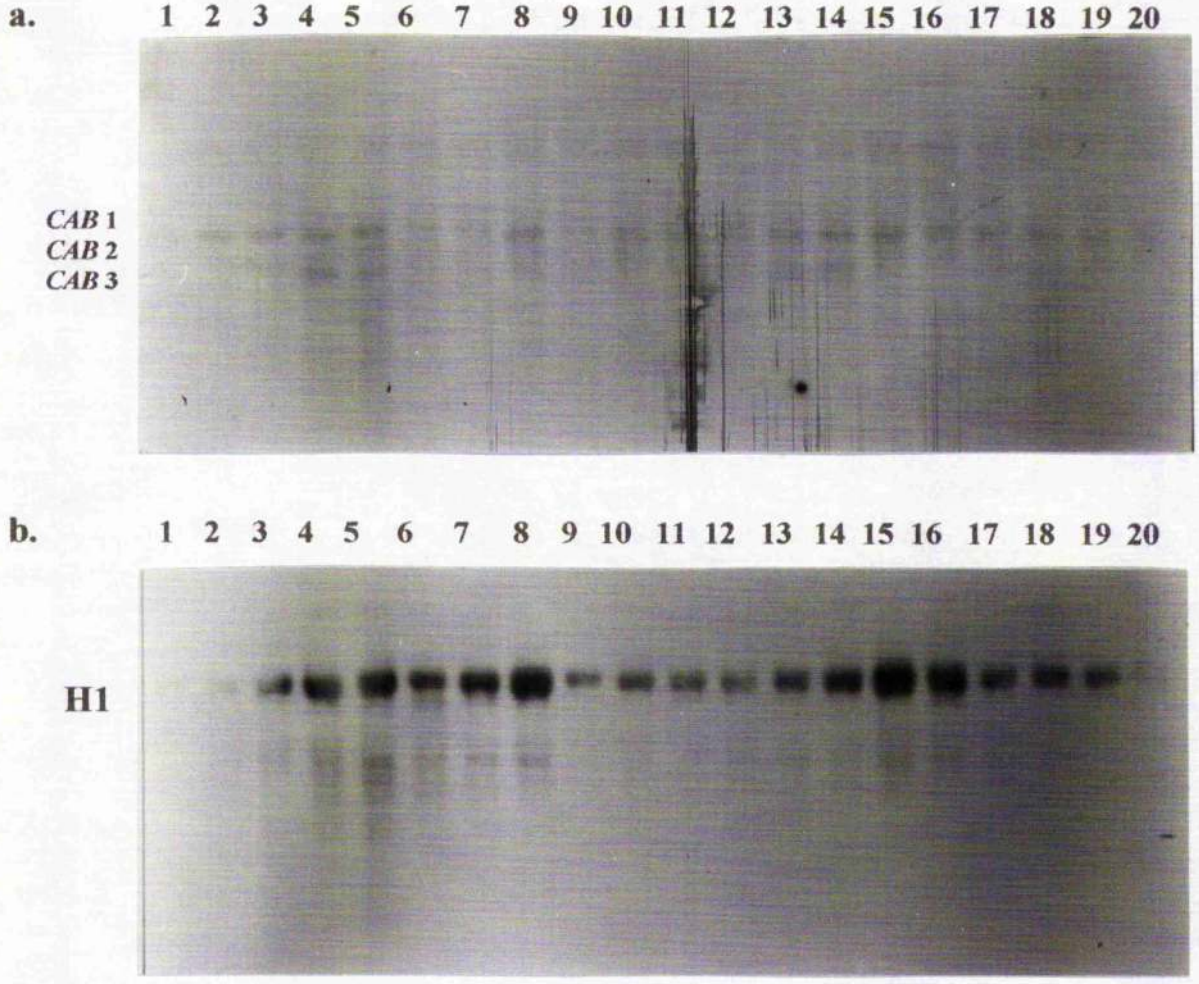
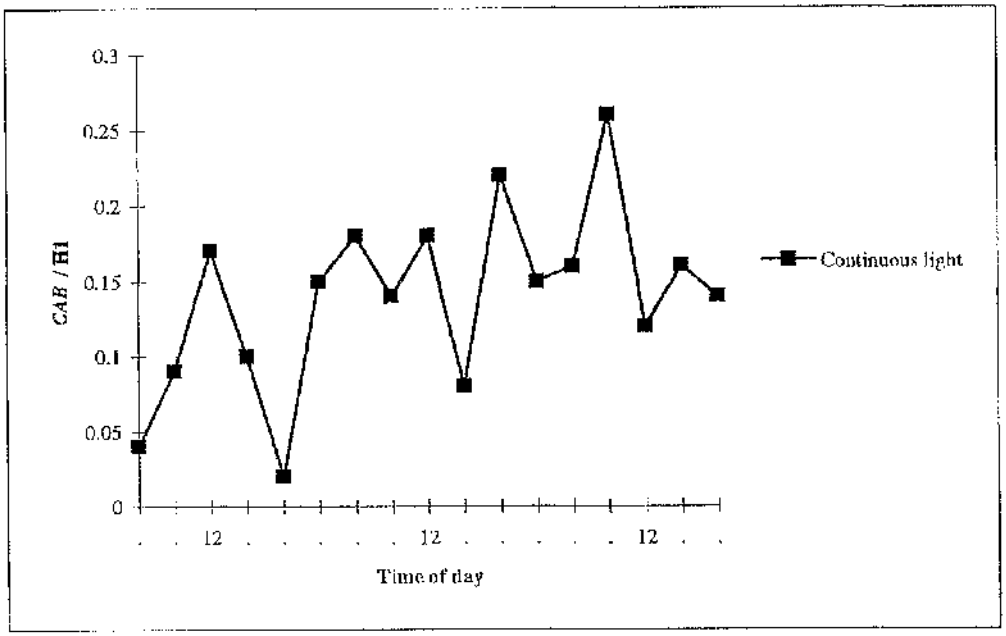

a.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
CAB 1
CAB 2
CAB 3
b.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
H1

**Figure 5.8** **Graphs showing *Bryophyllum fedtschenkoi CAB* gene expression of transcripts *CAB* 1 and *CAB* 3 in continuous light conditions**

Figures 5.8a and 5.8b show graphs of the average values for the *CAB*/H1 ratios (values determined by autoradiograph scanning, section 2.14.6) for the *CAB* 1 (1.0 kb to 1.1 kb) and *CAB* 3 (0.6 kb to 0.7 kb) transcripts, as described in Figure 5.7, with time.

a


0.3
0.25
0.2
0.15
0.1
0.05
0
CAB / H1
12
12
12
Time of day
Continuous light

b

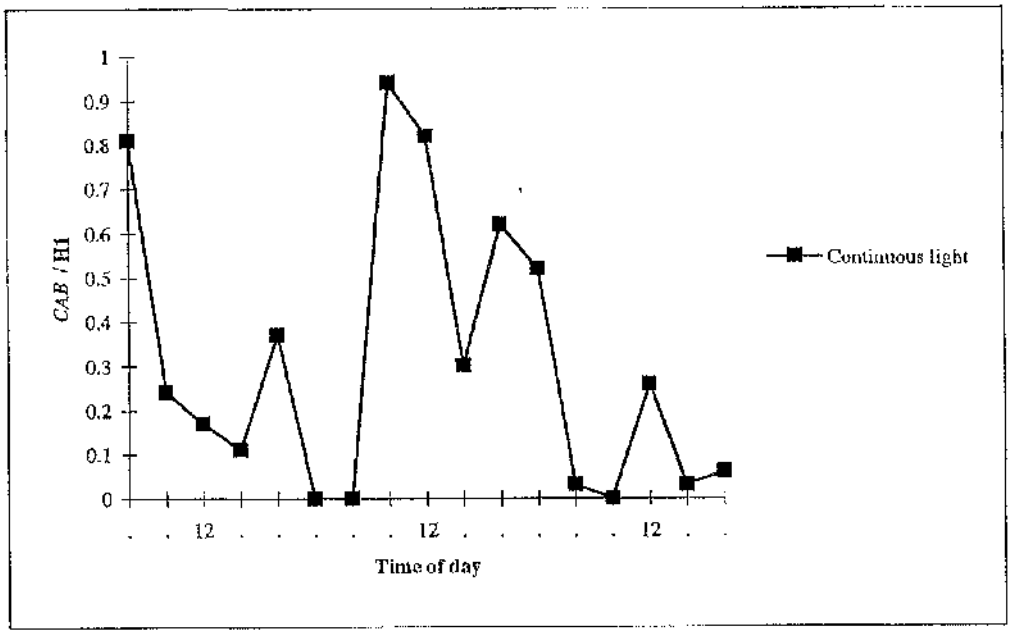

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CAB / H1
12
12
12
Time of day
Continuous light

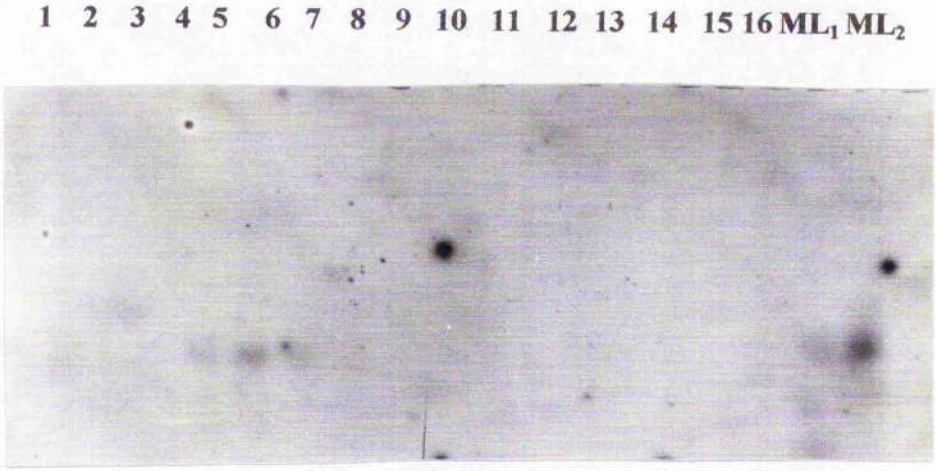
**Figure 5.9 Time course showing *Bryophyllum fedtschenkoi CAB* and H1 gene expression in continuous darkness, normal air and at a temperature of 15°C**

*Bryphyllum fedtschenkoi* plants were removed from the growth room at the end of the light period and placed into a dark room, at a temperature of 15°C. Leaf samples were taken, under a green safe light, every 4 hours and snap frozen in liquid nitrogen. RNA was extracted from the leaf tissue by using the Chang *et al.* procedure (section 2.4.2), run on a formaldehyde denaturing agarose gel (section 2.8.2) and northern blotted (section 2.11.1). The filter was hybridised with the *Bryophyllum fedtschenkoi CAB* cDNA (specific activity not calculated, DE81 strip used as in section 2.13.4.1) and an H1 cDNA (specific activity $8 \times 10^8$ dpm/μg) by using the Denhardts approach (section 2.14.1.1). The most stringent washing conditions applied were in 1 x SSC/0.1 % (w/v) SDS at a temperature of 60°C to 65°C for 10 minutes. Figure 5.9a shows the result of the hybridisation analysis with the *CAB* cDNA. Figure 5.9b shows the result of the hybridisation analysis with the H1 cDNA and identifies a transcript of size 3.7 kb to 4.0 kb. The GIBCO BRL 0.16 kb - 1.77 kb and 0.24 kb - 9.5 kb RNA markers were used. The different lanes are shown below. After the plants had been in these continuous dark conditions for two days they were returned to the normal growth room. Duplicate leaf samples were taken over the next two days at the middle of the light period and are identified as $ML_1$ and $ML_2$.

| Lane No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 1600 | 2000 | 2400 | 0400 | 0800 | 1200 | 1600 | 2000 | 2400 |

| Lane No. | 10 | 11 | 12 | 13 | 14 | 15 | 16 | $ML_1$ | $ML_2$ |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 0400 | 0800 | 1200 | 1600 | 2000 | 2400 | 0400 | 1200 | 1200 |

a.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 ML1 ML2
CAB

b.

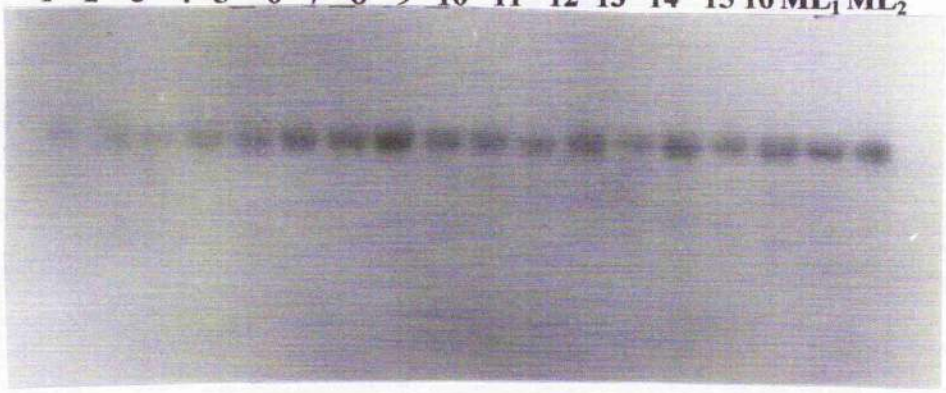

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 ML1 ML2
H1

**Figure 5.10 Time course showing *Bryophyllum fedtschenkoi* $CO_2$ gaseous exchange, *CAB* and H1 gene expression in conditions of normal air, continuous darkness and a constant temperature of 5°C**

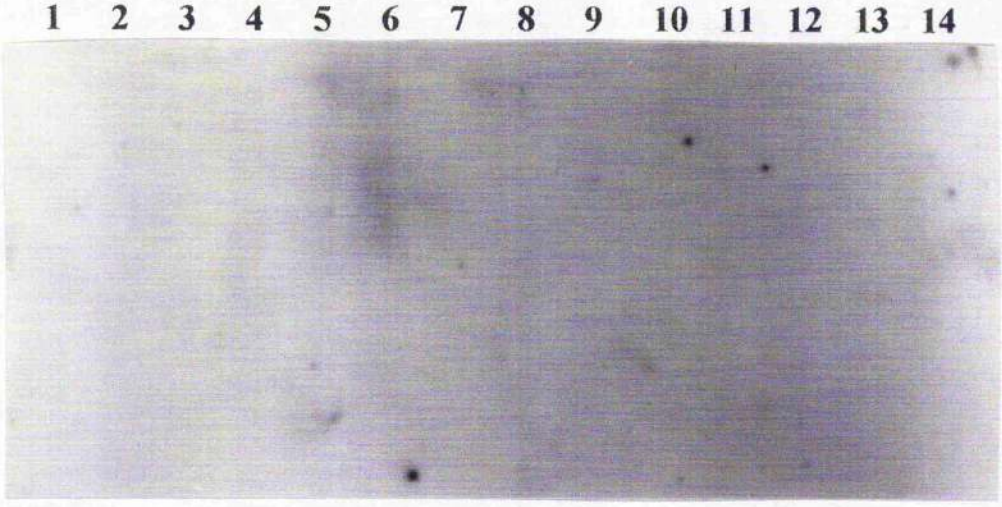
Pairs of *Bryophyllum fedtschenkoi* leaves were detached at the end of a normal light period and placed into a sealed air-tight container in conditions of normal air, continuous darkness and a constant temperature of 5°C. At specific time points, identified below, leaf samples were removed from these containers, under a green safe light and snap frozen in liquid nitrogen. RNA was extracted (section 2.4.2), run on a formaldehyde denaturing agarose gel (section 2.8.2) and northern blotted (section 2.11.1). The filter was hybridised at 42°C using Denhardts method (section 2.14.1.1) with the homologous *Bryophyllum fedtschenkoi CAB* cDNA (specific activity 1.4 x $10^9$ dpm/μg) and subsequently with the H1 cDNA (specific activity 8 x $10^8$ dpm/μg). The most stringent washing step was in 1 x SSC/0.1 % (w/v) SDS at a temperature of 55°C to 60°C for 10 minutes duration. Figures 5.10a and 5.10b show the results of these hybridisation analyses. The H1 transcript identified is 3.7 kb in size. No *CAB* transcripts are present. The GIBCO BRL 0.24 kb to 9.5 kb and 0.16 kb to 1.77 kb RNA ladders were used as markers. The different lanes are shown below. Lane 14 represents the samples after 4 hours of illumination, at what would be the start of the normal light period, at the end of the analysis. Lanes 1 to 13 are all in continuous darkness, normal air and a constant temperature of 5°C. Figure 5.10c shows the $CO_2$ gaseous exchange of three pairs of *Bryophyllum fedtschenkoi* leaves, as recorded by using the IRGA, over the duration of the time course. The pairs of leaves had a fresh weight of 2.6 g to 2.9 g and a length of 4.7 cm to 5.3 cm. The final point recorded

is after 4 hours of light. The flow rate of air passing across the leaves was 6.0 l/h.

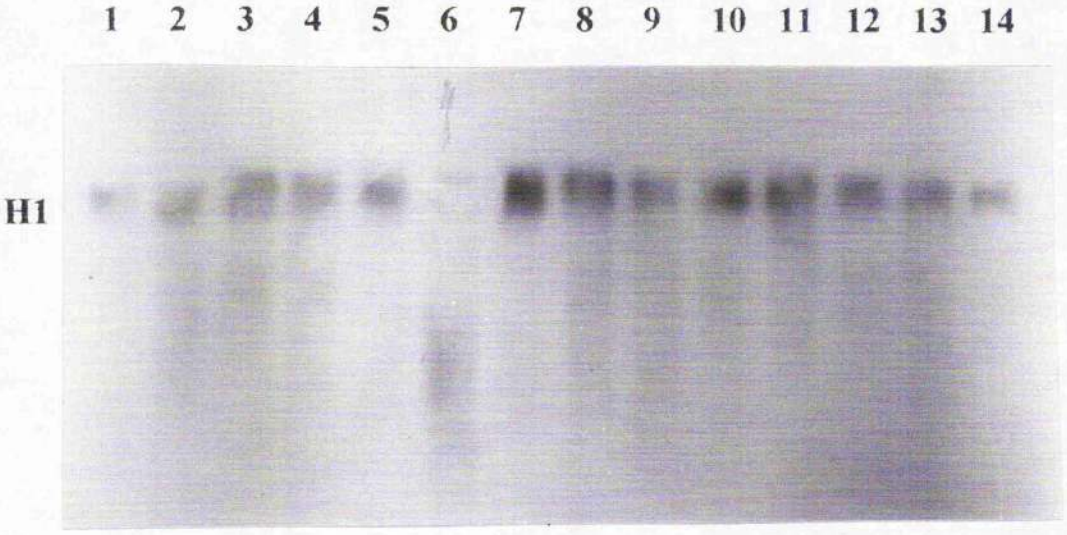
| Lane No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Time (h) | 1600 | 2000 | 2400 | 0400 | 0800 | 1200 | 1600 |

| Lane No. | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|
| Time (h) | 2000 | 2400 | 0400 | 0800 | 1200 | 1600 | 1200 |

a


1 2 3 4 5 6 7 8 9 10 11 12 13 14

b


1 2 3 4 5 6 7 8 9 10 11 12 13 14
H1

c

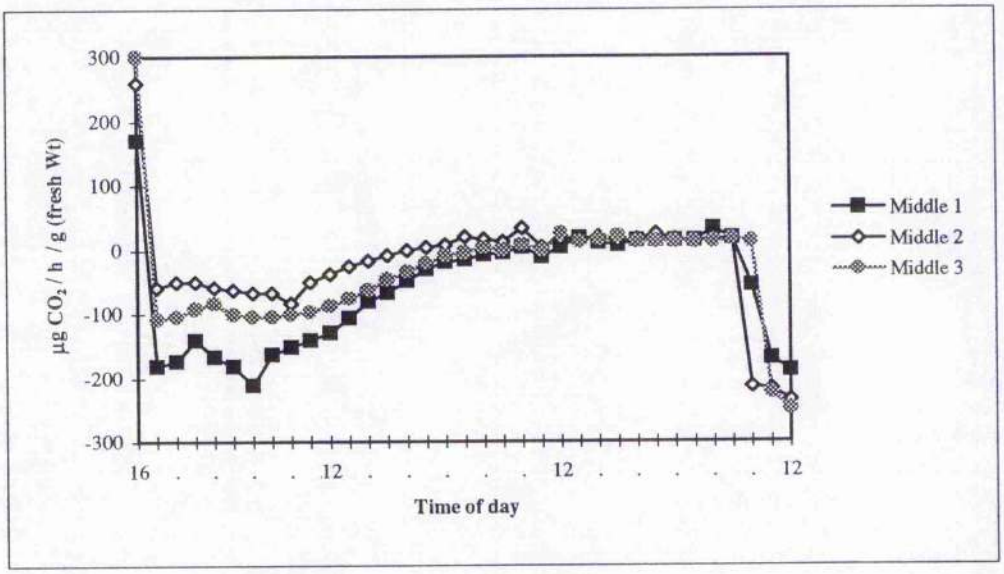
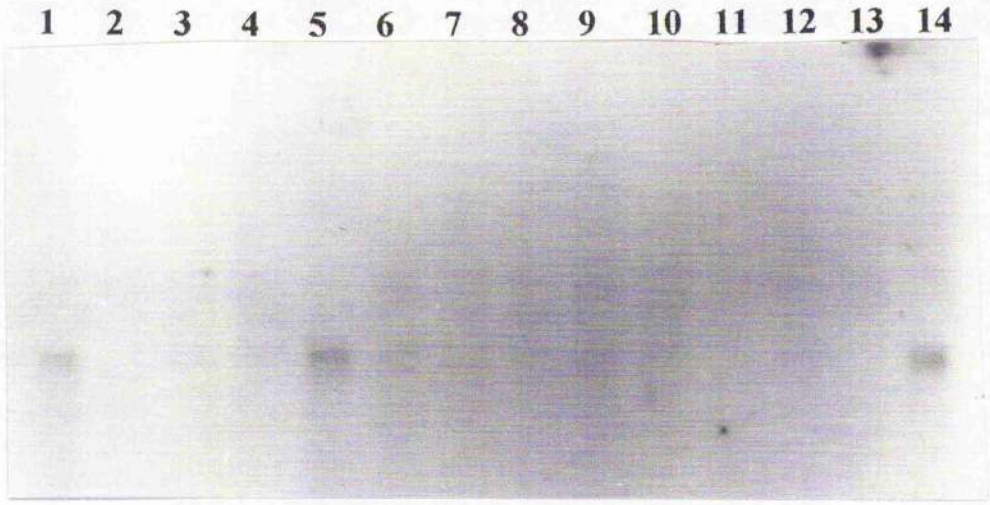

300
200
100
0
-100
-200
-300
µg CO2 / h / g (fresh Wt)
16 . . . . 12 . . . . . 12 . . . . . 12
Time of day
Middle 1
Middle 2
Middle 3

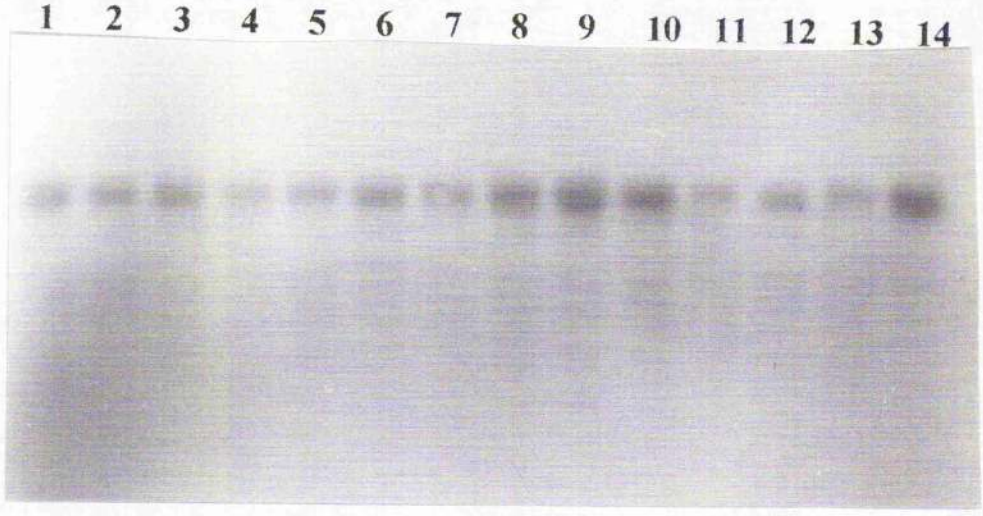
**Figure 5.11 Time course showing *Bryophyllum fedtschenkoi* $CO_2$ gaseous exchange, *CAB* and H1 gene expression in conditions of normal air, continuous darkness and a constant temperature of 27°C**

Pairs of *Bryophyllum fedtschenkoi* leaves were detached at the end of a normal light period and placed into a sealed air-tight container in conditions of normal air, continuous darkness and a constant temperature of 27°C. The sampling procedure, analysis of transcripts and $CO_2$ gaseous exchange were identical to those used in Figure 5.10. Figures 5.11a and 5.11b show the results of the hybridisation analyses with the homologous *Bryophyllum fedtschenkoi CAB* cDNA and the H1 cDNA respectively. The *CAB* and H1 transcripts identified are 0.8 kb to 1.1 kb and 3.7 kb in size respectively. The GIBCO BRL 0.24 kb to 9.5 kb RNA ladder and migration of ribosomal bands were used as markers. The different lanes are identical to those shown in Figure 5.10. Lane 14 represents the samples after 4 hours of illumination, at what would be the start of the normal light period, at the end of the analysis. Lanes 1 to 13 are all in continuous darkness, normal air and a constant temperature of 27°C. Figure 5.11c shows the $CO_2$ gaseous exchange of three pairs of *Bryophyllum fedtschenkoi* leaves, as recorded by using the IRGA, over the duration of the time course. The pairs of leaves had a fresh weight of 2.7 g to 2.9 g and a length of 5.0 cm to 5.2 cm. The final point recorded is after 4 hours of illumination. The flow rate of air passing across the leaves was 6.0 l/h.

a


1 2 3 4 5 6 7 8 9 10 11 12 13 14
CAB

b


1 2 3 4 5 6 7 8 9 10 11 12 13 14
H1

c

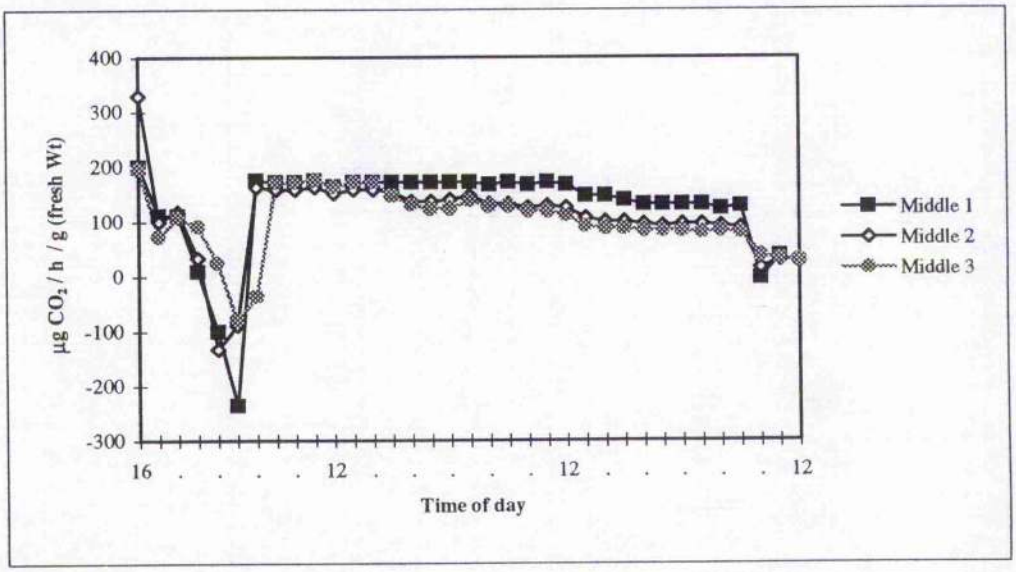
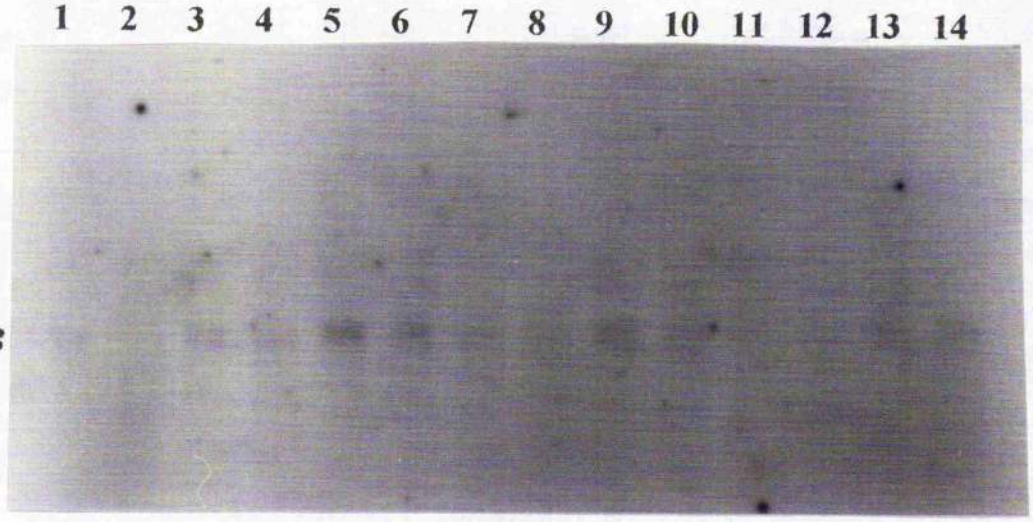

400
300
200
100
0
-100
-200
-300
µg CO2 / h / g (fresh Wt)
16 . . . . 12 . . . . . 12 . . . . . 12
Time of day
Middle 1
Middle 2
Middle 3

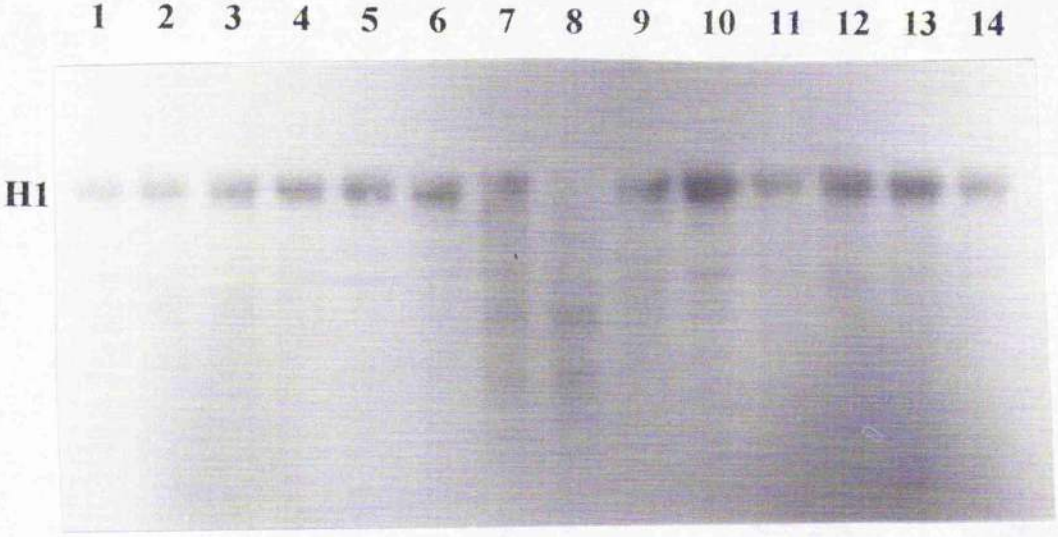
**Figure 5.12 Time course showing *Bryophyllum fedtschenkoi* $CO_2$ gaseous exchange, *CAB* and H1 gene expression in conditions of normal air, continuous darkness and a constant temperature of 35°C**

Pairs of *Bryophyllum fedtschenkoi* leaves were detached at the end of a normal light period and placed into a sealed air-tight container in conditions of normal air, continuous darkness and a constant temperature of 35°C. The sampling procedure, analysis of transcripts and $CO_2$ gaseous exchange were identical to those used in Figure 5.10. Figures 5.12a and 5.12b show the results of the hybridisation analyses with the homologous *Bryophyllum fedtschenkoi CAB* cDNA and the H1 cDNA respectively. The *CAB* and H1 transcripts identified are 0.8 kb to 1.1 kb and 3.7 kb in size respectively. The *CAB* and H1 transcripts are labelled accordingly. The GIBCO BRL 0.24 kb to 9.5 kb RNA ladder and migration of ribosomal bands were used as markers. The different lanes are identical to those shown in Figure 5.10. Lane 14 represents the samples after 4 hours of illumination, at what would be the start of the normal light period, at the end of the analysis. Lanes 1 to 13 are all in continuous darkness, normal air and a constant temperature of 35°C. Figure 5.12c shows the $CO_2$ gaseous exchange of three pairs of *Bryophyllum fedtschenkoi* leaves, as recorded by using the IRGA, over the duration of the time course. The pairs of leaves had a fresh weight of 1.9 g to 2.7 g and a length of 4.7 cm to 5.4 cm. The final point recorded is after 4 hours of illumination. The flow rate of air passing across the leaves was 6.0 l/h.

a


1 2 3 4 5 6 7 8 9 10 11 12 13 14
CAB

b


1 2 3 4 5 6 7 8 9 10 11 12 13 14
H1

c

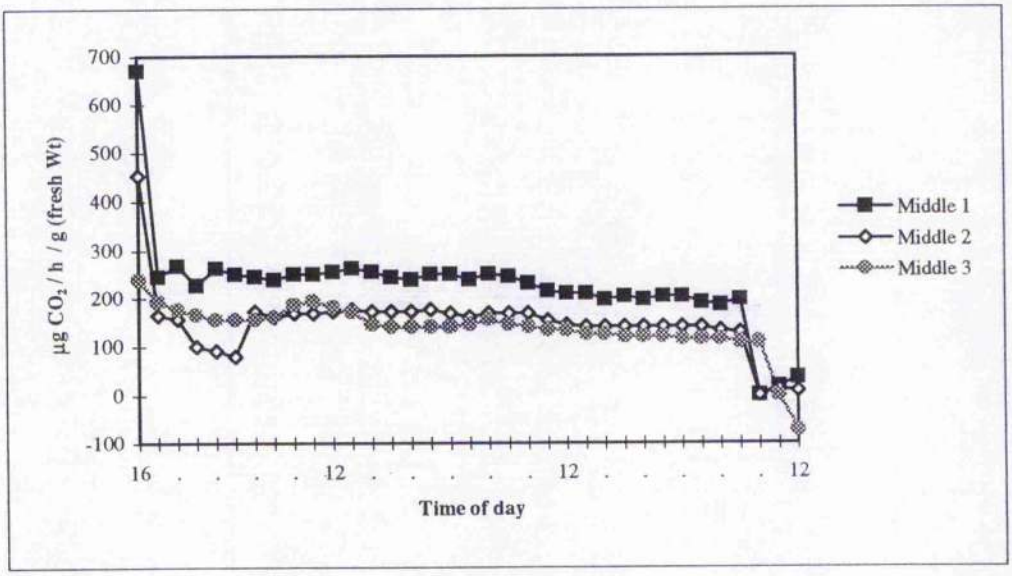
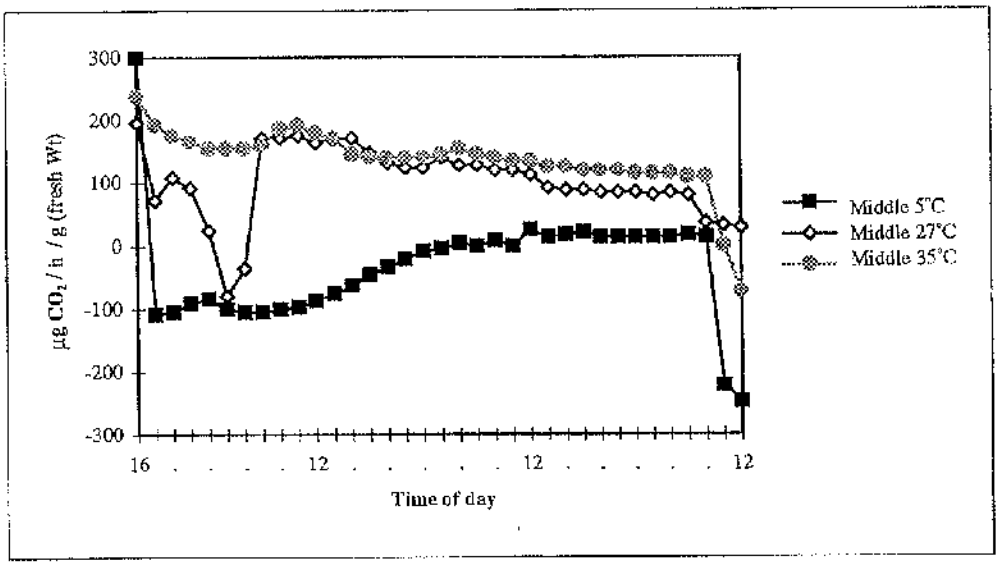

700
600
500
400
300
200
100
0
-100
µg CO2 / h / g (fresh Wt)
16 . . . . 12 . . . . . 12 . . . . . 12
Time of day
Middle 1
Middle 2
Middle 3

**Figure 5.13** **Carbon dioxide gaseous exchange in three pairs of detached middle sized leaves of *Bryophyllum fedtschenkoi* under conditions of continuous darkness, normal air and a constant temperature of 5°C, 27°C or 35°C**

Figure 5.13 compares the $CO_2$ gaseous exchange results from one of the three pairs of leaves shown in Figures 5.10c, 5.11c and 5.12c. The differences in the gaseous exchange patterns can be related to the different patterns of *Bryophyllum fedtschenkoi CAB* gene expression seen at these different temperatures.


300
200
100
0
-100
-200
-300
µg CO2 / h / g (fresh Wt)
16
12
12
12
Time of day
Middle 5°C
Middle 27°C
Middle 35°C

# Chapter 6. Discussion and conclusions

## 6.1 Final discussion

In this thesis I have shown that the *Bryophyllum fedtschenkoi CAB* cDNA, which I isolated, encodes for a type I LHC II polypeptide. I also show that in *Bryophyllum fedtschenkoi* there is a *CAB* gene family. I identified *CAB* gene expression, measured by the steady-state level of *CAB* transcripts, in *Bryophyllum fedtschenkoi* and found that a circadian rhythm of expression occurs under specific constant conditions. The extremes of temperature, and leaf size also affect *Bryophyllum fedtschenkoi CAB* gene expression.

The rhythm of *CAB* gene expression that occurs in *Bryophyllum fedtschenkoi* shows a number of similarities to the circadian rhythms of carbon dioxide metabolism that occur in detached leaves of *Bryophyllum fedtschenkoi*. Both rhythmical patterns are found to occur under two types of constant environmental conditions, which are $CO_2$ free air and continuous darkness, and normal air and continuous light, with each rhythm expressing a similar number of cycles. A continuous cycling rhythm is not shown, in either case, under conditions of continuous darkness and normal air, but each does appear to show a endogenous mechanism of control where a single cycle of $CO_2$ fixation or *CAB* gene expression occurs. In the constant conditions of $CO_2$ free air and continuous darkness, and normal air and continuous light, with both at a temperature of 15°C, the rhythm of $CO_2$ gaseous exchange in leaves of *Bryophyllum fedtschenkoi* continued for 3 to 4 days with a period length of 24 hours, and 10 days with a period length of 18 hours, respectively

(Wilkins, 1959, 1960, 1984; Anderson and Wilkins, 1989c). In the case of the rhythm of *CAB* gene expression under the same conditions of $CO_2$ free-air and constant darkness, and normal air and constant light, I found that the rhythm persists for at least 2 days with a period length of 26-28 hours, and for at least 3 days with a period length of 20 hours, respectively. The fact that, in each constant condition, there is a rhythm of *CAB* gene expression and similar period lengths are found to occur, suggests that both the rhythms of $CO_2$ metabolism and *CAB* gene expression are under the control of a similar oscillatory mechanism.

Since *Bryophyllum fedtschenkoi* shows endogenous rhythms of *CAB* gene expression it fits in with the patterns of *CAB* gene expression seen in other higher plants (Meyer *et al.*, 1989). In continuous light conditions a persistent rhythmical pattern of *CAB* gene expression occurs. This is similar to the rhythms identified by Nagy *et al.* (1988), Millar and Kay (1991), and Taylor (1990). In *Bryophyllum fedtschenkoi* the pattern of *CAB* gene expression in constant darkness and normal air shows the appearance of the *CAB* transcript during the subjective light period, the level of the transcript then decreases only reappearing after the trigger of a light pulse. Other studies find that a circadian rhythm of *CAB* gene expression occurs in continuous darkness but that the rhythm shows a rapid damping in amplitude (Nagy *et al.*, 1988; Millar and Kay, 1991; Kellman *et al.*, 1993). In *Bryophyllum fedtschenkoi* this may be the result of a number of factors. Firstly, in the same conditions the rhythm of the $CO_2$ gaseous exchange pattern shows a single peak of fixation, which then drops, until a constant level of $CO_2$ output is reached that steadily declines. The reason for this occurring is that the malate builds up to very high levels in the cells and eventually inhibits the PEPc whether it is in a phosphorylated or dephosphorylated form, but note also the

PEPc kinase vanishes and never re-appears (Carter *et al.*, 1995a), and the PEPc becomes dephosphorylated and stays so (Nimmo *et al.*, 1987). Therefore, no rhythm of $CO_2$ metabolism occurs. Theoretically the decrease in the levels of the *Bryophyllum fedtschenkoi CAB* transcript could also be linked to the changing malate levels such that the rising levels of malate either inhibit the synthesis or promote the degradation of the *CAB* mRNA. Since *CAB* gene expression occurs over a time period (0400 h to 1200 h) when high levels of malate are present in the cell this is unlikely, but it is still possible that a build up of malate has an effect on the operation of the rhythm, even though it is not the "driving force". The synthesised malate from dark $CO_2$ fixation is not utilised in photosynthesis and so may inhibit the complete circadian cycle. Alternatively, in *Bryophyllum fedtschenkoi*, the lack of a circadian rhythm in the levels of the *CAB* transcript in constant darkness and normal air could simply be a connection to light. Since there is no light the massive dampening of the rhythm is a result of reducing levels of active phytochrome. This would follow the theory proposed by Nagy *et al.* (1988), who argued that the clock operates as a gate that opens and closes rhythmically allowing phytochrome to induce *CAB* gene expression at specific times. This would be consistent with the continuous rhythm of *CAB* gene expression in leaves of *Bryophyllum fedtschenkoi* that occurs in continuous light and normal air.

*Bryophyllum fedtschenkoi* is a CAM plant and the appearance of a circadian rhythm of *CAB* gene expression in $CO_2$ free air and continuous darkness, and its absence in continuous darkness and normal air leads to the argument that the *CAB* rhythm is linked to the rhythm of $CO_2$ metabolism. Harris and Wilkins (1978), and Wilkins (1989) found that the rhythm of $CO_2$ metabolism could be entrained and reset by red light.

Wilkins (1983) advanced a hypothesis to account for the characteristics of phase shifts that are induced in leaves of *Bryophyllum fedtschenkoi*, placed in constant darkness and $CO_2$ free-air, by high temperature and light stimuli in terms of the leakage of malate from the vacuole to the cytoplasm through gates in the tonoplast that are open only during exposure to these stimuli. A similar hypothesis was proposed for the effects of red light. In continuous light and normal air, Wilkins (1984) and Anderson and Wilkins (1989a, 1989b) hypothesise that the variation of malate levels is achieved through its synthesis and breakdown. It is thus possible that if the circadian rhythms of *CAB* gene expression and $CO_2$ metabolism are linked, that the level of malate is, in some way, controlling the production of these rhythms. However, the effects of PEPc phosphorylation and PEPc kinase activity in the operation of the circadian rhythms should also be considered.

An investigation of the effects of red light and far red light on the circadian rhythm in *CAB* gene expression would identify if the two rhythms of *Bryophyllum fedtschenkoi* were linked and if a single oscillatory mechanism controlled their expression. It can be hypothesised that phytochrome is one of the photoreceptors involved in the expression of *CAB* genes and probably is linked to the oscillator (entrainment). The reason for this belief is the mass of information and experimental work of others which shows that phytochrome is connected in some manner to *CAB* gene expression. In one example, Tavladoraki *et al.* (1989) showed that a single pulse of red light entrains etiolated *Phaseolus vulgaris* leaves such that a circadian rhythm of *CAB* mRNA accumulation occurs. Therefore, in this latter case, the circadian oscillator is under phytochrome control. A red light pulse given to the detached *Bryophyllum fedtschenkoi* leaves is likely to trigger *CAB* gene expression

in conditions of continuous darkness and normal air. It may also prolong the rhythm that occurs in the conditions of continuous darkness and $CO_2$ free-air. These effects, if they are linked to phytochrome control, should be reversed by the use of a far-red light pulse. Thus in *Bryophyllum fedtschenkoi* if phytochrome is involved in the production of the rhythm of *CAB* gene expression, and this rhythm is linked to that of $CO_2$ metabolism, then the *CAB* rhythm should show phase shifts similar to the $CO_2$ rhythm (Harris and Wilkins, 1978; Wilkins, 1989). This would identify if the two rhythms are controlled by the same oscillator.

The results also show that *CAB* gene expression is affected by temperature. In constant darkness and normal air, the pattern of *CAB* gene expression was altered by the extremes of temperature (5°C and 35°C). *CAB* gene expression was totally inhibited at low temperatures (5°C) whereas at high temperatures (35°C) *CAB* gene expression was detectable but the expression showed no clear pattern as the *CAB* transcript appeared and disappeared a number of times. Normally under these conditions a continuous rhythmical pattern does not occur in either *CAB* gene expression or $CO_2$ gaseous exchange. However, the pattern of *CAB* transcript accumulation and degradation shows a similarity to the pattern of PEPc kinase activity under similar conditions. Carter *et al.* (1995a, 1995b) show the effects of temperature on the circadian rhythm of $CO_2$ fixation in *Bryophyllum fedtschenkoi*. At low temperatures (5°C) the PEPc kinase activity is stabilised and at high temperatures (35°C) it is rapidly reduced, these factors link into the control of PEPc activity. In comparison to *CAB* gene expression of *Bryophyllum fedtschenkoi* the exact opposite is seen to that of the PEPc kinase. Consequently, at ambient biological temperature (10°C to 30°C) regimes both the rhythmical patterns that occur follow a controlled expression cycle

(previously discussed). The pattern of $CO_2$ gaseous exchange at 30°C in leaves of *Bryophyllum fedtschenkoi* also begins to show a circadian nature. This rhythm becomes increasingly erratic at the higher temperature of 35°C and is consequently similar to the pattern of *CAB* gene expression at this high temperature as it shows an erratic pattern of expression with no discernible rhythm. However, at the low temperature of 5°C there is still an extended period of $CO_2$ fixation. Carter *et al.* (1995a, b) found that the malate sensitivity and activity of PEPc increase with temperature, and this leads to the differing patterns of $CO_2$ gaseous exchange that occur. Could these effects control *CAB* gene expression in *Bryophyllum fedtschenkoi* leaves? Anderson and Wilkins (1989b) show that, at the end of a normal day, there is always a residual level of malate in leaf cells. At different temperatures, with the onset of darkness, Carter *et al.* (1995a, b) show that the malate in the cytoplasm will regulate PEPc and this in turn will affect the relative level of malate synthesised by $CO_2$ fixation. At the low temperature of 5°C $CO_2$ fixation begins immediately and continues for some time. The reason for this latter occurrence is that the PEPc kinase becomes active (Carter *et al.*, 1991), is stable at this lower temperature, and phosphorylates the PEPc. This phosphorylation decreases the sensitivity of PEPc to inhibition by the rising levels of cytoplasmic malate (Nimmo *et al.*, 1984, 1986, 1987) and $CO_2$ fixation continues. In this condition the levels of malate are maintained at a higher level and this may link to inhibiting *CAB* gene expression. Low temperatures do also affect *CAB* mRNA synthesis as Martino-Catt and Ort (1992) show, by its inhibition, in the plant *Lycopersicon esculentum*. At the high temperature of 35°C there is no net period of $CO_2$ fixation in *Bryophyllum fedtschenkoi*. The sensitivity of PEPc is increased, there is no PEPc kinase activity and the residual malate inhibits $CO_2$ fixation. However, the amount of malate that is

synthesised, during the short periods of PEPc activity, is also very small but capable of inhibiting PEPc activity. When these small amounts of malate are transported to the vacuole periods of PEPc activity can occur and this accounts for the appearance of a circadian rhythm. Wilkins (1962, 1983) states that the tonoplast of *Bryophyllum fedtschenkoi* is increasingly permeable to malate at higher temperatures and any malate that is transported into the vacuole can then immediately leave it. Nonetheless, at this high temperature the lower levels of malate may allow the erratic pattern of *CAB* gene expression to occur. The cycle of malate may trigger a photosynthetic like response and *CAB* mRNA accumulation. Therefore, in *Bryophyllum fedtschenkoi*, *CAB* gene expression is likely to be linked to the oscillatory mechanism of control through the environmental controls of temperature and the level of malate.

Nagy *et al.* (1988) and others discuss the consequences for the plant of establishing a rhythmic expression pattern for the *CAB* genes. Plants coordinate the expression of the chlorophyll a/b binding protein with the key enzyme of chlorophyll biosynthesis (protochlorophyllide reductase) and so this plays a role in the maintenance and assembly of the photosynthetic apparatus. In this way *CAB* genes are important in setting up the light harvesting capacity of the plant so that an effective growth cycle is maintained and coordinated with the environment. The action of light and temperature affect the expression of *CAB* genes. Light can exert two effects on the expression of light inducible genes; induction of circadian rhythmicity in mRNA levels (Tavladoraki *et al.*, 1989) and the continual enhancement of transcript levels (Apel and Kloppstech, 1978). This latter effect is shown by the persistent rhythmical expression of *CAB* mRNA in continuous light and normal air by *Bryophyllum fedtschenkoi* leaves whereas in periods of constant darkness *CAB* transcript levels are

periodically undetectable. Beator *et al.* (1992) showed that, in *Hordeum vulgare*, a heat shock treatment leads to the induction of circadian oscillations in *CAB* mRNA levels. They go on to say that aspects like stability of the thylakoids against external effects of light and extreme temperatures will be under circadian control as is photosynthesis (Freedeen *et al.*, 1991; Hennessey and Field, 1991). It is known that phosphorylation of certain polypeptides occurs in LHC II (Allen, 1992) and that this is a response to optimize energy use, by the plant, under different light intensities and wavelengths. Therefore, temperature may affect this stability so ultimately affecting the expression of the *CAB* genes.

It is possible to hypothesise how the circadian rhythms of *Bryophyllum fedtschenkoi* are centrally coordinated. In *Bryophyllum fedtschenkoi* a complex signal transduction network may be in operation where the environment can interact with the gene expression apparatus. Phytochrome activation by light may initiate this transduction network. Since phytochrome was shown to affect the rhythm of $CO_2$ metabolism (Wilkins, 1989) and may affect the *CAB* rhythm, it may also be involved in coordinating the two rhythms. In *Bryophyllum fedtschenkoi* $CO_2$ fixation is a dark period reaction and photosynthesis is a light period reaction. Therefore, malate is constantly synthesised and processed. The circadian clock must be coordinated to these time variations. Beator *et al.* (1992) proposed that heat shock treatments cause effects similar to those stimulated by light. Therefore, if we follow the theory of Beator *et al.* (1992) and the fact that CAM plants grow in a diurnally varied temperature environment, in *Bryophyllum fedtschenkoi* the high temperature of 35°C probably reinforces the effects of light in setting up the characteristic CAM cycle. However, at these high temperatures in

constant darkness and normal air the synthesis of *Bryophyllum fedtschenkoi CAB* mRNA is erratic, but this could be linked to the findings of Carter *et al.* (1995a, b) where a low level of malate is present and is erratically synthesised. In similar constant conditions at the low temperature of 5°C, an extreme nighttime temperature, the *CAB* mRNA is not detected. At this low temperature the level of malate remains high and this may feedback inhibit the circadian rhythms of *CAB* and $CO_2$ metabolism. The build up of malate has an effect on the operation of the rhythms of *Bryophyllum fedtschenkoi.* Light and temperature may be linked to its synthesis and degradation. Therefore, the mechanism of control seen in the various transduction pathways, which involve light and temperature, of the $CO_2$ and *CAB* rhythm may also be coordinated by the synthesis and degradation of malate.

Beator and Kloppstech (1994) draw a model of how circadian rhythmicity affects gene expression. The central feature is the circadian oscillator or the clock. This clock is connected through a number of signal transduction pathways with the environment. It also shows that during development a switch from differing photoreceptors may occur and that the clock controls gene expression at a number of levels. Differences in developmental control are apparent in *Bryophyllum fedtschenkoi* between the rhythms of *CAB* gene expression and $CO_2$ gaseous exchange. The leaves that show maximal *CAB* expression are smaller than those which show the clearest rhythms in $CO_2$ release/uptake. In the larger leaves, *CAB* gene expression occurs but transcripts accumulate to a much lower level of expression as compared to the smaller leaves. The small *Bryophyllum fedtschenkoi* leaves that show circadian cycling in *CAB* gene expression show different patterns of $CO_2$ release/uptake to these larger leaves. A circadian rhythm in $CO_2$ metabolism occurs but the amplitude

is greatly reduced and the rhythm dampens out rapidly. This emphasises that, as the *Bryophyllum fedtschenkoi* leaves develop morphologically, a long term mechanism of control is also present in the operation of the circadian rhythms.

## 6.2 Concluding remarks

I have shown that there is a circadian rhythm of *CAB* gene expression in *Bryophyllum fedtschenkoi* and that this rhythm does show some characteristic features that relate it to the rhythm of $CO_2$ metabolism and also to the *CAB* gene expression rhythms of other higher plants. The interesting features are that a complex network of reactions may be at work in *Bryophyllum fedtschenkoi* that is controlled by a single oscillator mechanism. The clock of *Bryophyllum fedtschenkoi* coordinates the reactions of the CAM cycle so that efficient photosynthetic reactions occur through the light and $CO_2$ fixation reactions through the night. The advantage of this circadian mechanism of control is that the cycle of events is operating on an efficient level so that the plant is prepared for its environmental conditions over an ambient range of temperatures. I have shown the existence of a *CAB* rhythm and presented preliminary evidence linking it to the $CO_2$ rhythm, but further work is needed so that the nature of the clock in *Bryophyllum fedtschenkoi* can be addressed.

## 6.3 Future work

In order to determine if there is a definite link between the rhythms of $CO_2$ metabolism and *CAB* gene expression in *Bryophyllum fedtschenkoi* the rhythms of *CAB* gene expression need to be analysed more extensively. The effects of temperature variation and light treatments (red/far red light

pulses, dark/light pulses) on the phase of the rhythms of *CAB* gene expression should be assessed to identify if there are similarities to those of the $CO_2$ rhythm. Also the level of control of the *CAB* rhythm should be analysed, by use of *in vitro* transcription, to find out if control is at the level of transcription, as other studies with other higher plants, have already found. The Type I LHC II *CAB* cDNA sequence of *Bryophyllum fedtschenkoi* needs to be completed, its regulatory elements located, and then a more detailed analysis of the molecular mechanism of the control of the *CAB* gene expression can be carried out. Transformation of *Kalanchoe laciniata* was achieved by Jia *et al.* (1989) and so the production of transgenic *Bryophyllum fedtschenkoi* is possible. If this is achieved the elucidation of the molecular mechanism of the circadian clock, its interaction with receptors, and its role in the regulation of metabolism and development can eventually be discovered.

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