THE MODE OF ACTION OF CYTOKININ IN THE MOSS *PHYSCOMITRELLA PATENS*

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ABSTRACT

Different aspects of the mode of action of cytokinin in the moss Physcomitrella patens were studied. In the search for a cytokinin receptor, a particulate fraction was isolated which had cytokinin-binding activity. Cytokinin-binding activity is described as the amount of binding of a radioactive cytokinin that is prevented from binding by excess unlabelled cytokinin. The cytokinin-binding activity was only detected with a tritiated cytokinin of high specific activity. Two assays were used, an equilibrium dialysis and a centrifugation assay. These assays were used to show that the cytokinin-binding activity was heat-labile and can be solubilised by the detergent Triton X100, but not by acetone. Cytokinin-binding activity was higher in phosphate-starved tissue which consists mainly of caulonemata, the target cells for cytokinin action. A 13,000-80,000g pellet appeared to contain a component which has some of the characteristics expected for a cytokinin receptor that was a membrane bound protein.

Mutagenised spores were screened for cytokinin non-responding mutants. No such mutants were obtained from 25,000 plants. A temperature-sensitive mutant, ove 409, was isolated which produced normal leafy shoots at 17°C, but many abnormal buds at 24°C. This mutant was found to over-produce isopentenyladenine at the higher temperature. The level of cytokinin production in wild type and ove 78 was also found to be temperature-dependent. The change in cytokinin production by ove

409 was over twice that with wild type and ove 78, and dropped to wild type levels at 17°C. By the use of protoplast fusion, ove 409 was found to be recessive to wild type and in the same complementation group as ove A78.

The role of light was studied in the induction of the gametophore. In the presence of supplied cytokinin, bud induction was caused by red light around 613 to 687nm. When the cytokinin-induced bud induction occurs in the dark after exposure to red light, bud induction is reduced by a short exposure to far-red light or a dark period before the addition of the cytokinin. This indicates that phytochrome is involved in bud induction. However, another factor appears to be involved in bud induction as this was greater when the cytokinin was applied to the cultures 2 hours after the exposure to red light.

The possible role of calcium ions in bud induction was investigated. Calcium ions have been reported to induce the first stages of buds in the moss Funaria hygrometrica. Using the calcium ionophore A23187, calcium ions were shown to induce chloronemal branching in Physcomitrella patens. Therefore calcium ions appear to have a role in branching, but not in the differentiation of a bud.

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ABBREVIATIONS

BAP	6-benzyl aminopurine
CPM	counts per minute
DPM	disintigrations per minute
ft-c	foot-candles
GCMS	combined gas chromatography mass spectrometry
HPLC	high performance liquid chromatography
IPA	isopentenyladenosine
2iP	isopentenyladenine
MIM	multiple ion monitoring
P_{fr}	phytochrome which is converted to P_r by far-red light
P_r	phytochrome which is converted to P_{fr} by red light
S	Svedberg coefficient
SEM	scanning electron microscopy

CHAPTER I

Introduction

The term "hormone" is usually applied to substances produced by an animal in one part and transported to one or more other parts to have an effect at a low concentration. The term is now widely used to refer to small organic molecules in plants that have an effect on plant cells at very low concentrations. These substances are often referred to as plant hormones or plant growth substances. Although there are some similarities between animal and plant hormones there are some important differences.

Animal hormones versus plant hormones

There are a large number of animal hormones produced in any one organism. Over 40 hormones have been identified in mammals. They are synthesised in specific organs and transported to clearly defined tissues to evoke a specific response. These hormones have their effect at very low concentrations of 10^{-10} to 10^{-12}M. The target tissue must have a method of distinguishing the hormone from all the other fluid components. This is believed to be performed by a receptor with a high affinity and specificity for that hormone. Animal hormones range from simple ring structures, such as adrenaline, through small proteins such as glucagon with 29 amino acid residues, up to follicle-stimulating hormone with over 200 amino acid residues.

The larger hormones of animals usually bind to specific receptors on the outer surface of the target cell which then

initiate a cascade mechanism to amplify the signal and induce the specific response. The small steroid hormones move into the cytoplasm before binding to their receptor and this complex then migrates to the nucleus to affect transcription. Many receptors for animal hormones, both steroids (Williams and Gorski, 1972; Jensen and DeSombre, 1972) and polypeptides (Cuatrecasas, 1972), have been identified and the mechanism of the response clarified (see also review by Cuatrecasas, 1974).

In contrast, the mechanisms of plant hormone action are very poorly understood. Although the principle of a hormone being produced by one group of cells and having an effect on other cells containing a receptor could be applied to plants, the identification of a hormone-receptor system is made more complex by the lack of clearly defined organs in a plant for synthesising and responding to hormones. Plant hormones tend to be synthesised throughout the plant, but there are localized areas of increased activity such as meristems. There are only five classes of plant hormones that have so far been identified. These are auxins, cytokinins, gibberellins, abscisic acid and ethylene. With the exception of the gibberellins, there are only one or two hormones from each class present in any one species. Another important difference is that plant hormones affect several parts of the plant and do not have the same cell type specificity of animal hormones. Plant hormones also tend to have a large number of effects on an individual cell, some of these effects being brought about by different plant hormones. Plant hormones are often described as helping to promote a particular response, rather than causing the response.

Most of the classical plant hormone work was done by supplying hormones at levels that were much higher than the levels of the endogenous hormone. This can cause abnormal growth in a number of different ways. However, some of the responses might not occur with normal levels of hormones. The multiple effects of plant hormones, whether real or artefactual, and the inability to isolate sites of hormone production and receptor organs, have made it difficult to study the effects of plant hormones on development.

Hormone receptors

For a plant cell to respond to a hormone, it has to be able to detect the presence of that hormone in a highly specific manner. This is probably done by a specific receptor for the hormone (for reviews see Venis, 1973; Kende and Gardner, 1976; Venis, 1977c; Dodds and Hall, 1980), which is then activated to induce a biochemical process. With animal hormones, all the receptors that have been isolated so far have been proteins. If plant hormones are analogous to animal hormones, the most likely receptor for plant hormones is a protein, which could have the diversity to bind in a specific manner, although other possibilities cannot be excluded. Crude fractions and some purified proteins have been isolated from a number of different plant species for all five classes of plant hormones. These appear to bind specifically the particular class of hormone in question. Auxin-binding fractions have been reported in maize (Batt and Venis, 1976; Batt *et al.*, 1976; Cross and Briggs, 1978 and 1979; Dohrmann *et al.*, 1978; Hertel *et al.*, 1972; Lembi *et al.*, 1971; Ray, 1977; Ray *et al.*, 1977a and b; Venis, 1977a

and b; Venis and Watson, 1978), cucurbits (Jacobs and Hertel, 1978), tobacco pith callus (Vreugdenhil et al., 1979), dwarf bean (Wardrop and Polya, 1977), soyabean (Ihl, 1976), and pea epicotyls (Jacobsen, 1982). Cytokinin-binding fractions have been reported in wheat (Erion and Fox, 1981; Fox and Erion, 1975; Keim and Fox, 1980; Moore, 1979; Polya and Bowman, 1979; Polya and Davies, 1983; Polya and Davis, 1978), moss (Gardner et al., 1978), tobacco (Sussman and Kende, 1978; Takegami and Yoshida, 1975, 1977; Yoshida and Takegami, 1977), and chinese cabbage (Berridge et al., 1970). Gibberellin-binding fractions have been reported in lettuce (Stoddart, 1979a, b). Abscisic acid-binding fraction has been reported in onion (Hocking et al., 1978) and Vicia (Hornberg and Weiler, 1984). Ethylene-binding fractions have been reported in bean (Bengochea et al., 1980a, b; Thomas et al., 1984), and tobacco (Sisler, 1979). Before a binding site can be classed as a receptor, there has to be a biochemical connection identified which couples the binding of the hormone to a specific physiological response.

The hormone-receptor interaction can be considered essentially as a substrate-enzyme interaction and thus under the same kinetics. With protein receptors that initiate a biochemical process, the hormone-receptor interaction can be thought of as an enzyme-cofactor interaction, where the binding of the cofactor activates the enzyme, which then catalyzes the first reaction in the biochemical pathway. The reaction between hormone (H) and receptor (R) can be considered in the following reaction where k_1 and k_2 are the rate constants for association and dissociation respectively.

$$H+R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HR \qquad (1)$$

The dissociation constant K_D, and the affinity constant K_A can be defined;

$$K_D = \frac{k_2}{k_1} = \frac{[H][R]}{[HR]} \qquad (2)$$

$$K_A = \frac{k_1}{k_2} = \frac{[HR]}{[H][R]} \qquad (3)$$

The K_D is analogous to the Michaelis constant (K_m) in enzyme kinetics. If half the binding sites are occupied, [R]=[HR] and so K_D=[H]. Therefore K_D is the hormone concentration at which half the binding sites are occupied. Equation (2) can be rearranged to give:

$$\frac{K_D[HR]}{[H]} = R_{total} - [HR] \qquad (4)$$

If equation (1) is the equilibrium attained by a hormone-receptor mixture, then a plot of bound/free ([HR]/[H]) plotted against bound ([HR]), should give a straight line. Such a plot is known as a Scatchard plot (Scatchard, 1949). A Scatchard plot can be used, in an assay for hormone binding, to show if there is more than one type of hormone binding site present. The slope of the straight line of a Scatchard plot for a single type of binding site will be the reciprocal of the dissociation constant, and the intercept with the abscissa will be the concentration of the binding sites.

The Scatchard plot will only give a straight line with a slope of the reciprocal of the dissociation constant if there is only one type of binding site present. If there are two types of binding site, the Scatchard plot will give a curve which is the sum of the two straight lines which would be obtained from the two binding sites on their own. The intercept with the abscissa will give the total number of binding sites. The two straight lines, and hence the binding parameters from such a Scatchard plot can be calculated (Weder et al. 1974). The parameters obtained from a Scatchard plot may be inaccurate and this is made clear by plotting moles of bound ligand against the logarithm of the concentration of free ligand (Klotz, 1982). Such a plot should give an S-shaped curve with the inflection point at half the number of binding sites. Klotz shows that the data from published Scatchard plots, when plotted on a semi-logarithmic graph, do not reach an inflection point, and that even if the last point is assumed to be the inflection point, it gives a much larger number of binding sites than was originally obtained from the original Scatchard plot. If (a) the binding sites for a particular ligand are being assayed in a biological extract, (b) there is more than one type of site, (c) there is more than one site per binding molecule where the affinity of one site is dependent on whether any of the other sites are occupied, or (d) there are a large number of non-specific sites, the determination of the dissociation constant or the number of binding sites can be unreliable from a Scatchard plot. Therefore, before such parameters can be determined, a hormone-binding component has to be purified.

There are a number of different methods of measuring the

equilibrium of a small ligand that is bound to large molecules, such as proteins, and free ligand. The most direct method is to use equilibrium dialysis. The macromolecule is confined by a membrane which allows the passage of the ligand. The λ^{cell} is left for the amount of free ligand to equilibrate each side of the membrane. If the ligand binds to the macromolecule, at equilibrium, there will be more ligand on the macromolecule side of the membrane. This method was described by Klotz et al. (1946). For a hormone-receptor mixture, the amount of hormone on each side of the membrane can be determined by using a radioactive hormone. Other methods of measuring this equilibrium between a ligand and a macromolecule have been devised. An ultrafiltration method (Lever, 1972) can be used where the free ligand is removed by collecting the macro-molecule on a nitro-cellulose filter. Alternatively, the free ligand can be removed by gel filtration chromatography (Hummel and Dreyer, 1962; Tze-Fei and Porter, 1983). If the macromolecule is in a particulate fraction, it can be removed from the free ligand by centrifugation (Lembi et al., 1971).

An alternative method of detecting a hormone receptor is to use a photoaffinity label. The synthesis of a variety of different photoaffinity labels for detecting cytokinin-binding is described by Mornet et al. (1979), (see also Colman, 1983 for general review on purine nucleotide photoaffinity labels). These are azidopurines that have cytokinin activity, but when exposed to ultraviolet light a reactive intermediate is produced which then covalently binds to surrounding molecules. Therefore, such a photoaffinity label can be added to a biological system, where

it should bind to any specific binding sites. It can subsequently be activated and should bind covalently to the molecules that specifically bind to cytokinin. The label can then be used to locate the cytokinin-binding molecules, during purification. Any molecules to which the photoaffinity label is found to be bound could then be tested as a possible receptor.

Plant hormone binding

Most plant hormone binding assays have been performed using either equilibrium dialysis or centrifugation. The plant hormones most studied with respect to receptors are auxin and cytokinin. One of the first reports of active auxin binding was in a particulate fraction of corn coleoptiles (Hertel et al., 1972). They showed that radiolabelled NAA is displaced from the particulate fraction by 1-NAA and IAA, but not by inactive analogues such as 2-NAA or indol-3-yl propionic acid. More precise kinetics of auxin binding in corn coleoptile membranes are given by Batt et al. (1976). They showed evidence, by Scatchard plots, of two types of auxin binding sites. They showed also that the binding sites could be solubilised by treating the pellet with Triton X100. The two sites were studied for their interaction with various auxin analogues. Binding of NAA to site 1 was competitive with inactive auxin analogues as well as active auxins and anti-auxins. These two binding sites have been separated on a sucrose gradient (Batt and Venis, 1976). They report that site 1 appears to be associated with the dictyosomes or endoplasmic reticulum, and that site 2, which is the site reported by Hertel (1972), is associated with the plasma membrane. They conclude that this site is the stronger candidate

for an auxin receptor as only active auxins and anti-auxins compete with NAA for binding sites.

The auxin binding sites from corn coleoptiles have been studied by using the auxin analogues 2-chloro-4-aminophenoxyacetic acid (CAPA) and 2,5-dichloro-3-aminobenzoic acid and by using p-mercuribenzoate (PMB), (Venis, 1977a). From these studies, Venis has suggested that the amino acids in the binding site could be histidine, aspartate/glutamate, tyrosine/lysine and cysteine. The auxin binding site 2, reported by Batt et al. (1976), has been purified further (Venis, 1977b). Venis states that further purification could be achieved if the binding fractions were solubilised by acetone. Venis showed that the binding activity was confined to a single peak on a DEAE-cellulose column. The binding activity was heat labile, non-dialysable and precipitated with 70% ammonium sulphate. The binding activity appeared to be associated with proteins with molecular weights of 40,000 and 47,300.

A supernatant factor (SF) which reduces the amount of auxin binding to corn coleoptiles has been reported (Ray et al., 1977a and b). This supernatant factor can be removed by washing the pellet in buffer. Ray et al. reported that the SF was responsible for the small amount of binding reported by Hertel (1972). This SF was not initially found in the binding fraction of Batt et al. (1976). The SF appears to be present only in some varieties of Zea mays. Ray et al. show that the SF can be added back to reduce the auxin-binding activity. This reduction in binding activity is a reduction in affinity , not a reduction in the number of binding sites. They showed that the specific

binding is heat labile and is removed by treating with detergent. As the assay was by centrifugation, it was not possible to distiguish between denaturation or solubilisation of the binding activity by the detergent. The amount of specific binding was reduced by reducing agents such as dithionite. Inhibition by dithionite was partially reversed by ferricyanide. Dithionite reduced the number of binding sites, but not the affinity for NAA of the unaffected sites.

A very different auxin binding site has been reported in tobacco pith callus (Maan et al., 1983). Auxin binding to a particulate fraction did not occur at $0^{o}C$, and was slower at $25^{o}C$ than auxin binding to corn coleoptiles at $0^{o}C$. Auxin binding was measured by a non-equilibrium filtration method. As the rate of binding was slow, the time course of the binding reaction could be measured. Maan et al. showed that the binding was not due to a single site or due to take-up of auxin into vesicles. From their data of the kinetics of the reaction, they propose a model where there are four binding sites and that binding can cause a conformational change to form a stable complex. This was the simplest model that fitted the data.

The first report of specific binding of cytokinin in vitro was to Chinese cabbage leaf ribosomes (Berridge et al., 1970). They showed that the binding affinity to the 83S ribosomes was correlated with the the cytokinin activity of a number of compounds. They inferred from the binding activity on the 83S ribosomes that cytokinin control of growth was at the level of the ribosomes. Takegami and Yoshida (1975) purified a 4000 molecular weight protein from tobacco leaves by affinity

chromatography which bound benzyl amino purine. This protein also specifically bound kinetin, and with lower affinity, adenine, but not adenosine. Fox and Erion (1975) reported 2 cytokinin-binding sites on wheat germ and tobacco ribosomes. From Scatchard plots, they show that there is one high affinity site and multiple low affinity sites per ribosome. The high affinity sites can be washed off the ribosomes with 0.5M KCl and still show high affinity binding.

Association of the cytokinin-binding protein with the ribosomes has also been reported by Takegami and Yoshida (1977). The cytokinin-binding protein was radiolabelled by incubating tobacco leaf discs on a solution containing ^{3}H-4,5-leucine and the cytokinin-binding protein isolated. The cytokinin-binding protein was found to bind to KCl-washed ribosomes in the presence of BAP. The amount of binding was reduced to 10% with unwashed ribosomes, which they claim was due to the ribosomes being already saturated with cytokinin-binding protein. They show that the cytokinin-binding protein only binds to the 40S subunit of the ribosome.

The first study of cytokinin-binding using a radiolabel with high affinity was in tobacco (Sussman and Kende, 1978). The tritiated BAP was sythesised by catalytically dehalogenating a p-bromo benzyl amino purine which was sythesised by Sussman and Firn (1976). By the use of this high specific activity radiolabel, two cytokinin-binding sites were detected in a particulate fraction, one of high affinity for BAP, the other of low affinity. These sites appeared to be different from the

cytokinin-binding sites associated with the ribosomes, reported by previous authors. Most of the cytokinin-binding was to the low affinity sites which were heat stable. The number of these low affinity sites increased by heating to 100°C. The reason stated for this increase is that denaturation of proteins could expose more non-specific binding sites. The few high affinity sites were only detected using the tritiated BAP. This high affinity binding was greatly reduced by heat and was shown to have a high specificity towards biologically active cytokinin analogues. Two inactive analogues, p-chloro BAP and p-bromo BAP, which showed the greatest non-specific binding, did not bind to the high affinity site.

Kinetin binding to a soluble glycoprotein with a molecular weight of 180,000 has been reported in wheat germ (Polya and Davis, 1978). Kinetin binding to this protein could be displaced by low concentrations of biologically active cytokinins but not by inactive adenine derivatives. One exception was that zeatin was less effective than N^6-dimethylallyladenine. The concentration of this cytokinin-binding protein is relatively high for a receptor, 27μM, compared with animal hormone receptors.

Using the same tritiated BAP as Sussman and Kende (1978), Gardner et al. (1978) detected cytokinin-binding in a particulate fraction of the moss Funaria hygrometrica. Biologically active cytokinins, including BAP, zeatin, IPA, and kinetin, competed with the tritiated BAP. Adenine, 9-methyl-BAP, and the ribosides of BAP, IPA and zeatin did not compete with the radiolabel. As well as the specificity of the binding for active

cytokinins, Gardner et al. showed that over half of the binding activity was removed by heat and that it was greatly reduced by treating the pellet with Triton. To see if the Triton was solubilising the binding fraction rather than denaturing it, they tested the binding of the supernatant by gel filtration. However, they reported that there was an interaction between the BAP and the Triton. The binding activity was small even with the high specific activity ^{3}H-BAP which could have been due to there being only 0.5-1.0 g fresh weight of tissue per assay. They stated that a Scatchard plot gave a curve which indicates that there is more than one type of binding site.

Polya and Bowman (1979) show that the cytokinin-binding fraction in wheat, described earlier (Polya and Davis, 1978), has a high affinity for non-purine cytokinins, such as DCMU and CMU, and for hydrophobic derivatives of urea, triazine, carbamate and tryptophan. As this cytokinin-binding fraction also has an unusually low affinity for zeatin, Polya and Bowman suggested that this protein may not be a receptor, but could have a cytokinin "buffering" or sequestering function. A similar protein from wheat germ has been reported by Moore (1979). This also shows an unusually low affinity for zeatin, but has a molecular weight of only 122,000. However, Moore suggests that the cytokinin activity reported by all three groups could be derived from the same in vivo moiety.

Erion and Fox (1981) report that the cytokinin-binding protein from wheat germ has a molecular weight of 183,000, made up of subunits with molecular weights of 34,000, 39,000, 53,000 and 59,000. They claim that the subunit of 15,000 reported by

Moore (1979) could be obtained by ageing for several weeks, and that the 5 bands obtained by Polya and Davis (1979) could be due their own stated lack of homogeneity. The high concentration of the cytokinin-binding protein, 27 nmol/g fresh weight reported by Polya and Davis and 15 nmol/g fresh weight reported by Erion and Fox, in wheat germ, is only found in the germinating seed and in female reproductive tissue, according to Erion and Fox (1981) using antibodies to the cytokinin-binding protein.

In summary, auxin binding in corn coleoptiles is relatively well characterised. Two separate auxin high affinity sites have been located in corn membranes, one of which appears to have the same specificity as auxin activity. This receptor has been shown to be a protein, and some of the amino acids in the binding site have been tentatively assigned. The auxin binding fraction from tobacco was also membrane bound but showed a much slower rate of binding. This binding fraction has not been so well characterised and a model for auxin binding is suggested on the kinetics of the reaction. The initial reports of a cytokinin-binding fraction were of a soluble fraction which was associated with the ribosomes. The soluble protein which binds cytokinin to ribosomes in wheat germ and tobacco has been reported by a number of groups. The different groups report different molecular weights ranging from 122,000 to 183,000, and different molecular weights and number of subunits. From the data given, it is not certain that these groups are looking at the same proteins or whether only some of the subunits are required for binding of cytokinin. Polya and Bowman have reported a broad ligand specificity and so suggest that this protein may be a cytokinin sequestering agent rather than a receptor.

The particulate cytokinin-binding fractions reported in tobacco and moss have so far not been greatly studied. Sussman and Kende reported two sites in tobacco, one of which showed specificity towards active cytokinins, was heat labile and, due to its low concentration, was only detectable with a radiolabel of high specific activity. A similar binding fraction was reported by Gardner et al. from moss, which also appeared to be specific for active cytokinins and was heat labile. However, this binding activity was not purified further to give a straight line on a Scatchard plot.

Detecting high affinity binding sites

Most plant hormone binding assays are performed using commercially available radiolabelled plant hormones, which have a fairly low specific activity. Using these radiolabelled compounds one is able to detect binding fractions which are relatively abundant, such as the auxin binding proteins in corn coleoptile membranes and the cytokinin-binding proteins associated with the ribosomes in wheat germ and tobacco. However, to detect plant hormone binding sites which are less abundant, such as the particulate sites in tobacco and moss, a plant hormone of much higher specific activity is required. A major problem in trying to detect a small number of high affinity sites is that the high background of less specific or non-specific binding sites produces Scatchard plots which are curved rather than linear. This makes the identification of high affinity sites difficult and means that the amount of unlabelled ligand required to displace the radiolabel is much greater than would be required for a purified binding site.

Even detecting and purifying a binding site which has a high affinity for the active hormones only, does not prove that the binding site is a receptor. For such a binding site to be identified as a receptor, binding of the plant hormone has to be shown to initiate a biochemical process which brings about the responses assigned to that particular plant hormone. Few of the reports of plant hormone binding have shown a link. Roy and Biswas (1977) reported an auxin-binding protein in coconut which promoted transcription. Linde et al. (1984) have shown that a soluble auxin binding protein, from tobacco callus, stimulates IAA-dependent RNA sythesis in vitro. The reports of cytokinin-binding to a protein which binds to a subunit of the ribosome have suggested that this then directly affects translation of mRNA.

One method of showing the relevance of a plant hormone binding site is to use a mutant which is defective with respect to the receptor. The most useful mutant would be one that did not possess a receptor and so would not show hormone binding and which lacked the response to that plant hormone. Mutants that have a defective receptor could also be used, provided there was a loss of binding activity and a loss of the response to the hormone. Other mutants that did not respond to a particular plant hormone but had a defect after the hormone-receptor interaction could be useful in identifying part of the biochemical process involved in the response to the plant hormone.

Physcomitrella patens

The moss Physcomitrella patens is potentially a good organism to use for the identification of a hormone receptor as it is a relatively simple green plant with three distinct types of tissue, the differentiation of which is under the control of auxin and cytokinin. The life cycle of the moss Physcomitrella patens has been described in detail (Engel, 1968; Ashton, 1974; Ashton and Cove, 1977), and so will only be described briefly here.

The haploid spore of Physcomitrella patens, which is about 40μm in diameter, will germinate in 2-3 days at 24°C, under continuous white light of $15Wm^{-2}$. The filaments produced, the primary chloronemata, consist of highly-branched filaments which are 20μm in diameter and 120μm in length. These cells contain a large number of plump chloroplasts and have perpendicular cross walls. After about 7 days, some of the cells at the tips of the filaments grow with fewer, spindle-shaped chloroplasts, and have oblique cross walls. These cells are called the caulonemata. Older caulonemal cells can sometimes produce a brown pigment and can lack obvious chloroplasts. Side branching from a caulonemal filament can give rise to three types of tissue. Most of the side branches develop into filaments that are morphologically identical to the primary chloronemata. These are called the secondary chloronemata. A few of the side branches give rise to more caulonemal filaments. The other side branches develop into a leafy shoot called the gametophore. There is usually only one gametophore per caulonemal filament. If a 4-week-old culture is transferred to 15oC, antheridia and archegonia are produced

on the gametophore. Fertilization is facilitated by irrigating the culture with distilled water. After 4 to 5 weeks, the diploid sporophyte is produced. The capsules contain about 4,000 spores. Physcomitrella patens can be maintained in the gametophytic stage by removing a small piece of protonema and subculturing this on a fresh plate of medium. This will undergo regeneration through primary chloronemata.

Plant hormones and development of Physcomitrella patens

Cytokinin has been known to induce bud formation in mosses for some time (Gorton and Eakin, 1957). The first report of a cytokinin being identified from moss tissue was the isolation of N^6-(Δ^2-isopentenyl)adenine from culture medium of callus cells of the moss hybrid Funaria hygrometrica x Physcomitricum piriforme (Beutelmann and Bauer, 1977). One useful feature of Physcomitrella patens is that the isolation of mutants is relatively easy and can be achieved by treating either spores or tissue with a chemical mutagen (Ashton and Cove, 1977). As mosses are haploid there is no masking of recessive mutant alleles. Mutants of Physcomitrella patens have been isolated and used to study the role of the two plant hormones, auxin and cytokinin, in the development of the moss (Ashton et al., 1979b). Ashton et al. describe the effect of auxin and cytokinin on Physcomitrella patens and the isolation of mutants that are resistant to exogenous auxin and cytokinin. Exogenous auxin is slightly inhibitory at 2.5-5.0μM. At this concentration, auxin causes inhibition of chloronemata but promotion of caulonemata. Auxin also slightly inhibits gametophore production but increases the number of rhizoids produced by any one gametophore.

Cytokinins have the effect of suppressing the production of both chloronemata and caulonemata. At 50-500nM, the gametophores are abnormal, the leaves are smaller and arise directly from a callus-like bud. At higher concentrations, almost every branch site develops into an abnormal bud.

Mutants that have an abnormal response to exogenous auxin and cytokinin are also described by Ashton et al. (1979b). Category 1 mutants produce only tightly packed chloronemata, are unaffected by exogenous auxin and will only produce a few abnormal buds if treated with cytokinin. These buds arise from the primary chloronemata. Category 2 mutants produce both chloronemata and caulonemata, bud do not produce gametophores. They can be repaired by 5nM-50μM BAP. Category 3 mutants are morphologically normal and respond to exogenous BAP. However, they are resistant, or less sensitive than wild type to exogenous NAA. Category 4 mutants produce mainly chloronemata and a few caulonemal type filaments but no gametophores. Ashton et al. suggest from these findings that category 1 mutants are blocked in the differentiation from chloronemata to caulonemata. Category 2 mutants, which are repaired by cytokinin, appear to be unable to produce normal levels of cytokinin. Category 3 mutants appear to be blocked in the uptake of auxin, while category 4 mutants appear to be affected in the synthesis of auxin. When a wild type culture is grown under drip-feed, all the tissue is chloronemal (Ashton et al., 1979a). The morphology of such a culture resembles that of category 1 mutants. Wild-type cultures can be induced to differentiate under drip-feed by supplying auxin and cytokinin in the drip-feed medium (Cove, 1984). When the drip-feed medium contains 10μM NAA, caulonemata are produced.

This is the phenotype of category 2 mutants. When supplied with 10μM BAP, a wild-type culture is unaffected. This concentration of BAP causes the production of a large amounts of abnormal buds on solid medium. When both NAA and BAP are supplied at 10μM, over-production of buds occurs. From the effect of auxin and cytokinin on the morphology of category 1 to 4 mutants, and the effect on wild type under drip-feed, it has been concluded that auxin is required for the differentiation of primary chloronemata into caulonemata, and that the target cells for cytokinin action are the caulonemata, cytokinin inducing the production of the gametophores. This is further supported by some mutants which have the morphology of the wild type grown on cytokinin. Under these conditions the wild type over-produces buds and hence the mutants have been termed ove's (Ashton et al., 1979a). There are several mechanisms that could give rise to these ove mutants (Ashton et al. 1979a):

a) an increase in synthesis of cytokinin by the normal or a de novo pathway;

b) a decrease in the degradation of cytokinin;

c) the production of a cytokinin of increased biological activity;

d) an increase in sensitivity to cytokinin;

e) a decrease in a cytokinin antagonist;

f) the loss of requirement for cytokinin for bud induction.

All of the ove mutants studied so far have been shown to over-produce cytokinin (Wang et al., 1980; 1981b). Two cytokinins have been identified. These are N^6-(Δ^2-isopentenyl) adenine and zeatin.

From the results mentioned above, Physcomitrella patens is a good organism for the study of plant hormone action, as both auxin and cytokinin appear to cause specific responses rather than helping to promote a variety of different responses, which is the case for many of higher plants (Skoog and Armstrong, 1970; Dodds and Hall, 1980). The gametophore production in the response to cytokinin occurs in a specific target tissue. If cytokinin is only required for the production of the gametophore in mosses, then any cytokinin receptor is likely to be present only in the target cells, the caulonemata. However, if cytokinin is also required for another process such as cell division, a receptor would be required in all dividing cells. This receptor could be the same or a different receptor from the one for gametophore production. Therefore any binding activity detected in moss tissue can be investigated by the use of mutants, such as category 1 mutants, to see if it is only present in the target tissue for bud induction or if it is also in other tissue. If more than one binding site is detected, they can be studied to see if they are both present in the target cells.

If cytokinin is only required for bud production, or if bud production has its own receptor, then it should be possible to obtain mutants that do not have this receptor, or have a receptor that is unable to bind cytokinin. Such mutants would be a subclass of mutants that would be able to produce caulonemal filaments, but would not produce any gametophores, even in the presence of exogenous cytokinin. Other such mutants would be ones that had a normal cytokinin receptor but were blocked in a biochemical process involved in the initiation of the gametophore.

Once a hormone has bound to a receptor, a biochemical process has to be initiated which results in the response attributed to that hormone. When the receptor in a sensitive moss caulonemal cell binds cytokinin, it has to induce a biochemical process to produce a gametophore. There have been a number of reports of calcium being involved with the action of cytokinin. The level of calcium has been reported to enhance the cytokinin response in delaying senescence in corn leaf discs (Poovaiah and Leopold, 1973), stimulation of ethylene production in mung bean (Lau and Yang, 1975) and bud induction in the moss Funaria hygrometrica (Saunders and Hepler, 1982).

Calcium gradients can be visualised with chlorotetracycline-fluorescence (CTC). Using CTC, Reiss and Herth (1979) have shown that there is a calcium gradient in the caulonemal tip cells of Funaria hygrometrica. This polarity is only present in the tip cell of caulonemata (Reiss and Herth, 1979; Saunders and Hepler, 1981). Saunders and Hepler (1981) also showed that there is bright fluorescence 12 hours after treating a filament with BAP. This fluorescence remains in the dividing bud. Saunders and Hepler (1982) used the calcium ionophore A23187 (Reed and Lardy, 1972) to induce the initial asymmetrical division of bud induction on caulonemata in the absence of cytokinin. They suggested that one action of cytokinin was to increase the level of intracellular calcium which may be a second messenger for cytokinin-induced bud initiation. If calcium levels play an important role in the mechanism of cytokinin action, then calmodulin is likely to be involved in the regulation of the free calcium in the cell. This increase of intracellular calcium may involve the disruption of microtubules in the early stages of

bud formation (Doonan, 1983). Inhibition of cytokinin-induced bud production in Funaria hygrometrica has been demonstrated by calmodulin inhibitors (Saunders and Hepler, 1984), supporting the role of calcium in bud induction. One report (Elliot, 1983) shows inhibition of the cytokinin-regulated response, and induction of betacyanin in Amaranthus tricolor in the dark, by calmodulin-binding compounds. Using the moss Funaria hygrometrica, Bopp (1984) reported that an isolated caulonemal cell can be induced by kinetin to undergo morphogenesis (from filament to bud and reverse) without undergoing cell division. Bopp also showed that the antiauxin, parachlorophenoxyisobutyric acid (PCIB), inhibits kinetin-accelerated cell division, but not bud formation. Due to these two observations, Bopp (1984) suggested that the asymmetric cell division reported by Saunders and Hepler (1982) is not a replacement for cytokinin and that the conclusion drawn by Saunders and Hepler that calcium influx is a primary effect of cytokinin cannot be made from their experiments.

Light and moss development

Physcomitrella patens is a good model organism with which to study other aspects in the mode of action of cytokinins. Robbins (1918) reported that the moss Ceratodon purpureus could assimilate organic carbon in the dark. Robbins showed that if supplied with glucose, the moss would produce reddish-brown protonema, but no buds were produced. Robbins concluded that light was necessary for bud production. Fries (1945) showed that bud primordia initiated in the light will continue to develop in the dark. Mitra et al. (1959) showed that the quality of light affected development in Pohlia nutans. A bud would develop in

white light or red light (580-700nm). With blue light (400-530nm), protonemal growth was normal, but no buds developed. With green light (460-600nm) there was no differentiation: all the filaments were caulonemal in appearance, similar to growth in the dark. The poor growth is probably due to poor photosynthesis under green light.

Unlike Physcomitrella patens, a caulonemal filament of Physcomitrium turbinatum does not undergo dedifferentiation when isolated (Nebel and Naylor, 1968a). Nebel and Naylor (1968b) showed that temperature and light intensity both played a role in the initiation of buds in Physcomitricum turbinatum. At 16°or 26°C caulonemal filaments take 19 days before a bud starts to develop with 30ft-c red light. As the light intensity is increased, the time taken before the bud starts to develop is reduced. Increasing the light intensity above 60ft-c does not reduce the time taken (13 days) before a bud starts to develop. At 26°C, the time taken for a bud to start to develop is reduced to 6 days as the light intensity is increased to 240ft-c. Higher light intensities do not reduce the time any further. Nebel and Naylor also showed that at 16°C the total duration of red light required over the 13 days was only 144 hours which is the same as that required at 26°C. This indicates that there are two processes involved. One process is temperature-dependent but occurs in the dark, the other process is dependent on the total amount of light received. Nebel and Naylor (1964) have also described a similar photoperiod response in the moss Physcomitrium pyriforme. With this moss, bud induction can be prevented by exposing the tissue to far-red light before it has been exposed to a total of 150 hours of red light at 240ft-c.

This indicates that phytochrome is involved in the initiation of gametophores.

Evidence of the role of phytochrome in bud initiation has also been shown in the moss Funaria hygrometrica (Simon and Neaf, 1981). They show that bud induction in the presence of kinetin is dependent on the exposure to red light, and that the effect is partially reversed by far-red light. Simon and Neaf show that growth, measured as increase in dry weight, in blue light and red light is the same as in white light, but that bud induction does not occur in blue light. This supports the evidence given by Jahn (1964) that under blue light an inhibitor of bud initiation is produced and that buds can be produced under blue light if the culture is transferred onto fresh medium to prevent the build up of the inhibitor. Bud induction in the absence of light in the moss Funaria hygrometrica has been reported (Chopra and Gupta, 1967). Buds were induced by kinetin and sucrose. Chopra and Gupta concluded that the non-photosynthetic light requirement was replaced by kinetin.

In the formation of a bud by cytokinin in Physcomitrella patens, there is a light requirement. In the absence of light, even in the presence of exogenous cytokinin, no buds will develop (Cove et al., 1978). The light is required only for the initiation of the bud. Once the bud has been initiated in the light, it will continue to grow in the dark but the stem will be etiolated and the leaves grow as small scales. One hour of exposure to red light (6mmol quanta $m^{-2}s^{-1}$ of 660nm) causes normal gametophore development. However, if the red light is followed by 15 minutes of far-red light (715nm), the gametophores develop as though they

are in the dark. Cove et al. (1978), argued that this indicated that phytochrome is involved in the mode of action of cytokinin. The interaction of light, particul arly red and far-red light and cytokinin, can be studied in more detail in the initiation of buds.

In the following work, both the mode and the mechanism of action of cytokinin are investigated in the moss Physcomitrella patens. The mechanism of action is studied by competition assays using a radiolabel to detect a possible receptor in various crude preparations of wild-type moss tissue. Characteristics of the cytokinin-binding activity are investigated. The cytokinin-binding activity is also studied in various mutants affected in bud production. The mode of action is investigated by studying the quantity and quality of light required for bud induction on cultures treated with cytokinin. The role of phytochrome is also investigated in the initiation of the gametophore. The cytokinin production of a number of mutants was investigated, in particular ove 409, which was found to be temperature-sensitive with respect to cytokinin over-production. This mutant was studied genetically by protoplast fusion.

CHAPTER II

Materials and Methods

1. Culture Conditions

Physcomitrella patens cultures were grown on a solid or liquid modified Knops medium[1] (Wang, personal communication). The standard medium contained the following:

$Ca(NO_3)_2 \cdot 4H_2O$	59mg/l
$MgSO_4 \cdot 7H_2O$	250mg/l
KH_2PO_4	250mg/l
$FeSO_4 \cdot 7H_2O$	10mg/l
KNO_3	1.036g/l
TES	1ml/l

Final concentration of trace elements (TES)

H_3BO_3	614µg/l
$MnCl_2 4H_2O$	389µg/l
$NiCl_2 6H_2O$	59µg/l
$Al_2(SO_3)_3 K_2SO_4 24H_2O$	55µg/l
$CoCl_2 6H_2O$	55µg/l
$CuSo_4 5H_2O$	55µg/l
$ZnSO_4 7H_2O$	55µg/l
KBr	28µg/l
LiCl	28µg/l
$SnCl_2 2H_2O$	28µg/l
KI	28µg/l

Modifications

a) Nitrogen source

Unless tissue was being grown for caulonemata or gametophore production, ammonium tartrate (920mg/l) was included in the medium to promote the production of chloronemata.

b) Carbon source

When cultures were grown in the dark, the medium was supplemented with 0.5% (w/v) sucrose as a carbon source. Other carbon sources at various concentrations were also tried. These were glucose, myo-inositol and acetate.

c) Supplements

These were usually added to all media, except where prototrophs were being selected. Adenine was only added to the medium of those strains which were specifically adenine-requiring.

p-amino benzoic acid	250µg/l
nicotinic acid	1mg/l
thiamine-HCl	100µg/l
adenine	67.5mg/l

d) Mannitol medium

Mannitol was included in the medium as an osmoticum for the regeneration of protoplasts. The concentration of the mannitol was 60g/l.

e) "Starvation" medium

If the level of phosphate is reduced, with or without reducing the nitrate, in the standard Knops medium chloronemal growth is greatly reduced or stopped. This was done to enrich for caulonemata. Three starvation media were used:

(i) KH_2PO_4 was omitted and replaced by 137mg/l KCl

(ii) KH_2PO_4 was reduced to 12.5mg/l

(iii) KH_2PO_4 was reduced to 12.5mg/l and the KNO_3 was omitted.

f) Solid medium

The medium was solidified with 12g/l oxoid agar no. 1 when solid medium was required.

The medium was adjusted to pH 6.5 with NaOH, solid medium was dispensed into bottles in 400ml lots and autoclaved at 15 psi for 20 minutes. Ten and 15 litre batch culture vessels were autoclaved at 15 psi for 1 hour.

Axenic cultures were maintained as spot inocula on minimal Knops medium containing supplements. Larger amounts of tissue were obtained by growing the moss as an homogenate on medium overlaid with cellophane so as to prevent the filaments growing into the medium. Tissue was homogenised in an M.S.E. homogeniser for 10-15 seconds in sterile distilled water, and three millilitres of the suspension put on each plate which had been overlaid with a cellophane disc. This method of culture for Physcomitrella patens is a modification of that of Grimsley et al., (1977a). Tissue was grown in liquid culture either in conical flasks or large batch cultures that were aerated with air

sterilised by two miniature in-line filters (IF 32, Microflow, Dent and Hellyer, Walsorth Road, Andover, Hampshire). Flasks containing from 15ml to 1 litre of medium were kept on an orbital shaker rotating at between 60-100 rpm. Batch cultures of 10 and 15 litres were used for the production of large amounts of tissue or for cytokinin production. The 10 litre vessels were fitted with a condenser (to reduce water loss) and a collecting vessel so that samples could be taken to examine and measure growth rate. Growth rate was measured by weighing a Whatman 2.5cm GF/A filter wrapped in foil that had been dried in an oven at 80°C for at least 2 days. A known volume of culture was collected on the filter, which was then reweighed after being dried in the oven for 2 days (Wang et al., 1981b).

Cultures were routinely grown at 24°C under 10-15Wm^{-2} white light provided by fluorescent tubes (Ashton and Cove, 1977). For the induction of gametogenesis, cultures were transferred to 15°C. When required, a temperature gradient was obtained by heating one end of an aluminium block (20x50x1000mm) using a small thermostatically controlled refrigerator heater. This was placed in a constant temperature room at 10°C. Cultures were placed in 60ml specimen containers containing 20ml of Knops medium. The ridge on the base of the jar was removed with glass paper so that there was direct contact of the jar with the aluminium bar. The jars were placed on the bar and left for 5 weeks for the culture to grow. Just before the jars were removed, the temperature of the agar near the culture was measured with a thermocouple. Using this bar a temperature range of 26 to 16°C was obtained.

2. Spore production

Spores can be produced either by selfing or by crossing two strains. The strains to be crossed were inoculated next to each other in a 60ml specimen jar containing 40ml of Knops medium with the appropiate vitamins. Strains to be selfed were treated in the same way but only one inoculation was done. The cultures were grown at 24°C for 4 weeks. They were then transferred to 15°C for 4 weeks to induce the production of the antheridia and archegonia. The cultures were then irrigated with 10ml of a 20-fold concentrated solution of the vitamins that were required by those strains, as they will not produce viable capsules when irrigated with sterile distilled water (Courtice et al., 1978). For most strains being selfed, capsules were produced about 6 weeks after irrigation. The crosses were re-irrigated if no capsules were visible. When capsules were produced, they were removed from the culture, squashed in 1/2ml of sterile distilled water per capsule and stored at 4°C until required.

3. Protoplast isolation

Protoplasts were normally isolated enzymatically, as described by Grimsley et al. (1977a). Tissue was grown as an homogenate for 5-7 days on ammonium tartrate plates overlaid with cellophanes. Up to 5 plates of tissue were added to 10ml of 2%(w/v) Driselase (Sigma). The Driselase solution was made by dissolving 0.4g of Driselase in 20ml of protoplast wash (PW), which contained 80g/l mannitol. This was then acidified to pH5.5 and, if necessary, centrifuged at 3,000g (4500 rpm in an M.S.E.

Minor) for 5 minutes. The supernatant was filter-sterilized through a Millipore nitrocellulose 0.45 µm filter (Millipore U.K. Ltd., Middlesex) and added to the tissue. The mixture was incubated for 30 to 45 minutes at 20°C and then debris removed by passing the suspension through a 50µm mesh. The Driselase solution was removed by decanting after centrifuging the protoplasts at 100g (700 rpm in an M.S.E. minor) for 3-5 minutes. The protoplasts were rinsed twice by resuspending in protoplast wash and recentrifuging.

Other enzymes were used when attempts were made to obtain protoplasts from dark-grown caulonemal filaments, as Driselase was found to be ineffective. These were:

(a) cellulase and pectinase;

(b) cellulase, pectinase and rhomant P;

(c) meiselase;

(d) driselase and chitinase.

These enzymes were made up in a 5mM morpholinoethane sulphonic acid buffer at the optimum pH for the enzyme mixture.

4. Protoplast regeneration

Protoplast regeneration was on the normal Knops medium containing 60g/l mannitol, plus vitamins if required (see Chapter II section 1), and cellophane overlays. 1ml of protoplast suspension was added to 9ml of Knops medium containing 80g/l mannitol and 0.6%(w/v) agar. The soft agar was poured onto the cellophaned mannitol plates. Protoplasts were then regenerated under high intensity white light for 5 days. The cellophane and top layer were transferred to ammonium tartrate medium without

mannitol (as mannitol reduces the rate of growth) once the protoplasts had regenerated. The regenerated plants were then picked off when they had grown to about the ten-cell stage.

5. Protoplast fusion

The method of protoplast fusion was based on the method described by Grimsley et al. (1977b). The two strains to be fused had to have different vitamin requirements so that prototrophic hybrids could be selected. Protoplasts of the two strains to be fused were prepared as described above and resuspended in calcium protoplast wash (CaPW) containing 50 mM $CaCl_2$ and 80g/l mannitol. A proportion of each protoplast preparation was mixed together, centrifuged at 100g for 3-5 minutes and the supernatant removed. The protoplasts were left in 250µl CaPW. Protoplast fusion was induced by adding 750µl poly-ethylene glycol (600g/l PEG in 50mM $CaCl_2$). Fusion was allowed to take place for 40 minutes. After this time 1.5ml of CaPW was added. Ten minutes later, 10ml CaPW was added and another 10ml 10 minutes after this. After a further 10 minutes, the protoplasts were harvested by centrifugation at 100g for 3-5 minutes and resuspended in CaPW. The protoplasts were plated out to regenerate, as described above, on mannitol medium without vitamins to select for hybrids.

6. Hormone treatments and bud counts

Hormone treatments were performed on both protoplasts and homogenates. Protoplasts were prepared as described above and regenerated in a 5x5 repli-dish with auxin and cytokinin included in the medium. The concentration of the hormones, NAA and BAP, were 0, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M. The concentration of the agar was only 0.4% (w/v). This allowed the protoplasts to sink to the bottom of the wells. The dish could then be inverted so that protoplasts could be examined under the microscope.

For the treatments using homogenate tissue, 3 ml of wild-type homogenate was added to 17ml of Knops medium in a 100ml conical flask and grown for 7 days on an orbital shaker. The tissue was collected on a sterile 2.5 cm glass fibre filter (Whatman G/F-A). The tissue was then added to a flask containing 20 ml of fresh medium with 1 μM BAP. After the time of exposure to cytokinin the medium was again collected on a glass fibre filter, washed with 600ml sterile distilled water and added to fresh medium without cytokinin. After 2 weeks, the number of buds in each flask was chrome-counted. The chrome-counting was performed using 1ml of tissue, adding 3ml of 10% chromic acid, homogenising for 10 seconds in an M.S.E. homogeniser and adding another 3ml of water. The homogenate was used to fill slides to a depth of 1.4mm. Ten slides were made from each flask and buds were counted in 10 fields of view under the low power of a microscope. The diameter of each field of view was 1.75mm.

7. Mutant Isolation

The mutagen used was N-methyl-N′-nitro-N-nitrosoguanidine (NTG). Two methods of mutagenesis were used.

a) Spore mutagenesis

Spores were harvested by picking off mature capsules which were then crushed with a glass rod in 1/2 ml of sterile distilled water per capsule. The spores were centrifuged and resuspended in 10ml Tris-maleate buffer (6g/l Tris-(hydroxymethyl)-aminomethane and 6g/l maleic acid) pH 6.0. To this was added 1mg NTG, and the mixture incubated for 45 minutes with intermittent shaking. The spores were washed three times in sterile distilled water. The spore suspension was stored at $4^{o}C$ and plated out, when required, to produce about 200 surviving spores per 9cm petri dish, after testing the survival rate.

b) Somatic mutagenesis

This method was used for strains that were sterile and so could not produce spores. Tissue from up to 5 homogenate plates was added to 10ml Tris-maleate buffer containing 1mg NTG as in spore mutagenesis but only incubated for 30 minutes as protonema is more susceptible to NTG than are spores. The tissue was then collected on a 50µm mesh and washed with about 200ml sterile distilled water. Protoplasts were prepared from the mutagenised tissue and allowed to regenerate as described in Chapter II, sections 3 and 4 above. For screening, the plants were then

picked off and placed on Knops medium containing ammonium tartrate and vitamins.

8. Mutant screening

Young plants derived from NTG treated spores or protoplasts, as described above, were picked off and tested for their inability to produce buds even in the presence of exogenous cytokinin. The young plants were inoculated onto Knops medium supplemented with vitamins and sucrose. Each 9cm petri dish was inoculated with 16 mutants for testing. These were grown for 7 days under continuous white light of 10-15 Wm^{-2}. The plates were then transferred to the dark and placed on their edges for 14 days. This produced caulonemal filaments which had grown negatively geotropically along the surface of the agar. The plates were then returned to white light, with the plate horizontal, and 2.5 ml of 20 μM BAP added to each plate so as to give a final concentration of 1 μM. After three days the plates were screened for mutants that produced dark-grown caulonemata but did not have any abnormal buds on the filaments.

9. Extraction of endogenous cytokinins

a) Isolation

All solvents used in the extraction of cytokinins were redistilled before use. Cytokinins were usually extracted from the liquid culture medium from a 10-litre batch culture. The tissue was separated from the medium by filtration. The medium was then dried down in a rotary evaporator to about 200-300ml. It was

then adjusted to pH8 with NaOH and partitioned 5 times against an equal volume of water-saturated n-butanol. The bulked butanol phases were dried down and redissolved in 2ml of 35% ethanol. Undissolved salts were removed by centrifugation. The sample was injected onto a Sephadex LH20 column (Pharmacia Ltd., Milton Keynes), (Wang et al., 1980) that had been equilibrated for 2 days using 35% ethanol (v/v) as the solvent. The column was eluted with 35% ethanol at a flow rate of 30ml/h and 50x30ml fractions were collected for assay.

A portion of each fraction was then used in a bioassay for cytokinin before the relevant fractions were combined for further analysis by HPLC and GCMS. If the medium was clean enough, such as from the one-litre cultures described in Chapter III section 3, the LH20 step was omitted and the samples were further purified on HPLC before being analysed by mass spectrometry.

b) Bioassay

Two bioassays were used for the assay of cytokinin. These were the Amaranthus bioassay as described by Biddington and Thomas (1973) and the soya bioassay as described by Wang et al. (1980).

(i) Amaranthus bioassay

Aliquots of the fractions to be assayed were dried down onto a Whatman no. 1 filter paper in a 20 ml scintillation vial. Amaranthus caudatus (catalogue no 1657, Samuel Dobie and Son Ltd., Llangollen) seeds were germinated for three days in total

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darkness on a filter paper in a 9cm petri dish with 4ml of distilled water. The buffer for the assay contained 0.71g/l Na_2HPO_4 and 0.68g/l KH_2PO_4 (pH6.8). Before use, 1mg/ml tyrosine was dissolved in the buffer by heating to 100°C for 15-20 minutes In dim light, 200µl of buffer was added to each vial and 10 Amaranthus seedlings with seed coat and root removed, were placed on the filter paper. Controls were also set up using 0, 1, 10, 100, and 1000ng BAP dried onto the filters in place of aliquots of the Sephadex fractions. The vials were placed in a glass casserole dish on moist paper towels. The dish was incubated in total darkness at 25°C for 24 hours. Four ml of distilled water was then added to each vial and the vial freeze-thawed three times to extract the betacyanins. The absorbance of the solution in the vial was measured at 542 and 620 nm on a Pye Unicam SP8-500 UV/VIS spectrophotometer. The difference of these two readings was then calculated and values in cytokinin-equivalents calculated for each fraction by reference to the BAP standard curve.

(ii) Soya bioassay

The soya bioassay was a modified version of that used by Miller (1968). Soya callus was maintained on the following medium

KH_2PO_4	300mg/l
KNO_3	1g/l
NH_4NO_3	1g/l
$Ca(NO_3)_2.4H_2O$	500mg/l
$MgSO_4.7H_2O$	71.5mg/l
$MnSO_4$	14mg/l
KCl	65mg/l
Na-Fe EDTA	13.2mg/l
$ZnSO_4.7H_2O$	3.8mg/l
H_3BO_3	1.6mg/l
KI	0.8mg/l
$Cu(NO_3)_2.3H_2O$	0.35mg/l
$(NH_4)_6Mo_2O_{24}.4H_2O$	0.1mg/l
myo-inositol	100mg/l
nicotinic acid	1mg/l
pyridoxine-HCl	0.4mg/l
thiamine-HCl	0.4mg/l
sucrose	30g/l
NAA	2mg/l
BAP	1mg/l

This medium was adjusted to pH5.8 and solidified with 10g/l agar. The soya callus was subcultured every two weeks, and maintained in the dark at 24oC.

For bioassay, the portion of the LH20 fraction to be assayed was dried down in a 5cm deep, 7cm diameter Pyrex crystallizing dish with lid. Standard dishes contained 0, 10, 20, 50, and 100µg/l BAP. To this was added 40ml of the above medium with the BAP omitted. The dishes were then autoclaved at 15 psi for 20 minutes. When cool, 10 x ca. 10mg pieces of soya callus were placed in each dish and incubated in dim light at 24°C for 3 weeks. The pieces were then weighed on an electronic balance connected to an Apple micro-computer. The mean weight in mg was calculated and plotted against fraction number.

c) High Performance Liquid Chromatography

HPLC was carried out using a LDC dual pump chromatograph (Laboratory Data Control, Stone, Staffs.) fitted with a Gradient Master controller. Gradients between water (pH 7 with triethylammonium bicarbonate) and acetonitrile were used for separating cytokinins on columns of Hypersil ODS (Shandon Southern Ltd., Runcorn, Cheshire). Fractions were collected as appropriate and used for analysis by mass spectrometry.

d) Mass Spectrometry

Mass spectrometry was carried out in collaboration with Dr. T.L. Wang and Mr. J. Eagles (Food Research Institute, Norwich) using either an AEI MS 30 dual beam or a Kratos MS 80 mass spectrometer. Low resolution spectra were produced by direct probe insertion of the samples.

Combined gas chromatography-mass spectrometry multiple-ion-

monitoring (GCMS MIM) was performed using permethyl derivatives as previously described (Wang et al., 1981b). The column temperature used was 225°C for 2iP and 230°C for zeatin. For quantification, ions at m/e 262 (trimethyl-^{14}N-zeatin), 266 (trimethyl-^{15}N-zeatin), 232 (dimethyl-^{14}N-2iP) and 236 (dimethyl-^{15}N-2iP) were monitored. Levels of endogenous cytokinins were calculated by determining the $^{14}N/^{15}N$ ratio and referring to a standard curve (Scott and Horgan, 1980).

10. Binding assays

The methods for the assay of a cytokinin receptor were derived from the method of Gardner et al. (1978). The tissue for cytokinin work was grown on medium containing calcium, and potassium nitrate as the sole nitrogen source, so as to promote the production of caulonemal filaments, the target cells for the cytokinin in development. Tissue was grown from an homogenate either on cellophaned plates or in 15 litre liquid culture. All tissue was grown for 14 days before use. Some liquid cultures were grown on low levels of phospate or low phosphate and nitrate so as to increase the percentage of caulonemal filaments which had side branch initials.

a) Extraction procedure

Throughout the extraction procedure and the assay of cytokinin binding, the extract was maintained at 0-4°C. Two extraction methods were used.

(i) Homogenisation using fresh material

The tissue was added to 4 volumes (w/v) of MES1 buffer, pH 6.4 (25mM 2-(N-morpholino) ethane sulphonic acid, 0.25M sucrose, 1mM $Na_2S_2O_5$ or $K_2S_2O_5$). In earlier experiments, the tissue was homogenised in an M.S.E. homogeniser and then hand homogenised in a glass homogeniser or a motorised teflon and glass homogeniser. In later experiments, the tissue, after being ground in the M.S.E. homogeniser, was homogenised in a glass bead homogeniser

(B. Braun, Melsungen AG), or a Bead-Beater (Biospec Products, P.O. Box 722, Bartlesville, Oklahoma 74003). The glass bead homogenisers enabled more tissue to be used. The homogenate was then filtered through 1 layer of Miracloth or 8 layers of cheese-cloth. If the Bead-beater had been used, the residue was re-homogenised in the Braun homogeniser and refiltered.

The filtrate was centrifuged at 4,000g (3,000 rpm in an M.S.E. 6 x 300ml rotor), or at 13,000g (9,000 rpm in a Sorval GSA rotor or 10,000 rpm in an M.S.E. 8 x 50ml rotor) for 30 minutes. The supernatant was centrifuged at 80,000g (26,000 rpm in a Beckman type 30 rotor or an M.S.E. 8 x 50ml rotor) for 30 minutes. The pellet was removed and resuspended in a small glass homogeniser in about 30ml of MES2 buffer pH 6.4 (25mM MES, 0.25M sucrose). This was then recentrifuged at 80,000g for 30 minutes and resuspended, in an appropiate volume for the binding assay (6-12ml), in MES2 buffer.

(ii) Homogenisation using frozen material

The tissue was frozen in liquid nitrogen and ground with a mortar and pestle. The tissue was kept frozen throughout the grinding. It was then taken up in MES1. Unless the 13,000g supernatant was going to be used, the tissue was suspended in 4 volumes (w/v) of MES1; otherwise it was suspended in only a small volume of MES1. The extract was the centrifuged in the same way as for the homogenised tissue.

b) Solubilisation of the cytokinin binding fraction

If the pellet was to be solubilised, it was treated for solubilisation without being resuspended first. Two methods were used to try to solubilise the cytokinin-binding fraction.

(i) Acetone method

This was the method described by Venis (1977a). The pellet was resuspended in 0.1 volume (based on original fresh weight of tissue) in MES2. The suspension was then added to 20-fold volume of Analar acetone at 4°C. The clumped precipitate was collected by low speed centrifugation,washed with acetone and dried in a stream of nitrogen. This pellet was re-extracted in 12ml of MES2, and then centrifuged at 38,000g (17,000 rpm in an M.S.E. 8 x 50 ml rotor) for 15 minutes. The pellet was resuspended in 5ml of MES2 and both the pellet and supernatant were used to assay for cytokinin-binding activity.

(ii) Triton method

This was the method of Thomas et al. (1984). The final pellet was resuspended by adding 1ml of 20% Triton X-100 in MES2. The resuspended pellet was homogenised in a glass hand-homogeniser and then mixed with 10ml of MES2. This was then centrifuged again at 80,000g for 30 minutes and the supernatant used in the cytokinin-binding assay.

c) Assay for cytokinin binding

Assays for cytokinin-binding activity were carried out using a radiolabelled benzylaminopurine (BAP). Earlier experiments

were done with a 13.4mCi/mmol ^{14}C-BAP or a 57mCi/mmol ^{14}C-BAP (Amersham International plc, Amersham, Buckinghamshire, UK.). Later experiments were performed with a tritiated cytokinin of a much higher specific activity, 12Ci/mmol, which was prepared as described in appendix 1. Displacement of the radiolabel was usually by BAP, but some assays were performed using zeatin and adenine as the displacement ligand. Two assay methods were used: a centrifugation assay, which could only be used on a particulate fraction, and an equilibrium dialysis method for particulate fractions and soluble fractions.

(i) Centrifugation assay

The assay was performed in 10ml polycarbonate centrifuge tubes. Each tube had 1ml of resuspended pellet, 1ml of MES2 containing the radiolabel and 1ml of MES2 containing the required amount of displacement ligand. For replicates, where possible, the mixture was made up in bulk and then divided into the replicate tubes. The tubes were then incubated on ice for between 0 and 24 hours. Most of the incubations were for 1 hour. The particulate fraction was recovered by centrifugation at 80,000g (31,000 rpm in a Beckman type 50 rotor or 32,000 rpm in an M.S.E. 10 x 10 ml rotor) for 30 minutes. The supernatant was then removed and the sides of the tube washed with 1ml of distilled water. After this treatment, the tube was the left upside down on a paper towel to drain for 10 minutes. The pellet was then resuspended for liquid scintillation counting.

(ii) Equilibrium dialysis

Equilibrium dialysis was performed in Teflon Dianorm cells, type Makrozellen GD 1S (M.S.E. Crawley, UK.). The membranes were either Visking tubing or Spectropor 2 (M.S.E. Crawley, UK.), which had a molecular weight cut-off of 10,000. Membrane discs of 46mm diameter were cut from the tubing. The membranes were soaked three times in distilled water for at least 30 minutes each, and then in MES2 three times for 15-30 minutes each. Stoppers were put into all the emptying holes of the cells. The membranes were then drained and streched across the lid of the dialysis cell. The base of the dialysis cell was subs equently placed on top, the cell turned over and placed in the carrier. 1 ml of the extract to be assayed was added to the left half (lid) of the cell, and 1/2ml of MES2 containing the radiolabel and 1/2ml of MES2 containing the displacement ligand in the right hand (base) half cell. All stoppers were replaced and the cells rotated at 12 rpm at $4^{o}C$ for 5 hours. The contents of each half cell were then drained into glass vials and a measured amount removed for scintillation counting.

d) Scintillation counting

Due to the chlorophyll content, the samples could not be counted directly. In earlier experiments, the green colour of the chlorophyll was removed by bleaching with 25µl of bleach (Boots sterilising solution, Boots PLC, Nottingham. or Domestos, Lever Bros.). Bleaching took 10-20 minutes. After this, a measured volume of the sample was put in a scintillation vial with 10ml of scintillant (1 litre toluene, 0.5 litre Triton X100,

4g PPO and 250mg POPOP). The bleach caused chemiluminescence, and the samples were left at $4^{o}C$ overnight before counting.

In later experiments, the samples were oxidized in a Harvey biological oxidizer OX300 (R.J. Harvey Instruments Corporation, Hillsdale, New Jersey) before counting. Initially the centrifugation pellets were transferred to the quartz boats by resuspending the pellets in 1ml of distilled water or a solution of 2% Triton X100. However, it was difficult to resuspend them in the water and the Triton was too volatile for the oxidizer. The most reliable method was to transfer the whole of the pellet in 3 x 1/2 ml aliquots of distilled water. This volume of water was too large for the oxidizer, and so the quartz boats were left in the fume cupboard overnight, for the samples to dry down, before being oxidized. Samples were oxidized for 3 minutes. The scintillant used in the oxidizer was tritium Cocktail (R.J. Harvey OX162) or Quickscint 1 (Zinsser Analytic (UK) Ltd.).

The samples were counted for 10 minutes in a Beckman LS 7500 or an LKB 1215 Rackbeta scintillation counter. Both machines were calibrated for the scintillant being used and for quench using the external standard ratio method.

11. Microscopy

Scanning electron microscopy was performed in collaboration with Dr. T.L. Wang and Mr.R. Turner (Food Research Institiute, Norwich) using a Philips scanning electron microscope equipped with a cryostage. Tissue was grown on Knops medium supplemented with vitamins for 4 weeks at the required temperature and

prepared for microscopy by freezing in a liquid nitrogen slush and coating with gold.

12. Light treatments

All treatments were performed using monochromatic light produced by interference filters (DAL type; Schott, Mainz. FRG). For earlier experiments, the monochromatic light apparatus was the same as described by Jenkins and Cove (1983). Illumination was provided by Aldis Tutor 2 projectors (Rank Audio Visual Ltd.,Brentford,UK), which were fitted with thyristor dimmer controls and 250W tungsten halogen bulbs (type M36; Thorn Lighting, London, UK). For later experiments the projectors were supplied by a regulated D.C. supply so that they were unaffected by mains fluctuations.

Deep (2cm), 5cm petri dishes, filled with 20ml Knops medium supplemented with sucrose, were inoculated with a small piece of wild-type protonema 1cm from the edge of the dish. These were grown under white light (10-15Wm^{-2}) for 8 days. The plates were then transferred to the dark, on their edges, with the plates orientated so that the inoculum was at the lowest point. The plates were incubated for either 14 or 21 days. This caused the growth of unbranched filaments that resembled caulonemata. These grew negatively geotropically. After this period the tips of the dark-grown filaments had grown into the centre of the dish. Under green (542nm monochromatic) "safe" light of about 2mWm^{-2}, hormone solutions were added to the plates. One ml of a 20μM solution was added, to give a final concentration of 1μM.

The plates were then put into individual cardboard boxes that had a 1 x 2 cm gap in the middle of one edge. The plates were orientated so that the dark-grown caulonemal filaments were perpendicular to the gap in the box. The plates were left for at least 1 hour for the solution to be absorbed by the agar. The first experiments were performed with horizontal illumination but when low light intensities were used the filaments did not bend so it was difficult to know which bud sites to count. The boxes were therefore placed on their edges under vertical illumination so that the dark-grown caulonemal filaments were perpendicular to the light direction. This caused the filaments to bend upwards, due either to a positive phototropic response, or, at lower light intensities, to a negative geotropic response. The light intensity was measured with a radiometer (type J16 with a J6512 probe; Tektronic U.K. Ltd., London,U.K.). The opening of the box was put into the position of the sensor of the probe, after the reading had been taken.

The plates were left under the monochromatic light for three days. They were scored by counting the number of buds produced at five cells before the bend, the last part of the caulonemal filament to grow in the dark before being transferred to the monochromatic light. Twenty filaments were counted in this way so as to give a total number of buds from 100 cells.

CHAPTER III

Isolation and analysis of mutants

Results and discussion

In the search for a cytokinin receptor, any fraction that is isolated from the wild-type moss which appears to bind cytokinin specifically has to be shown to be developmentally relevant. One method of achieving this is to study mutants that do not have a specific response to applied cytokinin. Some of these mutants may not possess a receptor that is able to bind cytokinin,and thus cannot respond. These mutants can be used to show that the loss of the specific binding of cytokinin is associated with the loss of the cytokinin response by comparison with the wild type.

The moss, Physcomitrella patens, is useful in this respect, as the induction and screening of mutants is relatively easy. Also, cytokinin plays an important role in the development of Physcomitrella patens. Cytokinin induces the production of the gametophore from a caulonemal side branch. Therefore, it may be possible to isolate mutants that are unable to produce gametophores in the presence of exogenous cytokinin. Some of these mutants may have a defective receptor, some no receptor at all and some may be blocked at a later stage in the production of the gametophore.

1. Isolation of cytokinin non-responding mutants

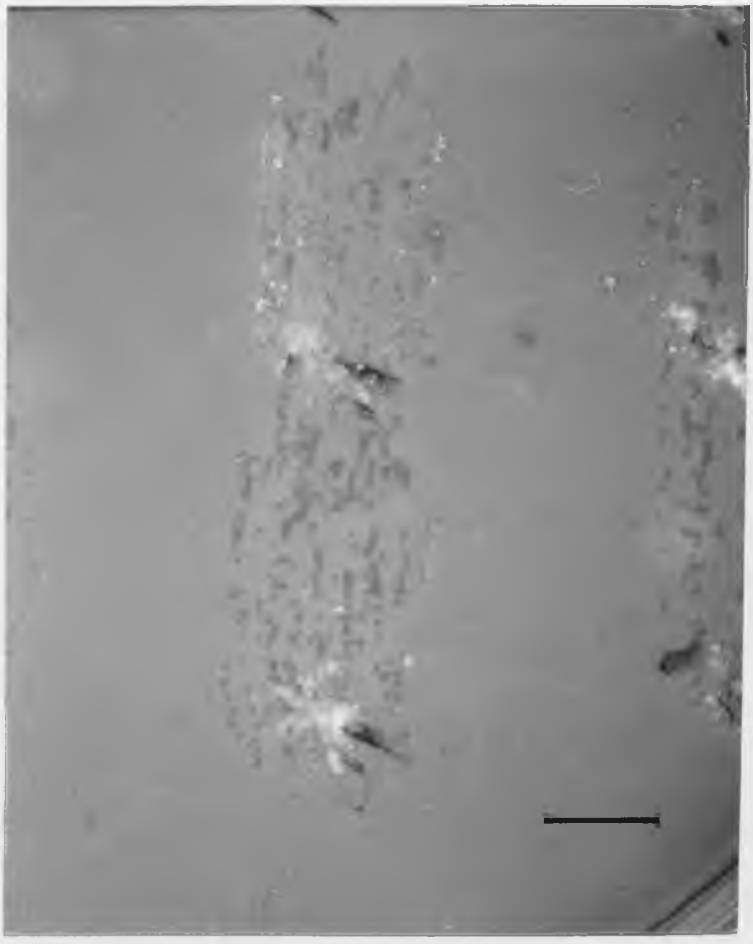
(i) Spore mutagenesis

Spores from pab A3 and thi A1 were treated with NTG and screened as described in Material and Methods. Of the 25,000 mutants picked off and tested in this way, none that produced dark-grown caulonemata failed to produce buds in the presence of 1 μM exogenous cytokinin. Typical colonies that have responded to the BAP are shown in figure 1. There are a number of possible explanations for not detecting any cytokinin non-responding mutants, viz:

a) insufficient mutants have been screened for the rate of occurrence of such mutants;
b) two genes need to be altered to make such mutants;
c) the receptor for cytokinin in bud induction is also required for an essential process such as cell division;
d) mutants that are unable to respond to cytokinin in the process of bud formation are also unable to produce dark-grown caulonemata.

From other mutation screening procedures, mutants are detected at a rate of about one in a thousand (Ashton et al., 1979b). Therefore screening 25,000 plants should produce some mutants of the type being sought. The lack of non-responding mutants is unlikely to be due to insufficient treatment with mutagen as only about 10% of the spores germinated and other types of mutant were isolated in the screening. If more than one gene has to be altered, due to there being replicate genes, then the rate of occurrence of such mutants would be

Figure 1. Mutant-screening plate.

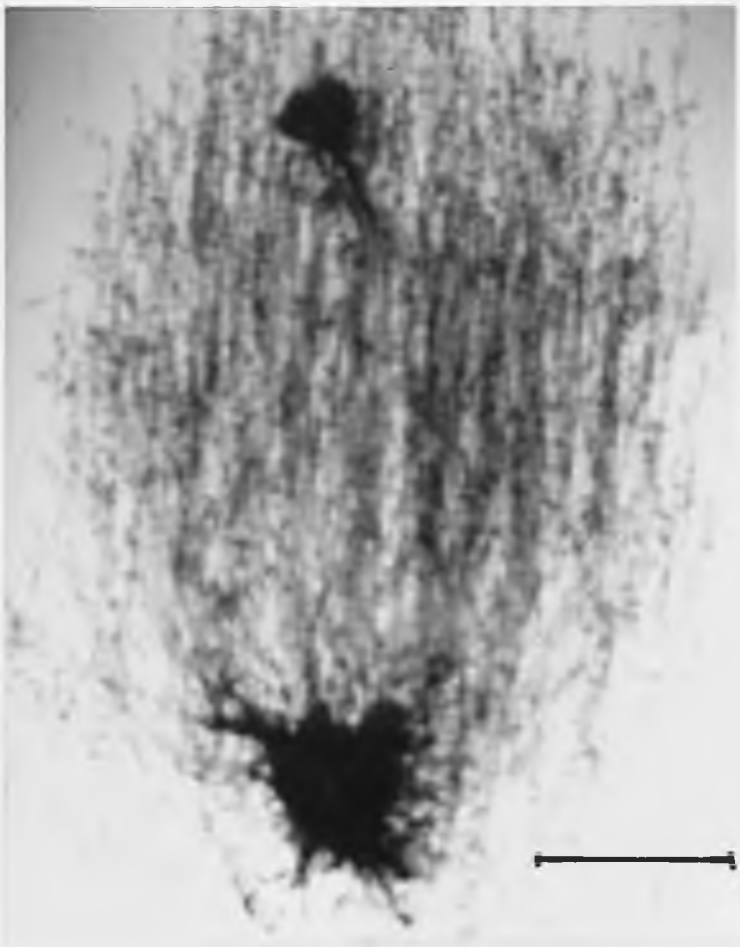


Mutant-screening for cytokinin non-responding mutants was performed on plants grown from NTG-treated spores. After 1 week's growth, the plants were left in the dark for 2 weeks, treated with 1μM BAP and then screened for bud production after 3 days under white light. (Bar=5mm).

greatly reduced. However, even if a double mutation event is required for the type of mutants being screened for, such double mutants could be expected to occur in the 25,000 mutants tested. Cytokinin may well be involved in an important process such as cell division, although at a lower concentration than for bud induction. If both processes are dependent on the same receptor, then a cytokinin non-responding mutant would be lethal. A common receptor that was also involved in the production of dark-grown caulonemata, although not lethal, would have been undetected by the screening procedure used. However, one mutant, bar 1, will produce a few dark-grown caulonemal filaments that are unable to to produce buds when treated with cytokinin. An interesting observation in support of the last possibility is that mutants that over-produce buds also produce more dark-grown caulonemata than wild type. Two mutants isolated, ove 408 and ove 409 were first suspected of being ove mutants because they produced much more dark-grown caulonemata than wild type.

However, other useful mutants were isolated from these mutation runs. As the screening procedure involved growth in the dark, geotropic mutants whose caulonemata lacked the response of dark-grown filaments were easily isolated. Some of these mutants were ageotropic, others were positively geotropic. Some mutants produced more dark-grown caulonemata than the wild type (figure 2). This is a characteristic of ove mutants, mutants that over-produce gametophores. Therefore, when the plates containing these mutants were transferred to the light, no exogenous cytokinin was added. These mutants, however, still produced a large number of abnormal buds. Two mutants in particular were extreme gametophore over-producers, and were designated ove 408

Figure 2. ove 409 grown in the dark.



Growth of ove 409 in the dark, showing more caulonemata than in wild type (compare with figure 1). (Bar=5mm).

and ove 409. These were held as stocks and as such were maintained at 15°C. After one month, the stock tube of ove 409 was observed to have produced some normal leafy gametophores. When this mutant was subcultured at 24°C, it again started to over-produce gametophores and so appeared to have a temperature-sensitive phenotype. This mutant, as with other ove mutants, may have arisen due to a number of factors which resulted in either too much cytokinin being produced or in the tissues being over-sensitive to normal levels of cytokinin. The levels of cytokinin in this mutant were therefore studied (see this chapter, section 3). ove 409 was also studied genetically (see this chapter, section 2).

(ii) Somatic mutagenesis

Somatic mutagenesis was attempted with ade 5, which requires adenine. This strain was used, in particular, since an ove mutant in an ade background would be useful in the study of cytokinin synthesis from radiolabelled adenine. About 10^6 protoplasts were prepared from NTG-treated tissue. None of the protoplasts regenerated. This was most likely due to the combination of high kill by NTG and to the fact that the level of adenine required for this strain is near the level that is toxic in the wild type. As this strain was difficult to regenerate from protoplasts, it was not used to isolate any further mutants.

2. Genetic analysis of ove 409

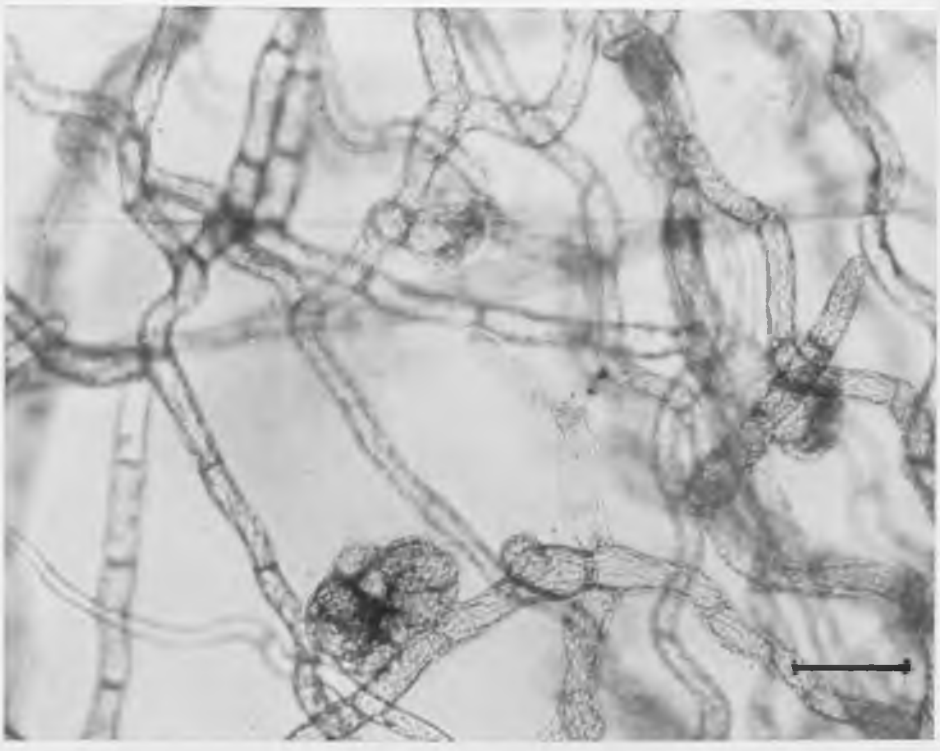
ove 409 appeared to be a mutant which was temperature-sensitive (see this Chapter, section 3) for bud production and

was therefore of interest. It was decided to study ove 409 genetically. As ove 409 can produce normal gametophores at 15°C, it might be possible for it to produce spores and to cross it with other mutants. Complementation analysis of ove 409 with other ove mutants can be done by protoplast fusion. Other ove mutants that have been analysed in this way have, so far, fallen into 3 complementation groups (Grimsley et al., 1979; Cove et al., 1980). However, there is only one mutant in complementation group C. This is ove C200 thi A1. This mutant has the same vitamin requirement as ove 409, and so it is not possible to detect ove 409/ove 200 hybrids by selecting for prototrophs. ove 409 was fused with ove 78 and also with ove 100, which are in complementation groups A and B respectively.

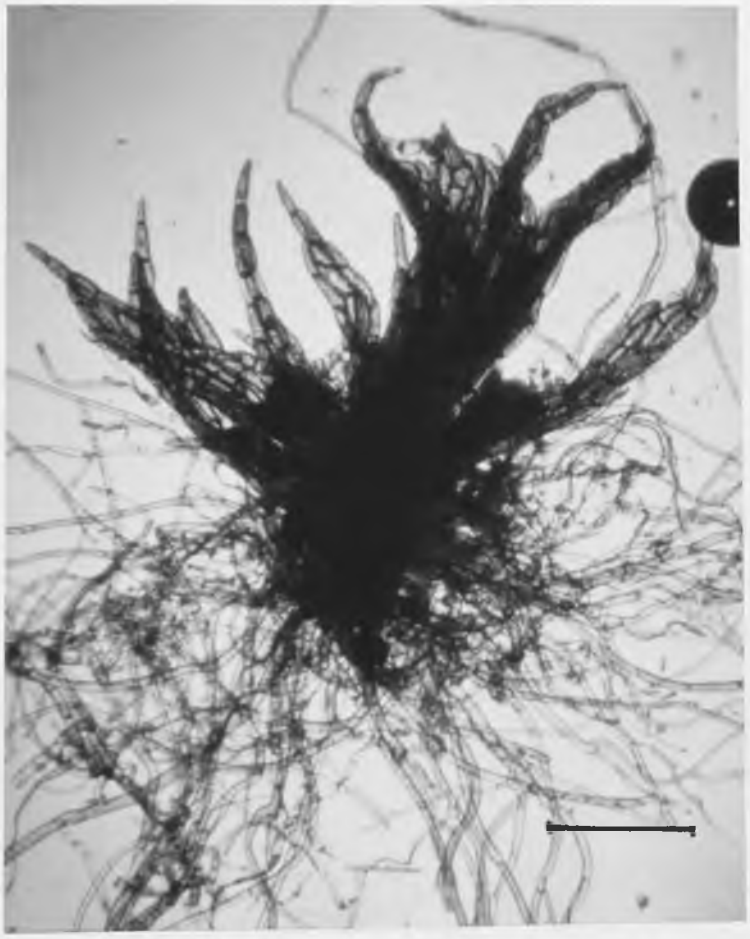
(i) Fusion with ove A78

ove 409 was fused by protoplast fusion with ove A78 as described earlier (Chapter II, section 5). Six prototrophs were produced. All of these hybrids produced abnormal buds after about six weeks. One of these is shown in figure 3a. These hybrids were also transferred to 15°C, as a hybrid of ove 409 that produces 'callusy' buds, due either to no complementation or to ove 409 being a dominant allele, should produce normal leafy shoots at the permissive temperature. After 8 weeks, all six hybrids stopped producing 'callusy' buds, but only three produced normal leafy shoots. A leafy shoot from an ove 409/ove 78 hybrid is shown in figure 3b.

Figure 3a and b. Somatic hybrid of ove 409 and ove 78



A somatic hybrid of ove 409 and ove 78 produced by protoplast fusion using polyethylene glycol. This hybrid is producing abnormal buds at 24°C. (Bar=100µm).



A somatic hybrid of ove 409 and ove 78 induced with polyethylene glycol. This hybrid is producing normal buds at 15°C. (Bar=500µm).

(ii) Fusion with pab A3 and pab A5

ove 409 was fused with pab A3 and also with pab A5 to see if the ove 409 phenotype was due to a dominant allele. However, neither fusion produced any prototrophs.

(iii) Fusion with ove 100

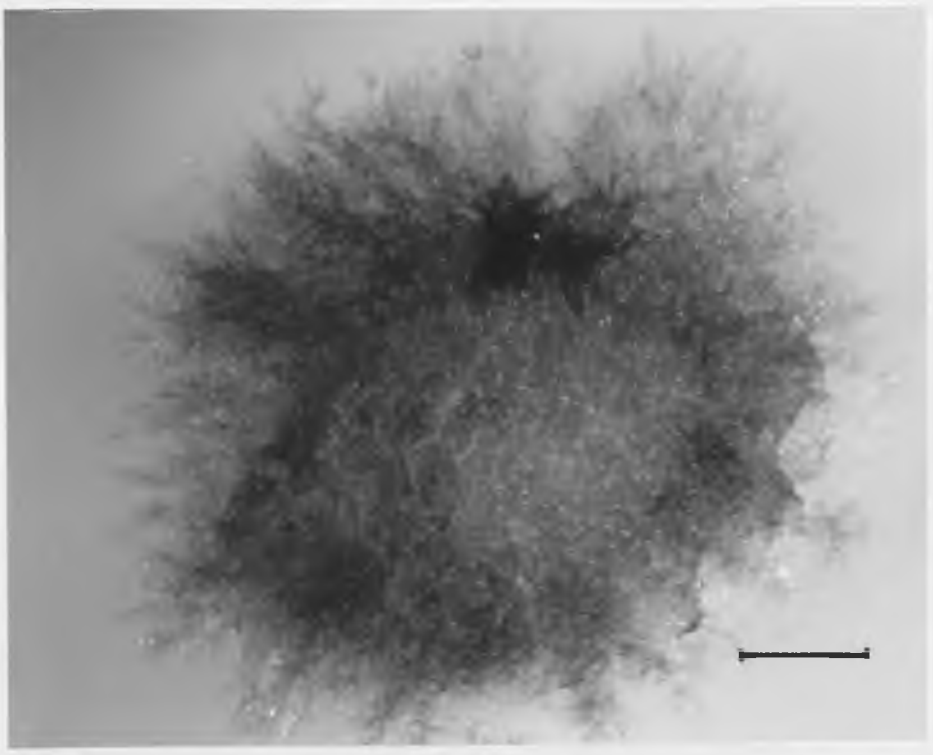
ove 409 was fused with ove 100 by protoplast fusion. Only one prototroph was produced. This produced some normal leafy shoots within three weeks at 24°C. These are shown in figures 4a and 4b.

(iv) Crossing of ove 409 with self-sterile mutants

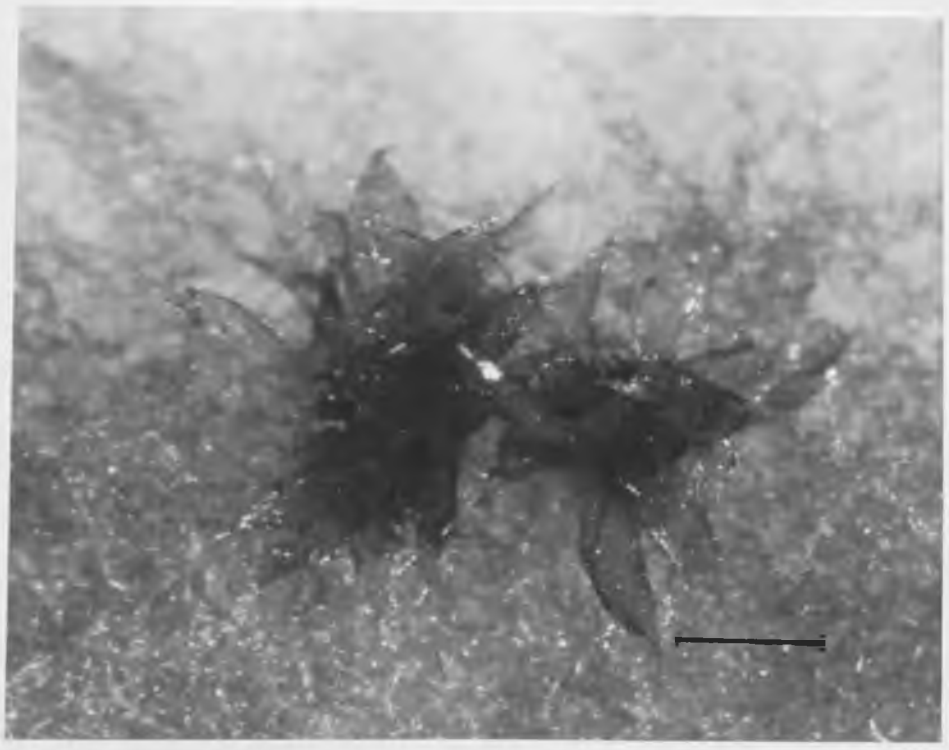
Cultures for selfing ove 409 and for crossing ove 409 with auxotrophic mutants to obtain ove 409 in a different background were set up as described in Materials and Methods (section 2). Even after 5 months, with the cultures being irrigated every month, no capsules were produced.

The attempts to cross ove 409 with other auxotrophic mutants, to isolate ove 409 in a different background so that it could be fused with ove 200, were unsuccessful. Also, the fusions of ove 409 with pab 3 and pab 5, to test for dominance, did not produce any hybrids. However, from the fusions with ove 78 and ove 100, the ove 409 phenotype is highly likely to be due to a recessive allele, and can be assigned to complementation group A, which is the group containing ove 78. Because ove 409 is temperature-sensitive for the production of cytokinin, it is potentially a

Figure 4a and b. Somatic hybrid of ove 409 and ove 100.



A somatic hybrid of ove 409 and ove 100. (Bar=2mm).



A somatic hybrid of ove 409 and ove 100. (Bar=500µm).

useful mutant for the isolation of secondary mutations. ove revertants could be isolated at 24°C and then studied at 15°C to see the effect of the secondary mutation without the one causing the over-production of cytokinin. This method could be used in the isolation of cytokinin non-responding mutants.

3. Temperature-sensitivity of ove 409

The production of normal leafy shoots by ove 409 at 15°C could either be due to a temperature-induced reversion, a temperature-sensitive response, or the colony being comprised of a mixture of two strains:- an ove which grows well at the high temperature but not at the low temperature, and a phenotypically wild type strain which grows at the low temperature. To check that ove 409's production of leafy shoots at 15°C was due to temperature-sensitivity and not reversion, cultures were grown at 15 or 24°C and then transferred to the other temperature. ove 409 continued to over-produce abnormal buds at the high temperature but normal leafy shoots at the low temperature. The response of ove 409 appeared to be temperature-sensitive so it was grown on a temperature gradient as described in Materials and Methods.

Table 1. *ove* 409 grown on a temperature gradient.

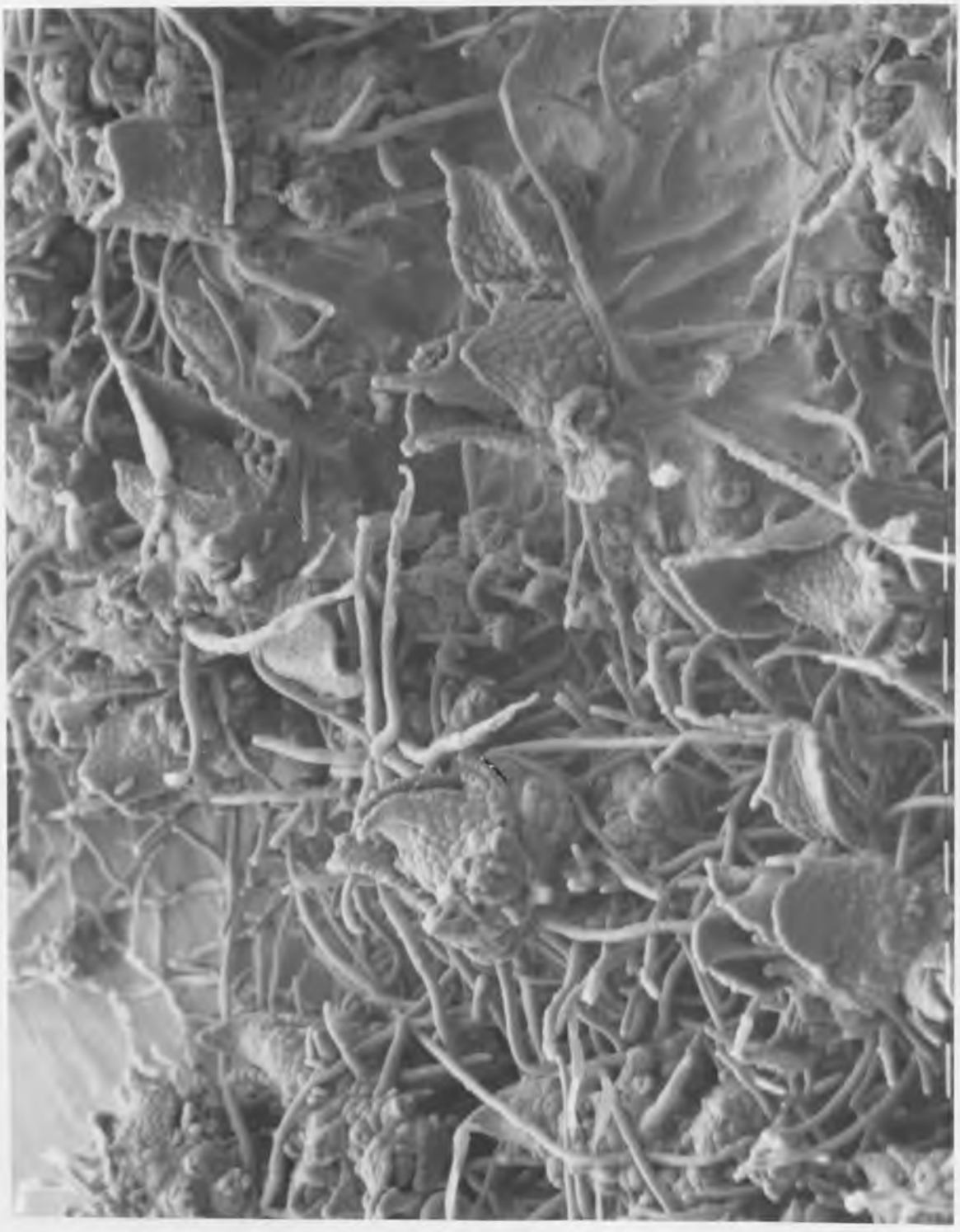
temperature °C	growth	phenotype
26	+	extreme *ove*
24	++	*ove*/intermediate *ove*
23	++	intermediate *ove*
22	++	intermediate *ove*
21	+++	slight *ove*
20	+++	slight *ove*
17	+++	wild type
16	++	wild type
14	++	wild type

Table 1 shows the results obtained by growing *ove* 409 on a temperature gradient between 14 and 26°C. +++ is 80 to 100% of the amount of growth of a wild-type spot inoculum grown for 5 weeks at 24°C. ++ is 40 to 80% growth of wild type. + is less than 40% the growth of wild type.

Table 1 gives the results obtained when ove 409 was grown at a range of temperatures from 26°C to 17°C; there is a gradual change in phenotype of ove 409 from an extreme ove to wild type. The extreme ove phenotype of ove 409 is shown in figure 5a. The intermediate ove phenotype is shown in figure 5b. ove 409 grown at 15°C is shown in figure 5c and wild type in figure 6. To check that ove 409 was not a mixture of two strains, it was grown at 15°C and protoplasts were isolated from the protonema. 50 regenerants were replica plated and cultured at the permissive and non-permissive temperature. All 50 regenerants phenotypically resembled ove's at the non-permissive temperature, but were wild type at the permissive temperature. Furthermore, ove mutants are usually more difficult to protoplast and regenerate than wild type. Thus, if ove 409 was a mixture, most of the isolated protoplasts would have been the wild type strain and therefore it would have been expected that most of the regenerating protoplasts would be wild type at both temperatures. As all 50 regenerants changed phenotype with temperature, ove 409 does appear to be a temperature-sensitive mutant for gametophore overproduction.

Figure 5a, b and c. The phenotype of ove 409 at different temperatures.

ove 409 grown for 5 weeks at 26°C. (Bar=1mm).



An SEM of ove 409 grown for 5 weeks at 23°C. (Bar=100μm).

An SEM of ove 409 grown for 5 weeks at 15°C. (Bar=100μm).

Figure 6. Wild type at 24°C.

An SEM of wild type grown for 5 weeks at 24°C. (Bar=100μm).

As mentioned in section 1 a (i) above, ove 409's ove phenotype could be due to ove 409 over-producing cytokinin or being over-sensitive to normal levels of cytokinin. These two possibilities can be subdivided (Ashton et al., 1979a; Wang et al., 1983) as follows:

increase in cytokinin activity

a) increased synthesis of cytokinin

b) decreased metabolism of cytokinin

c) production of a more active cytokinin

d) decrease in production of a cytokinin antagonist

change in sensitivity to cytokinin

a) loss of requirement of cytokinin for bud production

b) increase in sensitivity to normal levels of cytokinin

All the ove's that have been studied so far overproduce buds and overproduce cytokinin (Wang et al. 1981a). ove 409 was therefore studied for production of cytokinin. Cytokinin production was measured at both 15 and 24°C. This was carried out by growing ove 409, ove 78 and wild type in 1 litre of liquid medium in a 2 litre conical flask on an orbital shaker at the two temperatures for 6 weeks. The tissue was collected , weighed and freeze dried. The medium from these cultures was labelled with ^{15}N cytokinin as an internal standard (a gift from Dr. R. Horgan, Aberystwyth). To each flask was added 100ng of ^{15}N-zeatin and 100ng ^{15}N-isopentenyl adenine. The medium was then extracted for cytokinin as described in materials and methods. After butanol extraction, the samples were dried down into dram vials. They were then purified on HPLC using a gradient from 5→25% acetonitrile with an exponent of 0.5 in 15 minutes. Zeatin eluted after 5.5 minutes and 2iP after 13 minutes. The relevant

fractions for each sample were collected, dried down and permethylated. Quantification was carried out as described in Materials and Methods (section 8d) using GCMS under chemical ionisation conditions. The results of isopentenyl adenine are shown in Table 2. The zeatin samples could not be measured accurately as so little was present in the samples, and other ions interfered at similar m/e values.

In all three strains, the amount of isopentenyladenine per mg dry weight is higher at 24°C than at 15°C. ove 409's phenotype at 24°C is highly likely to be due to the over-production of isopentenyladenine. However, the amount of over-production of 2iP by ove 409 is much less than the over-production of 2iP by ove 78. The drop in production of ove 409 with the drop in temperature is enough so that at 15°C the amount of 2iP produced is within the range produced by wild type. This alteration in the level of 2iP would account for the change of phenotype of ove 409 from an extreme ove to wild type. Thus, ove 409's phenotype could be due to an increased synthesis of cytokinin by the normal or a de novo pathway, or a decreased metabolism of cytokinin. With wild type and ove 78, there is only a 5-6 fold change in 2iP production between 15 and 24°C. In the case of ove 409 there is a 14-fold change in 2iP production. This change in cytokinin production with temperature, especially the 5-6 fold change with wild type and ove 78, could be due to less cytokinin-producing tissue being present at 15°C. This would result in slower regeneration and differentiation, and a delay in the production of cytokinin. Temperature may also have an effect in differentiation and so at the lower temperature there is more primary chloronemata, and less cytokinin and auxin.

Table 2. Production of isopentenyladenine at 15 and 24°C.

strain	temperature	dry weight(g)	2iP(nM)	2iP/dry weight(ng/mg)
wild type	24	0.263	0.42	0.33
	15	0.303	0.07	0.05
ove 409	24	0.299	2.3	1.59
	15	0.358	0.19	0.11
ove 78	24	0.233	62.3	57.5
	15	0.265	15.5	11.6

Table 2 shows the production of isopentenyladenine by wild type, *ove* 409 and *ove* 78 at 15 and 24°C. The cytokinin was extracted from the medium of 1-litre cultures that were grown for 6 weeks. The amount of cytokinin produced was measured by mass spectrometry using ^{15}N-isopentenyladenine as an internal standard.

In the case of the 14-fold change of cytokinin of ove 409, there could be a temperature-sensitive mutation which is contributing to the change in cytokinin production.

4. Growth on starvation medium

When wild type is grown on phosphate-free agar medium, it forms mainly radially-growing caulonemata with the normal number of buds, about 1 per filament. Side-branch initials form at most of the potential sites, but these fail to develop further. When such a culture is supplied with 1 μM BAP, the side branch initials are induced to form callus-like buds. This type of tissue is therefore a good source of target tissue for cytokinin and hence potentially a good source of any cytokinin receptor (see chapter 5). Wild type was grown in a 1-litre phosphate-free liquid culture and produced long caulonemal filaments with normal gametophores (figure 7), but no side-branch initials. A possible explanation of the difference on solid and in liquid phosphate-free medium is that the agar could contain low levels of phosphate. Side-branch initials can be produced in liquid culture on a low phosphate and low nitrate medium. Both phosphate-free, and low phosphate with low nitrate, media were used to grow batch cultures for the assay of cytokinin binding (see chapter 5 section 2). bar 1 was grown in 1 litre of phosphate-free medium. After about a week it appeared to stop growing. bar 1 did not turn brown even after 2 months. Therefore, bar 1 could not be enriched for cytokinin target cells by this method.

Figure 7. Phosphate-free grown wild type.

Wild type culture grown in liquid medium without phosphate. The culture shows normal caulonemata and gametophores but very few chloronemata. (Bar=250μm).

5. Assay of cytokinin production

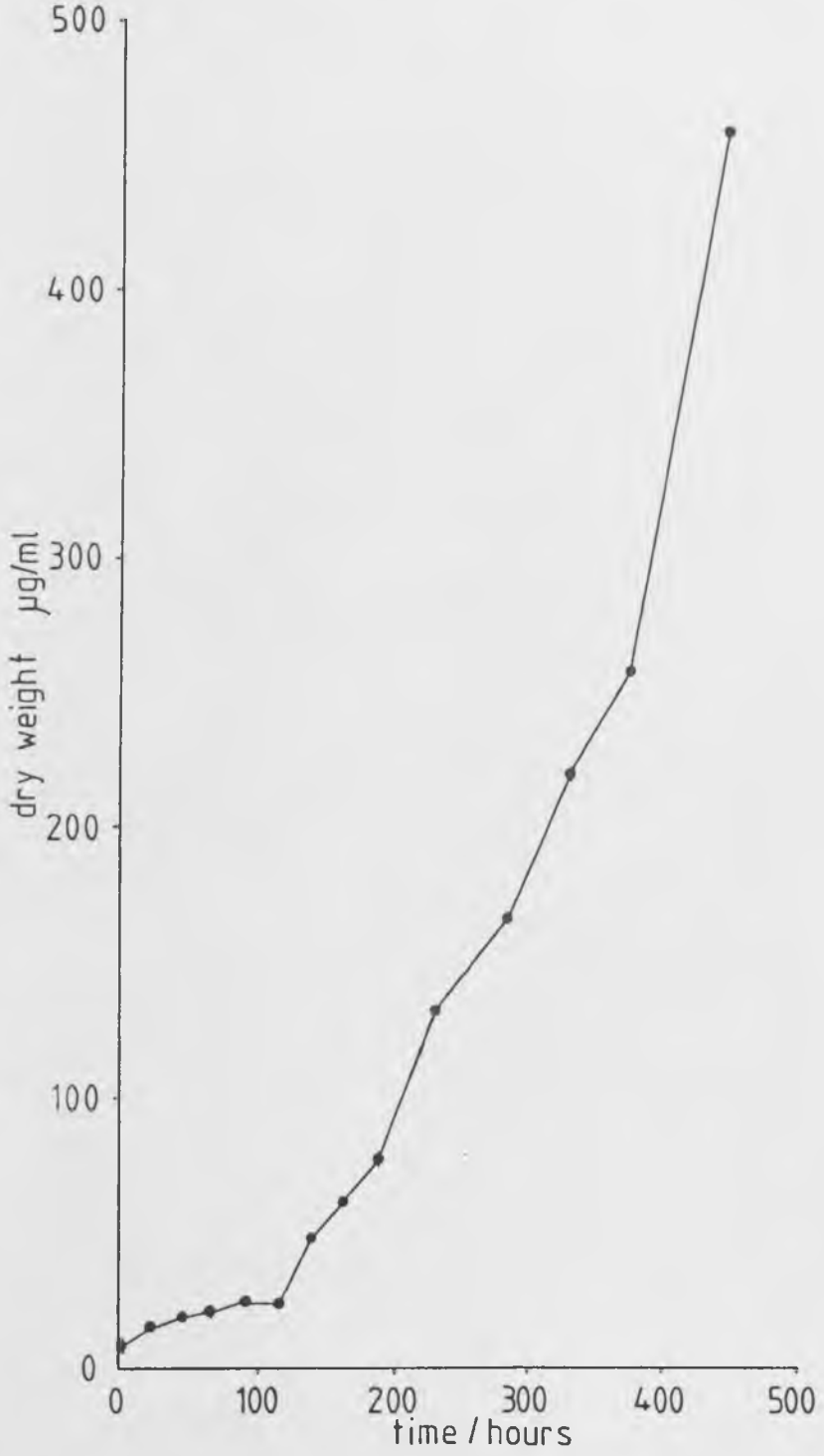
Batch tissue cultures were carried out in a 10 litre spherical vessel fitted with a condenser and a sample port (Wang *et al.*, 1981b). The medium used in these vessels was Knops minimal medium with vitamins. Cytokinins were extracted and assayed from the medium of the batch cultures (see Chapter II section 9). The *nar* mutants are strains that were originally isolated as NAA-resistant mutants (Ashton *et al.*, 1979b).

nar 429

The growth of *nar* 429 (figure 8) was non-linear. The very slow growth rate over the first 4 days, which then slowly increased, could have been due to the inoculum being over-homogenised. The tissue would then have required a few days to regenerate. The higher growth rate achived by *nar* 429 could be due to it growing as highly-branched chloronemata.

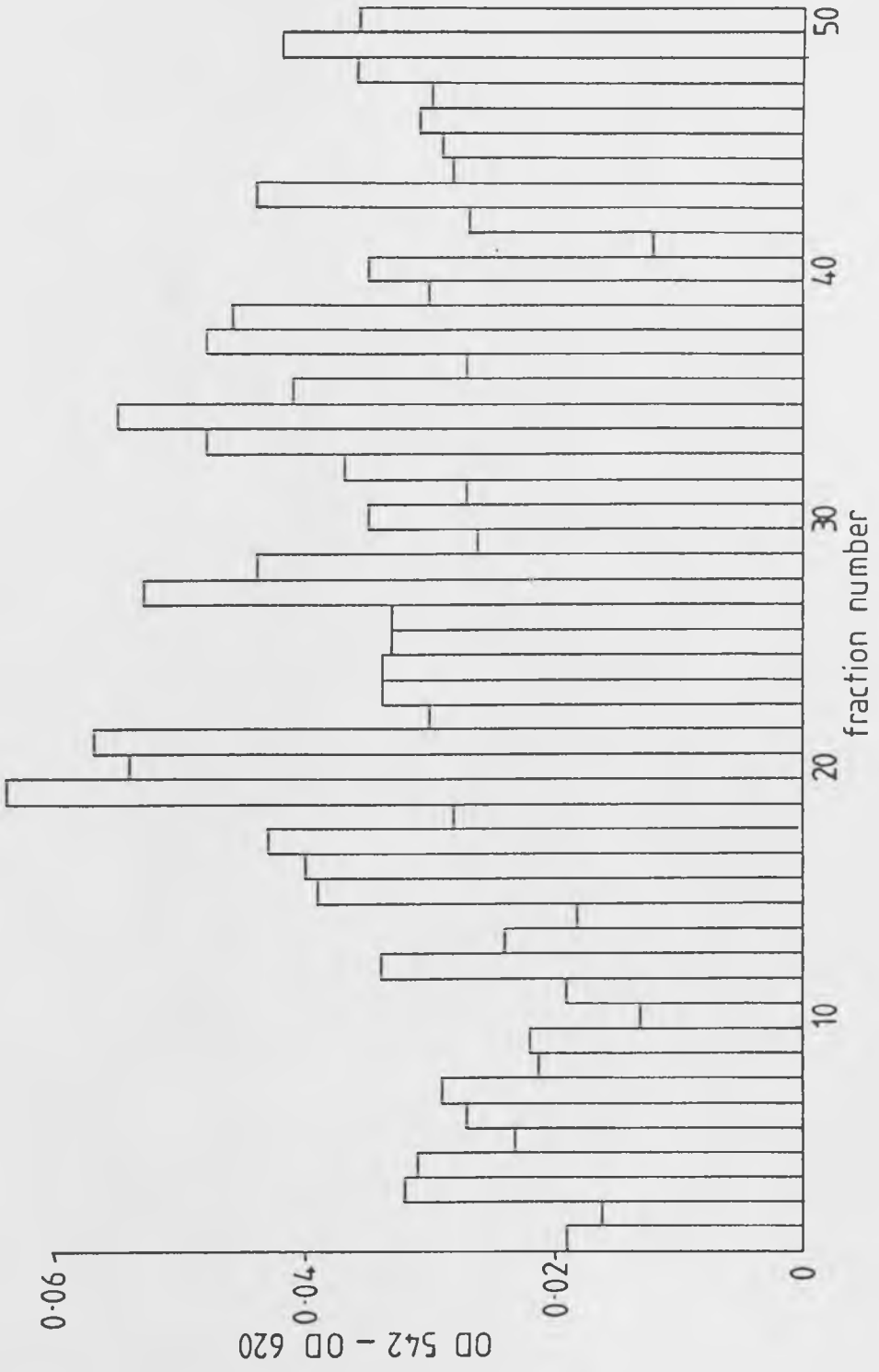
The medium from this batch culture was extracted for cytokinins as described in materials and methods. The whole of each 30ml fraction from the LH20 column was used in an Amaranthus bioassay. The results from the assay are given in figure 9. This shows that there is no detectable cytokinin produced by *nar* 429, using the amaranthus bioassay.

Figure 8. Growth curve of nar 429.



Increase of dry weight of nar 429 grown in a 10-litre batch culture. For further details see section 5.

Figure 9. Bioassay of nar 429.



Cytokinin bioassay of the culture medium of nar 429 from figure 8, using the Amaranthus bioassay described in Chapter II section 9 b (i). A standard vial containing 100 ng BAP gave an $OD_{542} - OD_{650}$ of 0·112.

nar 185

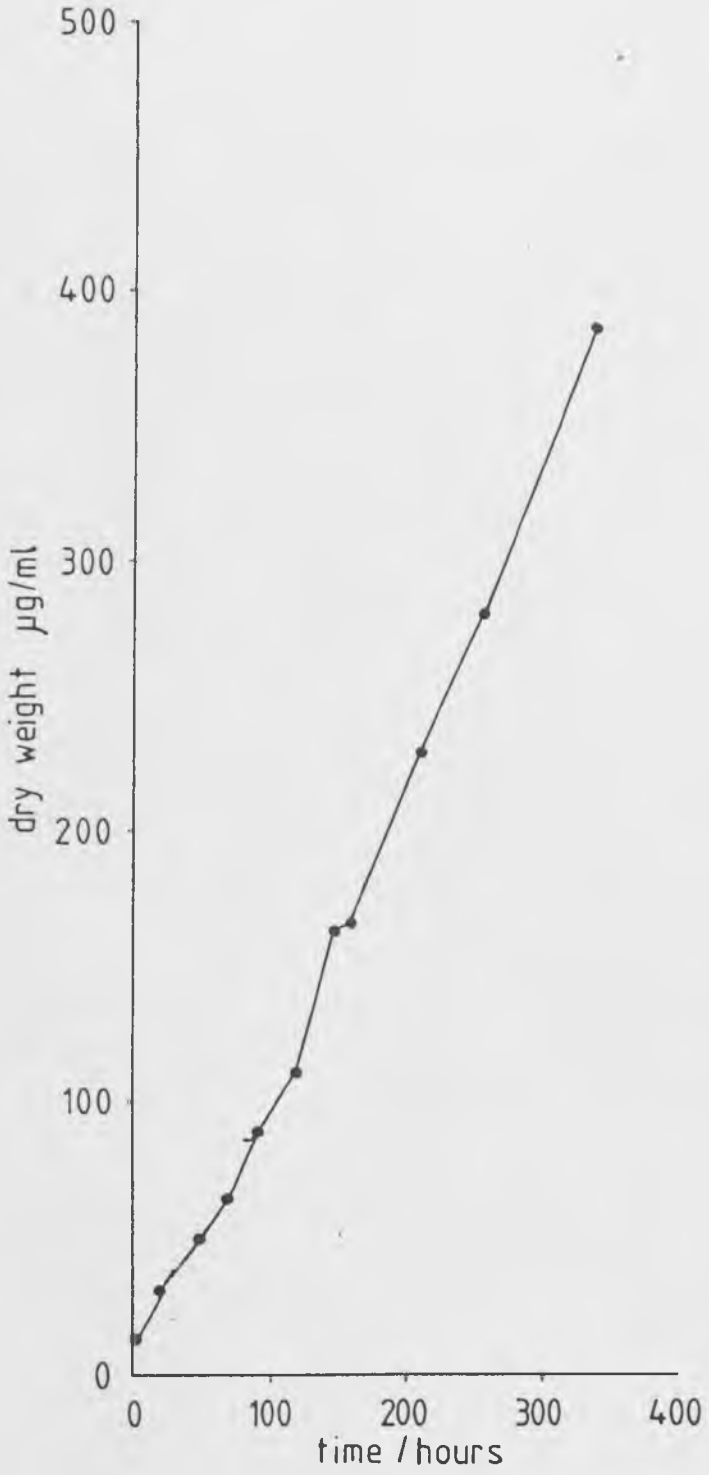
The growth rate of nar 185 (figure 10) was slightly less than wild type, producing about 30µg/ml/day. This mutant was treated in the same way as nar 429. The largest O.D. difference in the *Amaranthus* bioassay was 0.032, and so there was no detectable cytokinin produced by this mutant.

ore 6

ore 6 was originally isolated by N.H.Grimsley as a revertant of ove 130. ore 6 grew slower than wild type and had a short lag phase. From the linear part of the graph (figure 11), the growth rate was less than 20ug/ml/day. In liquid culture this mutant did produce abnormal buds.

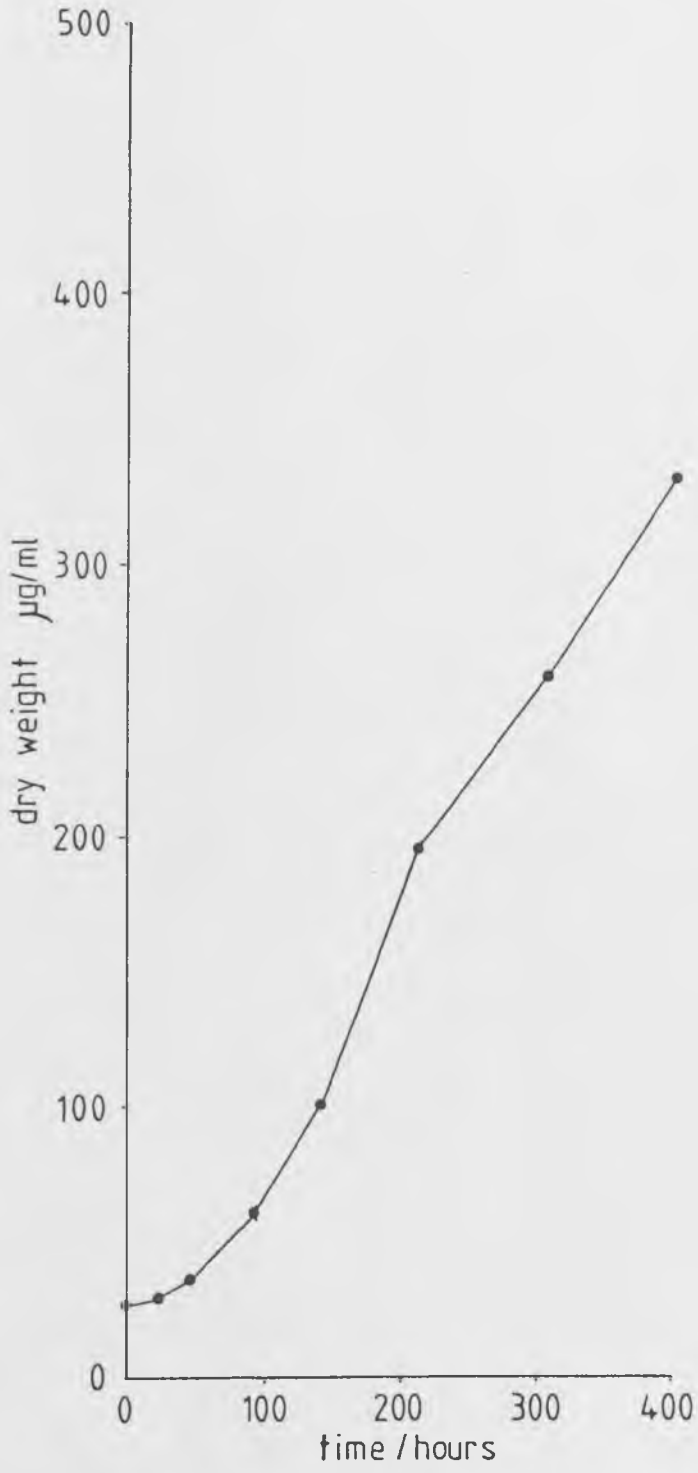
After extraction for cytokinins, the fractions were asssayed by the soya bioassay. Only 5ml from each fraction from the LH20 column was used for the assay. The results are given in figure 12. This shows a pair of peaks at fractions 19 and 22, and another pair at fractions 29 and 33. The second of each pair of peaks corresponds to zeatin and isopentenyl adenine respectivly. The position of the first of each pair of peaks is in the position of the corresponding ribosides, but these have not yet been identified in P.patens. Fractions 19-24 were pooled, as were fractions 28-34, for further analysis by gas chromatography mass spectrometry as described in Appendix II section 1. Fractions 19-24 gave the same spectra as zeatin and fractions 28-34 gave the same spectra as for isopentenyladenine. It can therefore be concluded that ore 6 overproduces both of these

Figure 10. Growth curve of nar 185.



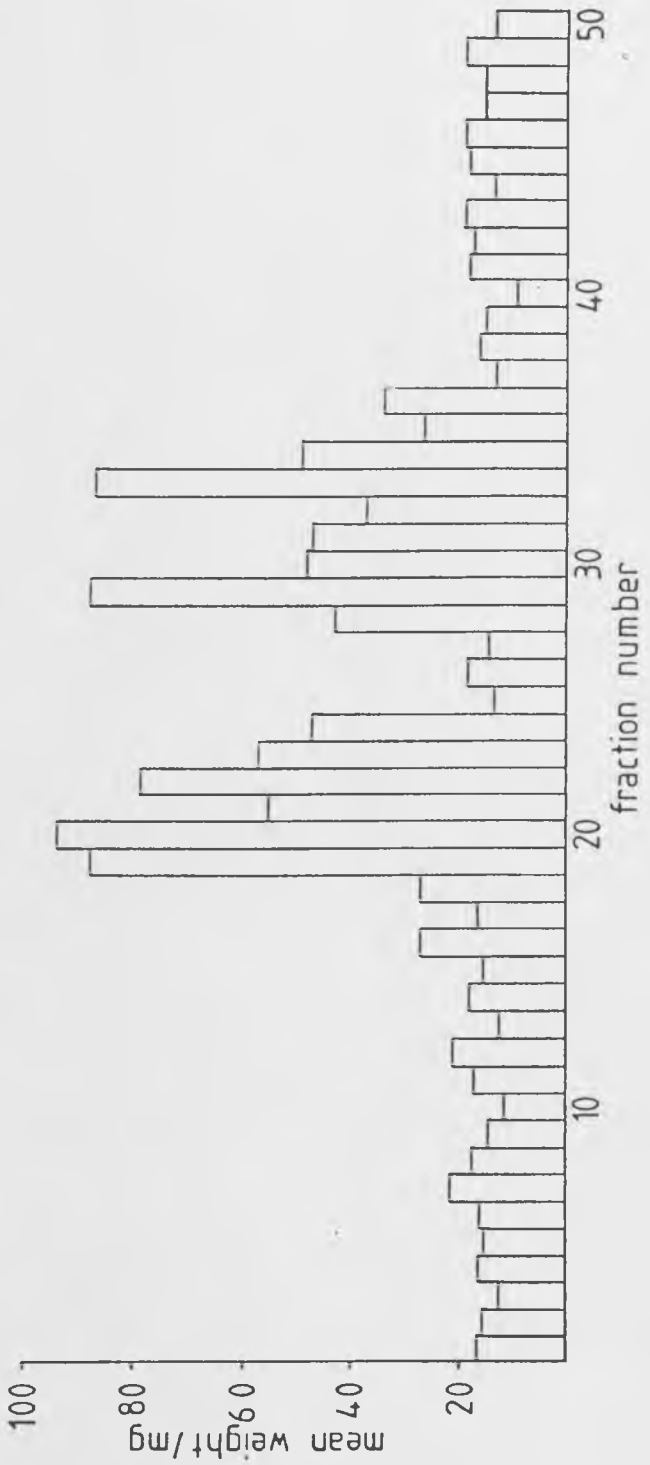
Increase of dry weight of nar 185 grown in a 10-litre batch culture. For further details see section 5.

Figure 11. Growth curve of ore 6.



Increase of dry weight of ore 6 grown in a 10-litre batch culture. For further details see section 5.

Figure 12. Bioassay of ore 6.



Cytokinin bioassay of the culture medium of the batch culture of ore 6 shown in figure 11, using the soya bioassay described in Chapter II section 9 b. (ii).

Mean weight of standards: no BAP: 14 mg 50 µg/l BAP = 242 mg.

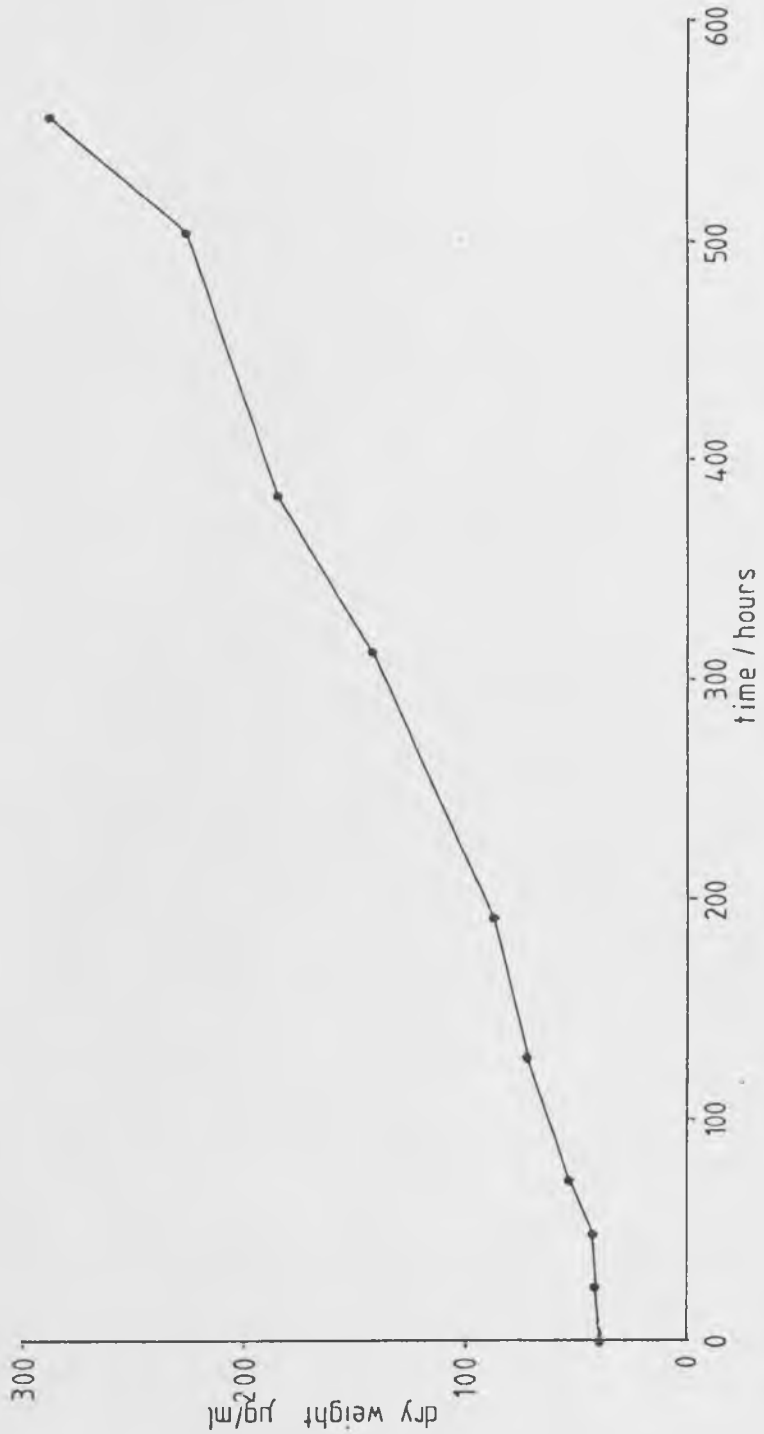
cytokinins. This mutant did produce many abnormal buds although bud production was slower than for ove 130 (Grimsley, personnal communication). Therefore, the original classification of ore 6 as an ove revertant could have been due to a mutation affecting growth but not the reversion of over-production of buds.

ove 409

ove 409 shows a short lag phase (figure 13), and has a growth rate of only 15µg/ml/day at 25°C. At this temperature ove 409 has a slow growth rate.

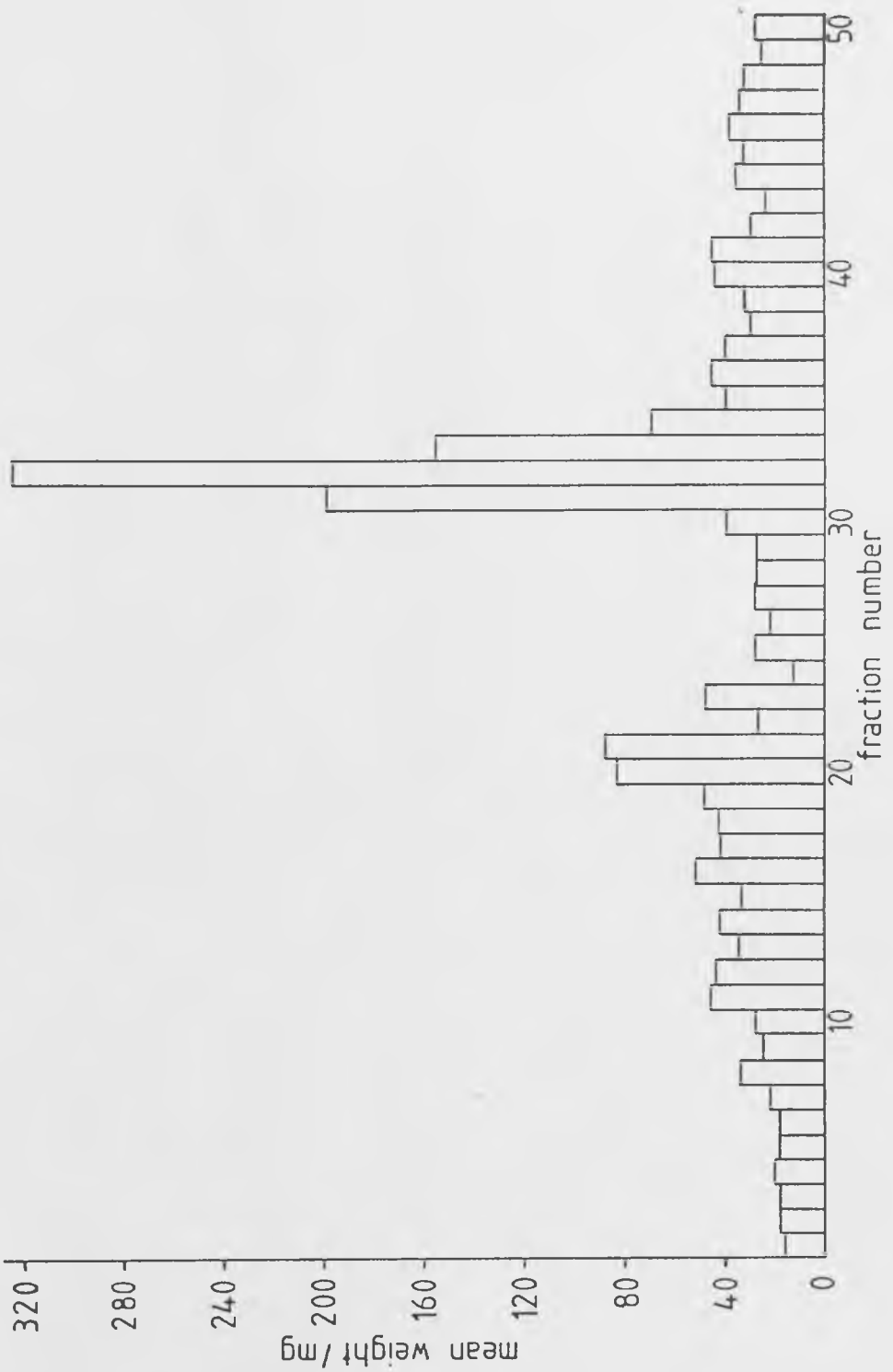
The medium from the 10-litre batch culture of ove 409 was extracted for cytokinins and 5ml of the 30ml fractions from the LH20 column were assayed using the soya bioassay. The results are given in figure 14. This shows a peak at fractions 20-21 and a larger peak at fractions 32-33. These fractions correspond to zeatin and isopentenyladenine, and so were pooled for further analysis. The mass spectra are shown in Appendix II section 2. The sephadex fractions 20-21 were not clean enough for analysis, but the fractions 32-33 gave the same spectra as authentic 2iP. It can therefore be concluded that ove 409 over-produces 2iP. The level of zeatin production could not be ascertained.

Figure 13. Growth curve of ove 409.



Increase of dry weight of ove 409 grown in a 10-litre batch culture. For further details see section 5.

Figure 14. Bioassay of ove 409.



Cytokinin bioasay of the culture medium of the batch culture of ove 409 shown in figure 13, by the soya bioassay described in Chapter II section 9 b. (ii).

CHAPTER IV

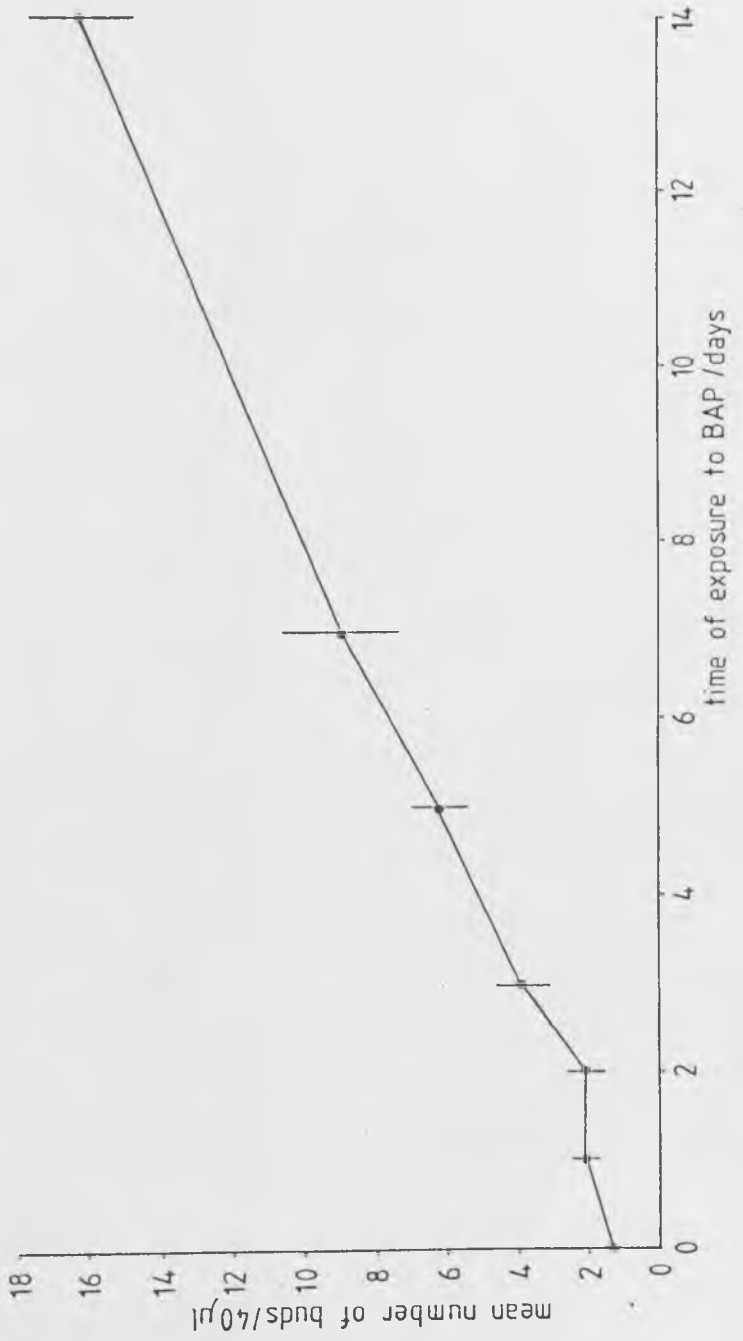
Characteristics of cytokinin action

Results and discussion

1. Time of exposure to BAP for bud induction

Brandes and Kende (1968) showed that bud induction could be reversed by removal of the cytokinin in the moss Funaria hygrometrica. Brandes and Kende concluded that cytokinin does not act as a trigger for bud induction but is required for a critical time period to allow the differentiation to stabilize. This effect was investigated in Physcomitrella patens. Wild-type cultures were exposed to cytokinin as described in Materials and Methods for 1 to 7 days, after growth in liquid culture for 7 days. The tissue was collected by filtration, added to liquid medium containing 1 μM BAP and, after set intervals, removed by filtration, washed with sterile distilled water and placed in fresh medium without BAP. The number of buds produced was counted 14 days after the transfer to the medium containing BAP. The results are shown in figure 15. There is very little increase in the number of buds with exposures of up to 48 hours. With longer exposures to BAP, there is an increase in the number of buds produced. As buds can be seen in a culture exposed to cytokinin for less than 72 hours, the effect of the cytokinin is removed by washing the culture. The increase of the number of buds after 72 hours could be due to bud induction on new tissue.

Figure 15. Bud induction against time of exposure to BAP.



All cultures were placed in medium containing 1 uM BAP after 7 days growth in cytokinin-free medium. The cultures were washed and placed into medium without BAP at different time intervals. All cultures were scored for the number of buds 14 days after exposure to BAP. For further details see section 1.

The 'time of exposure to BAP' experiments have shown that the level of cytokinin for bud induction has to be maintained for 72 hours to prevent dedifferentiation of the bud. The cytokinin receptor must therefore be present during the 72 hours that the bud can dedifferentiate by the removal of the cytokinin. The receptor is also likely to be present in all the cells of a cytokinin-induced abnormal bud and thus an OVE mutant could be a good source for the isolation of cytokinin receptors. To see if the continued bud induction over the 14 days was partially due to prevention of dedifferentiation, or only due to continued growth of caulonemal filaments and hence the production of more target cells, bud counts would have had to be done at different times over the 14 day period.

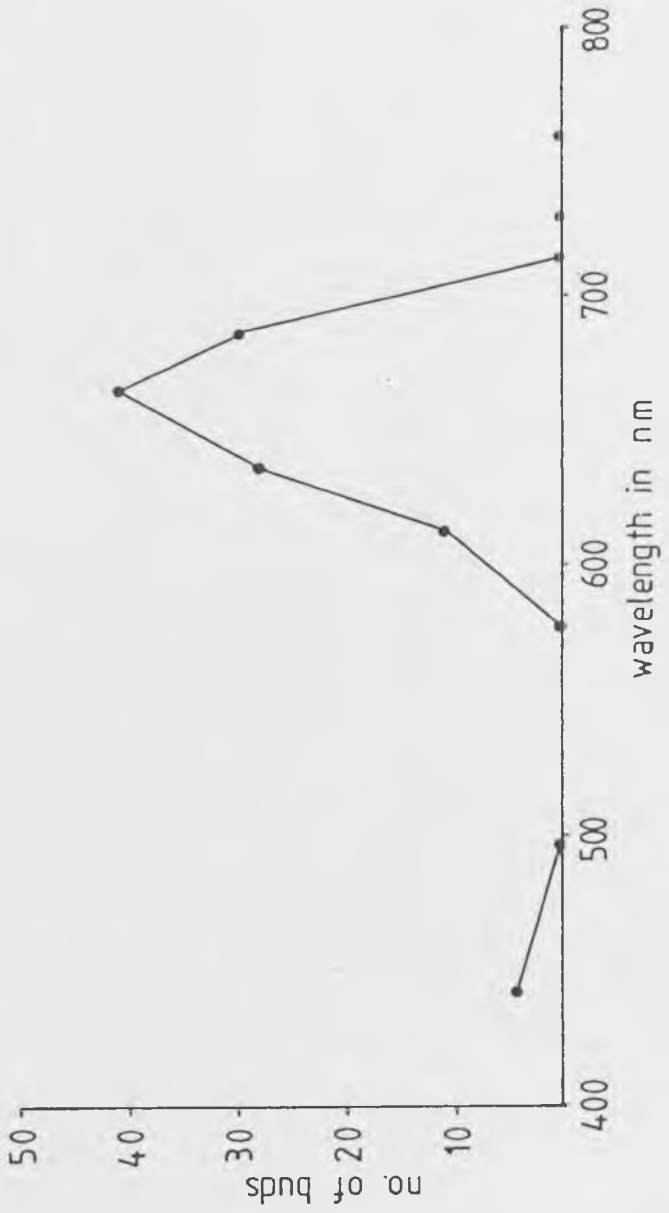
2. Bud induction under monochromatic light

As gametophores are not produced by P.patens in the dark, even with exogenous cytokinin, the quantity and quality of light required for bud induction was studied. In earlier experiments NAA was also added to some of the cultures. There was no detectable effect of the NAA on bud induction in the absence or presence of BAP, and so the NAA treatments were omitted. Bud induction in the absence of exogenous cytokinin was very small. The number of buds per 100 sites was usually zero or in single figures, the maximum being 13 with red light (665-668nm) at an intensity of 556nmol quanta $m^{-2}s^{-1}$. Bud induction at different wavelengths with a photon fluence rate of 556nmol quanta $m^{-2}s^{-1}$ is given in figure 16. This shows that the highest bud induction occurs around 665nm and quickly falls to zero at 613 and 715nm. However there is a small amount of bud induction with blue light

(442nm). This indicates that phytochrome could be involved in the induction of gametophores, as phytochrome-regulated responses are activated with red light of around 665nm. Bud induction by red light (665-668nm) in the presence of 1μM BAP is shown on figure 17. Each point on the graph is the average of all measurements at that light intensity. This appears to show a linear response of bud production to light intensity, and can be investigated further by exposing the plates to different light intensities for only part of the three-day period.

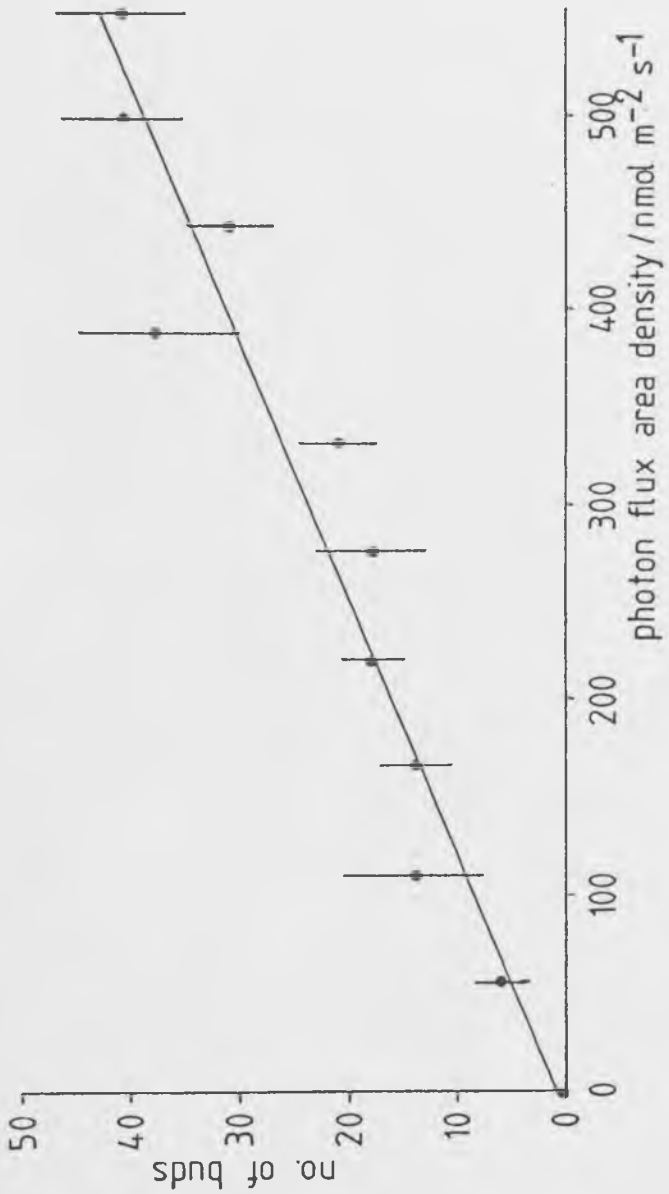
In order to see if phytochrome might be involved in light perception, attempts were made to reduce the amount of bud induction under 665nm light by simultaneous exposure to far-red light of 730 or 760nm. The results from these experiments were too variable for any conclusion to be drawn. Experiments were also performed to try to find out how much exposure to red light was required to cause bud induction during the three days' incubation with cytokinin, and whether this could be reversed by short exposures to far-red light. These results were also too inconsistent for any conclusions to be drawn, apart from the fact that at least 6 hours of red light at 556nmol quanta $m^{-2}s^{-1}$ appear to be required to cause bud induction.

Figure 16. Graph of wavelength of light on bud induction.



Cultures with dark-grown caulonemata were supplied with BAP and exposed to light of 556nM quanta $m^{-2}s^{-1}$ of different wavelengths. After three days exposure to the monochromatic light the cultures were scored for bud induction. For further details see section 2.

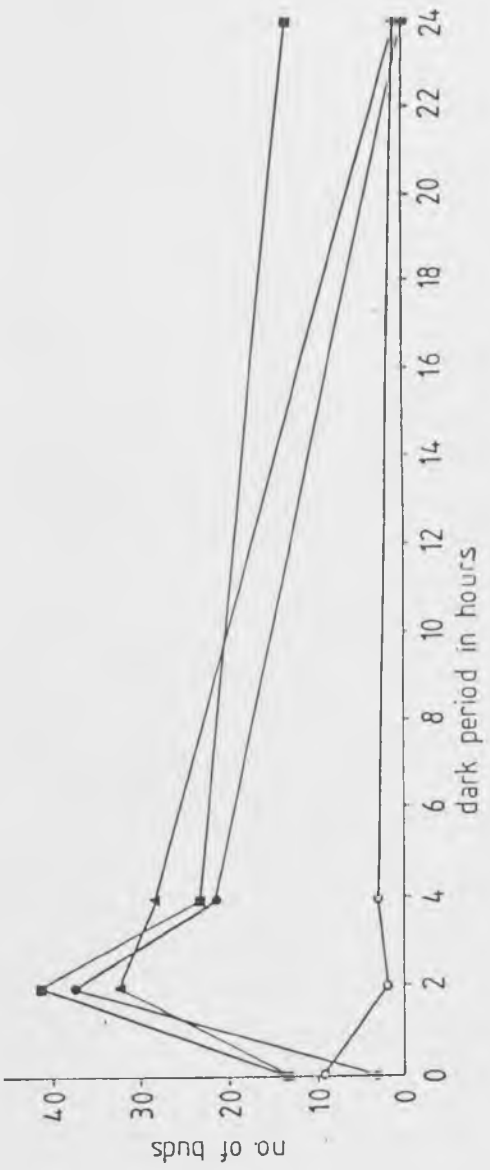
Figure 17. Graph of effect of red-light intensity on bud induction.



Cultures with dark-grown caulonemata were supplied with 1µM BAP and exposed to different light intensities of red light (665nm).

Cultures were also exposed to 556nmol quanta $m^{-2}s^{-1}$ red light (665-668nm) before the addition of 1 μM BAP. Plates were exposed to the red light for 24, 30 and 48 hours. Some of the plates exposed for 24 hours were then exposed to 1/2 hour far-red light (760nm) at an intensity of 556nmol quanta $m^{-2}s^{-1}$. The plates were left in the dark for 0, 2, 4 and 24 hours before the addition of the BAP. The results are shown in figure 18. In all cases there is only a small amount of bud induction when the BAP is added immediately after the exposure to the light. There is much more bud induction with a period of two hours in the dark before the addition of the BAP with the plates exposed only to the red light. This response is reduced after 4 hours in the dark and almost gone after 24 hours in the dark. Therefore this response to red light appears to be slowly lost if the plates are left in the dark before the addition of the BAP. The exposure to the far-red light after the red light appears to have prevented the bud induction with the subsequent addition of BAP. These results indicate that phytochrome could be involved as the conversion of P_{fr} to P_r occurs slowly in the dark and quickly in far-red light. The reduction of bud induction by far-red light and darkness before treating the cultures with BAP can be investigated further by reducing the intensity or the amount of red light exposure and altering the time of the dark period. Such experiments would indicate that the rate of bud induction is reduced by the dark period. The shorter dark periods before adding the BAP would show at which stage the amount of bud induction was at a maximum. This increase in bud induction when there is a dark period between the exposure to the red light and the addition of the cytokinin implies that there is another factor involved in the induction of the bud as well as

Figure 18. Cytokinin bud induction after light exposure.



Cultures with dark-grown caulonemata were exposed to 24, 30 and 48 hours red light (556 nM quanta $m^{-2}s^{-1}$, 665nm). Some cultures were then exposed to 1/2 hour far-red light (556 nM quanta $m^{-2}s^{-1}$, 760nm). After this, cultures were placed in the dark, and at different time intervals were supplied with 1μM BAP. All cultures were scored for bud induction 3 days after adding the BAP. (●-24h, ▲-30h, ■-48h red light. ○-24h red + ½ h far-red light.)

phytochrome and cytokinin.

In summary, these results show that light is required for the induction of buds in P.patens and that this light requirement is in addition to the cytokinin requirement. The most effective wavelength of light is 665nm. Wavelengths below 578nm, with the exception of 442nm, and above 715nm do not cause bud induction even with exogenous cytokinin. This red-light response may mean that bud induction is under the control of phytochrome. If this is the case it should be possible to reverse the effect with far-red light. However, due to the variation between experiments, it has not been possible to demonstrate the reduction of bud induction by simultaneous red light with far-red light. In some of these experiments there was no greater reduction of bud induction with the far-red light than with the red light on its own. Exposure to far-red light after the exposure to red light, but before the addition of BAP has shown that the amount of bud induction is reduced. This shows the possible involvement of phytochrome in the induction of buds in moss. The result also shows that the amount of bud induction appears to be greater if there is a 2-hour dark period after the exposure to red light, before the addition of the BAP. Far-red reversibility might be easier to demonstrate by changing the red filter for a far-red filter every few minutes and seeing if this produces fewer buds than a regime of red light and darkness.

The monochromatic light experiments suffer from a high degree of variation. There are a number of possible causes of this variation. The main causes are probably the positioning of the box after taking the light reading (as the light intensity changed as the light meter was moved around under the projector)

and the fact that some of the filaments were growing under the agar. Thus there would be a greater light loss from the gap in the box to these filaments than to those on the surface of the agar. It may be possible to improve this by not using the boxes and placing the dish horizontally under the vertical light. This would allow the light intensity at the surface of the agar to be measured and there would be only a small reduction in light intensity for the filaments growing just under the surface. However, fewer plates could be put under each projector in this orientation and it might be more difficult to see which part of the filament had grown under the monochromatic light. One possible solution may be to use polarised light at 45^{o} to the filaments. This may cause the filaments to bend either parallel or perpendicular to the direction of the polarised light.

3. Auxin and cytokinin effects on protoplast regeneration

Protoplasts were regenerated in the presence of cytokinin and auxin to see if they could be induced to regenerate to form caulonemata or gametophores. If hormones can affect protoplast regeneration in this way, it would show that the protoplasts contained the receptors for these hormones. This possibility was investigated by preparing protoplasts as described in Materials and Methods. They were then regenerated in a 5x5 repli-dish with auxin against cytokinin. The concentrations of the hormones were 0, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M. The concentration of the agar was only 0.4% (w/v). This allowed the protoplasts to sink to the bottom of the wells and for the dish to be inverted so that the protoplasts could be examined under the microscope.

There was good protoplast regeneration , about 60 to 80%, within 7 days in all wells. However, all regenerants were chloronemal. The only observed effect of the hormones was inhibition at the higher concentrations. Both NAA and BAP, at the higher concentrations, caused the cells to be shorter and wider than the chloronemal cells with less or no exogenous hormones. This lack of a developmental response of the protoplasts to exogenous cytokinin and auxin may be due to the cells having lost their cell walls, losing also the appropriate hormone receptors. Alternatively the procedure for preparing protoplasts may have selected for protoplasts from young chloronemal cells which have not yet developed the ability to respond developmentally to cytokinin. Attempts were therefore made to isolate caulonemal protoplasts, which should initially contain the receptor for cytokinin. These protoplasts should be able to respond to exogenous cytokinin unless they have to undergo a regeneration sequence through primary chloronemata.

A good source of caulonemal tissue without chloronemata is dark-grown filaments. These were obtained by growing spot inocula of wild type, on Knops medium supplemented with sucrose and overlaid with cellophane, for 1 week. The plates were transferred to the dark on their edges for 3 weeks. The dark-grown filaments were easily removed from the tissue that had grown in the light. The dark-grown filaments were put into a number of different protoplast preparations as described in Chapter II section 3. None of these enzyme mixtures had any visible effect on these dark-grown filaments. The filaments were left in the mixture of cellulase, pectinase and rhomant P for 7 days with no observable effect. To check that the enzyme mixture

had not killed the cells, the filaments were rinsed and placed onto Knops medium. Within 2 days, regeneration into highly-branched chloronema had occurred at almost every cell division, so it appears that these filaments have some component which prevents the enzymes digesting the cell wall. This could be due to a cell wall component of all caulonemata or of older or dark-grown caulonemata. If it is only dark-grown filaments that are resistant to these enzymes, it could be due to a complex saccharide formed when grown on sucrose. Therefore other carbon sources were tested for dark-grown filaments.

Four different carbon sources were tried at three different concentrations. They were sucrose, glucose, myo-inositol, and sodium acetate, at 5, 10, and 15mM. Wild-type spot inocula, 4 per plate, were grown, on Knops medium with these carbon sources overlaid with cellophane, for 1 week in the light followed by 3 weeks in the dark with the plates on their edges. In darkness there was good growth with all three concentrations of sucrose and glucose, only 5-10 filaments produced per inoculum on myo-inositol and no growth on sodium acetate. The filaments produced on glucose were also resistant to the protoplast enzymes used above.

The regeneration of protoplasts in the presence of NAA and BAP did not effect differentiation, showing either that protoplasts lose the ability to respond developmentally to cytokinin and auxin before they can regenerate, or that the protoplast isolation procedure selects for young chloronemal protoplasts which are unable to respond to cytokinin and auxin. As attempts to isolate protoplasts from caulonemal filaments

produced in the dark, to obtain filaments free of chloronemal cells, were unsuccessful with a variety of enzymes, it was not possible to determine whether caulonemal protoplasts, if they are able to regenerate, could respond to auxin and cytokinin or whether they have to dedifferentiate to chloronemata. Alternatively, sucrose or glucose may cause the production of complex saccharides in the cell wall, making it resistant to attack by the protoplast enzymes.

CHAPTER V

Development of the cytokinin binding assay

Results and discussion

Assays for cytokinin binding were performed using either a centrifugation method or equilibrium dialysis as described in Materials and Methods. Each set of results presented is tabulated with the variables for that experiment. In each table, the "fold excess BAP" is the ratio of unlabelled BAP to radioactive BAP. In tables of centrifugation assays, "DPM" is the amount of radioactivity in the pellet. "Mean" is the mean of replicate samples. In the tables for equilibrium dialysis, "DPM sample" is the amount of radioactivity in the dialysis half cell containing the moss tissue. "DPM other" is the amount of radioactivity in the dialysis half cell not containing the moss tissue. The "difference" is the difference in radioactivity in the two half cells. The "displacement", in both types of table, is the difference between that sample and the sample with the largest excess of unlabelled BAP. This figure is the number of counts in that sample which are displaced in the presence of excess unlabelled BAP.

The first experiments were carried out using the method of Gardner et al. (1978), except the samples were chemically bleached rather than oxidised before scintillation counting. These results gave apparent displaceable binding using wild type and NAR 87 (a typical result is shown in Table 3). This mutant was used as it appears to produce less or no cytokinin compared

to wild type and therefore, if cytokinin binding is irreversible, may show high cyokinin binding activity. The apparent binding was also obtained when either zeatin or adenine was used as the diplacement ligand. As adenine is a purine that does not have cytokinin or anti-cytokinin activity, it might be expected not to bind to a cytokinin receptor.

To check the reproducibility with the dialysis cells, all the cells were used with the same amount of radiolabelled cytokinin and no unlabelled cytokinin. These results showed a similar apparent displaceable binding (Table 4). The samples were then left at $4^{o}C$ overnight and recounted. These results (Table 5) show that there is only a small variation between replicates. The apparent displacement in all earlier experiments, e.g. Tables 3 and 4, was due to chemiluminescence caused by the bleach, which was gradually dying down while the samples were being counted. In all further experiments where the samples were chemically bleached, they were left in the fridge overnight before counting for the chemiluminescence to die away. The bleach also reduced the counting efficiency and so was added to all samples, except some of the controls. When samples were left overnight before counting, the proportion of counts that could be displaced was greatly reduced and did not show a reduction of counts with an increase in excess unlabelled BAP (Table 6).

One method employed to homogenise the tissue was to freeze it in liquid nitrogen and then grind with a mortar and pestle. Using this method, after the low speed centrifugation, the supernatant was a straw yellow colour instead of green. The

samples could therefore be counted without the need to bleach them. When the tissue was ground in liquid nitrogen, the 13,000g supernatant was used so as to detect any binding that might be present in the soluble fraction. Results from a binding assay after grinding in liquid nitrogen are given in Table 7. This shows that there is less binding to the protein side and that the small amount of displacement does not match the amount of excess unlabelled BAP.

All these (Tables 3-7) and other experiments, using 13.4 or 57mCi/mmol ^{14}C-BAP, showed that specific cytokinin binding could not be detected. These ^{14}C-BAP labels do not appear to have a high enough specific activity for specific binding to be detected. A tritiated BAP was therefore sythesised as described in Appendix 1. This tritiated BAP had a specific activity of 12Ci/mmol and was used in all further cytokinin binding studies.

As the tritiated cytokinin has a specific activity of over 200 times that of the highest ^{14}C-BAP, a much smaller amount was required in each assay and therefore the ratio of excess unlabelled BAP used could be higher and still remain physiologically relevant. Initial results using the tritiated BAP did not show any more binding (Table 8). The Braun glass bead homogeniser was used as this made it possible to grind up more tissue for each assay. A tissue oxidiser became available and this was used to oxidise the samples before counting; bleaching was therefore unnecessary. A typical result is given in Table 9. This shows only a small amount of binding which is displaced by unlabelled BAP. To test the reproducibility of the centrifugation assay with the tritiated BAP and the oxidiser, 5

replicates were done. As only 10 assays could be carried out at once, only 0 and 10,000-fold excess BAP were used. In case any binding activity was being removed by the 13,000g centrifugation, the low speed spin was reduced to 2,000g. These results are given in Table 10. Although this experiment shows very little cytokinin binding that can be competed with by unlabelled BAP, it shows good reproducibility.

The incubation of the assay mixtures was increased from 15 minutes to 1 hour. Results of a long incubation are given in Tables 11 and 12. These show a much higher amount of radiolabel bound to the pellet which can be displaced with excess unlabelled BAP. An assay was therefore carried out with incubations at $4^{o}C$ for 1 and 24 hours (see Table 13). This demonstrates that the displaceable binding that is present after 1 hour's incubation has been greatly reduced after 24 hours. The assay was repeated with 0, 1 and 2 hour incubations (see Table 14). The 0 hour incubation was, in fact, about 10 minutes - the time taken to set up the tubes and start the centrifuge. The highest displaceable binding is at 1 hour and is greatly reduced, to below the 0 incubation, at 2 hours.

As a higher amount of displaceable binding was obtained with a 1 hour incubation, different particulate fractions were tested (see Table 15). There were slightly more counts in the 13,000-80,000g pellet than in the 2,000-13,000g pellet. As the 2,000-13,000g pellet was larger and more difficult to resuspend than the 13,000-80,000g pellet and did not contain a higher cytokinin binding activity, all further assays were performed with the 13,000-80,000g pellet.

In developing the assay, once the chemiluminescence had been eliminated, it was shown that commercially available ^{14}C-BAP does not have a high enough specific activity to detect cytokinin binding activity in Physcomitrella patens, as it has to be used at relatively high concentrations with unlabelled cytokinin already present. The amount of tissue that can be used per assay has been greatly increased by the use of glass bead grinders to homogenise the tissue.

The reproducibility of both the centrifugation assay and the equilibrium dialysis assay appears to be good with one batch of tissue. However, there is variation in the amount of label binding to the pellet between assays. This could be accounted for by the degreee of homogenising of the tissue for each assay. The homogenisation could also account for the binding activity being present in both the 2,000-13,000g pellet and the 13,000-80,000g pellet. For the amount of homogenisation to be optimised so as to release as much of the cytokinin-binding activity into the smallest differential centrifugation fraction, without denaturing it, more assays have to be done with the same tissue.

The different incubation times have shown that the displaceable binding is relatively slow, taking an hour at 4^{o}C to reach a maximum. Much of this activity may be lost by 2 hours. The loss of cytokinin-binding activity could be due to the "receptor" being unstable, or if it is a protein, being attacked by a protease in the extract. Alternatively, the radiolabel might be broken down by a cytokinin-metabolising enzyme. The optimum time for the dialysis cells needs to be determined. However, because

of the variation between assays, the different assays would have to be done at the same time.

The best assay conditions so far achieved were:

(i) using about 10g fresh weight of tissue per assay,

(ii) grinding in a glass bead homogeniser,

(iii) using the 13,000-80,000g particulate fraction,

(iv) using the tritiated BAP of high specific activity and

(v) using the centrifugation assay with a 1-hour incubation on ice.

Table 3 Cytokinin-binding activity with ^{14}C-BAP.

Strain	wild type
Tissue/assay	6.6g fresh weight.
Extraction	glass homogenised
Fraction used	13,000-80,000g pellet
Radiolabel	13.4 mCi/mmol ^{14}C-BAP
Amount/assay	0.056µg (0·12 µM)
Ligand	BAP
Assay method	Equilibrium dialysis
Membrane	Visking tubing
Bleaching	20µl Domestos

Fold excess BAP	^{12}C/^{14}C-BAP	DPM sample	DPM other	difference	displacement
0	4	8969	6131	2838	1711
0.1	4.5	7651	6032	1619	492
1	9	7736	6192	1544	417
10	54	7573	6164	1409	282
100	504	7472	6345	1127	

The results of a cytokinin-binding assay performed as described in Chapter II section 10 are shown. "Fold excess BAP" is the ratio of unlabelled BAP to radiolabelled BAP added to the mixture. The ratio of unlabelled to radiolabelled BAP present in the mixture is dependent on the specific activity of the radiolabel used. This is listed in the table under "^{12}C/^{14}C-BAP". "DPM sample" is the number of disintegrations per minute in the dialysis half cell containing the moss sample. "DPM other" is the number of disintegrations per minute in the dialysis half cell not containing the moss sample. The "difference" is the difference in counts between the two half cells, which is the amount of the radiolabel bound by the sample. The "displacement" is the number of counts bound to the sample minus the number of counts bound to the sample with the highest ratio of unlabelled BAP to labelled BAP.

Table 4 Cytokinin-binding activity without displacement ligand.

Strain	wild type
Tissue/assay	1.4g fresh weight.
Extraction	glass homogenised
Fraction used	13,000-80,000g pellet
Radiolabel	13.4 mCi/mmol ^{14}C-BAP
Amount/assay	0.056µg (0·12 µM)
Ligand	-
Assay method	Equilibrium dialysis
Membrane	Visking tubing
Bleaching	20µl Domestos

Fold excess BAP	^{12}C/^{14}C-BAP	DPM sample	DPM other	difference	displacement
0	4	8113	5211	2902	1663
0	4	7361	5212	2149	910
0	4	7014	5093	1921	682
0	4	6661	5180	1481	242
0	4	6357	5118	1239	

The results of a cytokinin-binding assay without using a displacement ligand. The samples were counted immediately after bleaching with Domestos. For further details see Table 3.

Table 5 Cytokinin-binding activity without displacement ligand.

Strain	wild type
Tissue/assay	1.4g fresh weight.
Extraction	glass homogenised
Fraction used	13,000-80,000g pellet
Radiolabel	13.4 mCi/mmol ^{14}C-BAP
Amount/assay	0.056µg(0.12µM)
Ligand	-
Assay method	Equilibrium dialysis
Membrane	Visking tubing
Bleaching	20µl Domestos

Fold excess BAP	^{12}C/^{14}C-BAP	DPM sample	DPM other	difference	displacement
0	4	6186	5179	1007	60
0	4	6219	5152	1067	120
0	4	5953	5129	824	-123
0	4	6159	5185	974	27
0	4	6079	5132	947	

The samples that were counted for Table 4 were recounted after being left overnight. The displaceable counts obtained in Table 4 have been removed by leaving the samples at 4^{o}C overnight. For further details see Table 3.

Table 6 Displacement of cytokinin-binding by BAP.

Strain	wild type
Tissue/assay	7g fresh weight.
Extraction	glass homogenised
Fraction used	13,000-80,000g pellet
Radiolabel	57 mCi/mmol ^{14}C-BAP
Amount/assay	0.033µg (0.07µM)
Ligand	BAP
Assay method	Equilibrium dialysis
Membrane	Visking tubing
Bleaching	20µl Domestos

Fold excess BAP	^{12}C/^{14}C-BAP	DPM sample	DPM other	difference	displacement
0	0.2	6895	5395	1500	388
0.1	0.3	5615	4445	1170	58
1	1.2	7380	5742	1638	526
10	10	7229	5797	1432	320
100	100	7096	5984	1112	

The cytokinin-binding activity displaced by BAP after samples were left overnight before counting to allow the chemiluminescence to die away. For further details see Table 3.

Table 7 Cytokinin-binding activity in a soluble fraction.

Strain	wild type
Tissue/assay	4.6g fresh weight.
Extraction	ground in liquid nitrogen
Fraction used	13,000g supernatant
Radiolabel	57mCi/mmol ^{14}C-BAP
Amount/assay	0.033µg (0.07µM)
Ligand	BAP
Assay method	Equilibrium dialysis
Membrane	Spectrapor 2
Bleaching	-

Fold excess BAP	^{12}C/^{14}C-BAP	DPM sample	DPM other	difference	displacement
0	0.2	8418	7697	721	267
0.1	0.3	8418	7852	566	112
1	1.2	8416	7893	523	69
10	10	8375	7818	557	103
100	100	8342	7888	454	

Cytokinin-binding activity in the 13,000g supernatant. For further details see Table 3.

Table 8 Cytokinin-binding activity detected using a ^{3}H-BAP.

Strain	wild type
Tissue/assay	5.1g fresh weight.
Extraction	motorised teflon homogenised
Fraction used	13,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng (0·18 nM)
Ligand	BAP
Assay method	centrifugation
Bleaching	20µl sodium hypochlorite

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	1389	1287	366
0	1.5	1185		
1	2.5	1368	1257	336
1	2.5	1145		
10	11.5	1289	1315	394
10	11.5	1341		
100	102	1184	1137	216
100	102	1089		
1000	1002	928	921	
1000	1002	914		

Cytokinin-binding activity using the tritiated BAP. The assay mixtures were incubated on ice for 15 minutes before centrifugation. "^{1}H/^{3}H-BAP" is the ratio of unlabelled to tritiated BAP present in the mixture. "DPM" is the number of disitegrations per minute in the 80,000g pellet. "Mean" is the average DPM of the replicates. For further details see Table 3.

Table 9 Cytokinin-binding activity detected using a ^{3}H-BAP.

Strain	wild type
Tissue/assay	4.5g fresh weight.
Extraction	glass bead
Fraction used	13,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng (0.18-M)
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	2238	2244	389
0	1.5	2249		
1	2.5	1943	2095	240
1	2.5	2247		
10	11.5	2030	2056	201
10	11.5	2081		
100	102	2208	2112	257
100	102	2016		
1000	1002	1976	1855	
1000	1002	1733		

The assay for the above results was performed in the same way as in Table 8 except that the tissue was ground in a glass bead homogeniser.

Table 10 Reproducibility of the centrifugation assay.

Strain	wild type
Tissue/assay	10.6g fresh weight.
Extraction	glass bead
Fraction used	2,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng (0.18nM)
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	2260	2282	393
		2105		
		2570		
		2295		
		2178		
10000	10002	1718	1889	
		1975		
		1949		
		1806		
		1999		

The results above show the reproducibility of the centrifugation assay and the tissue oxidiser using the tritiated BAP. For further details see Table 3.

Table 11 Binding activity with a 1-hour incubation.

Strain	wild type
Tissue/assay	8.5g fresh weight.
Extraction	glass bead
Fraction used	2,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng (0.18 nM)
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	6691	6701	1083
0	1.5	6711		
1	2.5	6364	6330	712
1	2.5	6295		
10	11.5	5973	6262	644
10	11.5	6550		
100	102	6029	6260	642
100	102	6491		
1000	1002	5701	5618	
1000	1002	5534		

For the results above, the assay mixtures were incubated at 4^{o}C for 1 hour before centrifugation. For further details see Table 3.

Table 12 Binding activity with a 1-hour incubation.

Strain	wild type
Tissue/assay	11.1g fresh weight.
Extraction	glass bead
Fraction used	2,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng (0.18nM)
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	6414	6443	1055
0	1.5	6472		
1	2.5	6464	6664	1276
1	2.5	6864		
10	11.5	6335	6438	1050
10	11.5	6540		
1000	1002	5833	5844	456
1000	1002	5854		
10000	10002	5220	5388	
10000	10002	5556		

The assay for the above results was a repeat of the assay for Table 11.

Table 13 Effect of time on binding activity.

Strain	wild type
Tissue/assay	9.2g fresh weight.
Extraction	glass bead
Fraction used	2,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng (0.18nM)
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	time hours	DPM	mean	displacement
0	1.5	1	4360	4435	792
			4510		
1000	1002	1	3868	3829	186
			3789		
10000	10002	1	3535	3643	
			3751		
0	1.5	24	3141	3189	172
			3237		
1000	1002	24	2985	3090	73
			3194		
10000	10002	24	3049	3017	
			2984		

The above results show the cytokinin-binding activity with incubations of 1 and 24 hours at 4^{o}C before centrifugation. For further details see Table 3.

Table 14 Effect of time on binding activity.

Strain	wild type
Tissue/assay	8.5g fresh weight.
Extraction	glass bead
Fraction used	2,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng (0.18nM)
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	time hours	DPM	mean	displacement
0	1.5	0	3273	3269	629
			3264		
10000	10002	0	2655	2640	
			2624		
0	1.5	1	3836	3854	1093
			3871		
10000	10002	1	2658	2761	
			2863		
0	1.5	2	3988	3277	373
			2565		
10000	10002	2	2918	2904	
			2889		

The above results show the binding activity when the assay mixture is incubated on ice for 0, 1 and 2 hours. For further details see Table 3.

Table 15 Binding activity in different particulate fractions.

Strain	wild type
Tissue/assay	6.2g filtered fresh weight
Extraction	glass bead
Fraction used	2,000-13,000g pellet or 13,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng (0.18nM)
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP		DPM	mean	displacement
0	1.5	13,000g pellet	5997	6142	750
			6274		
			6156		
10000	10002		4950	5392	
			5216		
			6010		
0	1.5	80,000g pellet	3635	3734	878
			3833		
10000	10002		2742	2856	
			2969		

The results above show the cytokinin-binding activity in different particulate fractions. For further details see Table 3.

CHAPTER VI

The mechanism of action of cytokinin

Results and discussion

1. Using the cytokinin binding assay

The assay developed in the previous chapter has been used to determine the nature of the binding activity, to purify it further and to determine the type of tissue containing the activity. If the binding is due to a specific site on a protein it is likely to be lost by heat treatment. Non-specific binding is more likely to be heat-stable or even increase by heat treatment due to exposure of non-specific sites as proteins are denatured (Sussman and Kende, 1978). The heat-stability of the binding fraction was tested by boiling the extract for two minutes. The binding activity was greatly reduced by heat treatment (Table 16). If the binding activity that has been detected in the particulate fraction is mainly due to a single or a few specific binding sites, further study of these binding sites would be aided by solubilising them out of the membrane. Venis (1977a) has solubilised auxin binding fractions from corn membranes by treating them with acetone. This method (described in Chapter II section 5 b.) was used on a 13,000-80,000g particulate fraction from moss. As the acetone treatment did not appear to solubilise much of the pellet, both the re-extracted pellet and supernatant were tested for cytokinin-binding activity. Very little of the binding to either the pellet or the supernatant was displaced by excess unlabelled BAP (Table 17).

An alternative method is to solubilise membrane components with a detergent. This was achieved by using the Triton method of Thomas et al. (1984). The supernatant was obtained by treating the 13,000-80,000g pellet with Triton and then centrifuging at 80,000g. The pellet, in this instance, that was tested for cytokinin-binding activity was the 13,000-80,000g pellet which had not been treated with Triton. 85% of the cytokinin-binding activity of this pellet appears to be solubilised by this Triton treatment (Table 18). If facilities had allowed, the pellet obtained after treatment with Triton could also have been tested for cytokinin-binding activity. If all three fractions could have been tested, a useful check would have been to see if the total binding activity in the Triton-treated pellet and supernatant was equal to the binding activity in the untreated pellet.

As shown in Chapter III section 4, moss tissue grown in phosphate-free medium produces mainly caulonemata. The caulonemal filaments produce side branch initials and gametophores. There is usually only one gametophore per filament. The side-branch initials do not develop further, but will differentiate into buds if supplied with cytokinin. Such tissue may be enriched for a cytokinin receptor. The results in Table 19 are from a 15-litre liquid culture of wild type grown in phosphate-free medium (See Chapter II section 1 e.). The amount of tissue produced in this culture was much less than would be expected for a normal 15-litre culture. The tissue was tougher than usual and so was ground in 8 volumes of MES1 instead of 4 volumes. A relatively high amount of cytokinin-binding activity was obtained for this amount of tissue (activity with phosphate-

free culture is 630 DPM/g fresh weight, activity with tissue grown on phosphate is 100-140 DPM/g fresh weight).

As phosphate-free-grown culture of wild type appears to be a good source of cytokinin-binding activity, an attempt was made to solubilise the binding activity from a phosphate-free culture. This result is shown in Table 20. The pellet was the 80,000g pellet after treating with Triton. Most of the activity has remained in the pellet. This could be due to the phosphate-free tissue being tougher and not being broken up by the Triton so easily as normal tissue. However, the very small amount of counts bound to the sample indicate that the Triton is not responsible for the binding shown in Table 18. A Triton-BAP interaction was reported by Gardner et al. (1978), using a gel filtration method to assay for cytokinin-binding activity. This interaction prevented Gardner et al. detecting whether the Triton denatured or solubilized the binding activity they reported in a moss particulate fraction.

The culture grown without phosphate in Table 19 gave a higher amount of displaceable binding per gram of tissue than tissue on normal Knops medium, but was very tough. On a low phosphate and low nitrate medium, wild type grows as caulonemata with side branch initials, but these do not develop further. However, these side branch initials, and therefore this type of tissue, is potentially suitable for seeking cytokinin receptors. Such a culture was used to obtain the results shown in Table 21. This gave displaceable binding. The amount of cytokinin-binding activity was similar to that of a normal-grown wild type culture.

In the identification of a biologically meaningful cytokinin receptor, it is useful to compare cytokinin binding activity in mutants that do not respond to cytokinin. Showing that a mutant that does not have the cytokinin binding activity also does not have the ability to respond to cytokinin would support the hypothesis that such binding was involved in the hormone response. bar mutants were originally isolated as benzyl-adenine resistant mutants. bar 1 produces some caulonemata but does not produce buds even with exogenous cytokinin. This mutant therefore has the phenotype of a cytokinin non-responding mutant that was screened for as described in section 8 of chapter II. bar 1's phenotype is that of a mutant that has no functional cytokinin receptor, but there are other possibilities for this phenotype, such as a block in the response to cytokinin after the receptor. bar 1 was therefore used to see if it lacked the cytokinin-binding activity that has been obtained in the wild type. The results in Table 22 show that bar 1 does have a similar binding activity as wild type in the 13,000-80,000g pellet (activity is 330 DPM/g fresh weight).

The results in Table 23 were also obtained with bar 1, but included a dilution of the pellet to see if the binding activity of the pellet was limiting. This binding activity is similar to that obtained in the previous experiment (220 DPM/g fresh weight) and is reduced to almost half by the dilution of the resuspended pellet by a half before being used for the assay. This indicates that the extract is limiting.

Wild type has to be exposed to cytokinin for about three days to prevent a bud dedifferentiating into protonemata. If each cell of a bud has receptors for cytokinin, then mutants that

overproduce buds may have more receptors than wild type. ove 78 was used in a cytokinin-binding assay as ove's produce abnormal buds which may be a good source for cytokinin receptors. ove 78 showed displaceable cytokinin binding (see Table 24). This binding is reduced when the pellet is diluted, again demonstrating that the displaceable cytokinin-binding fraction is limiting. ove 78 appears to have similar cytokinin-binding activity as wild type (250 DPM/g fresh weight).

Using the cytokinin-binding assay developed in the previous section, the binding activity in the 13,000-80,000g particulate fraction in Physcomitrella patens appears to be heat labile, which suggests that the displaceable binding could be specific binding to protein. The binding activity is lost from both the pellet and the supernatant when treated with acetone. If the binding activity is due to an intrinsic membrane protein, removing the lipids with acetone could denature the protein and so result in no binding activity in the pellet or supernatant. When a membrane fraction is solubilised with a detergent, hydrophobic parts of a protein are less likely to be exposed and so are less likely to be denatured. The Triton method used appeared to solubilise most of the binding activity that was present in the particulate fraction. Although it was not possible to quantify the degree of binding for the amount of tissue used, the Triton treatment does appear to have enriched for binding activity as some of the particulate fraction was not solubilised by the Triton.

The phosphate-free culture had more binding activity per gram tissue than tissue grown on normal Knops medium. As this

tissue was mainly caulonemal, which is the target tissue for cytokinin with respect to bud differentiation, it is likely to have more cytokinin receptors than the chloronemata. However, this binding activity in the phosphate-free culture was not solubilised with Triton. So, either the binding activity in the phosphate-free culture is different from that of a culture grown in the presence of phosphate or is more difficult to solubilise from the tougher caulonemal tissue. The low phosphate and nitrate culture, which contained more side branch initials than the phosphate-free culture, did not show any more binding activity than a normal wild type culture and so did not appear to enrich for a possible cytokinin receptor.

bar 1 was tested for cytokinin-binding activity as it does not respond to exogenous cytokinin and so is a candidate for a mutant that does not have a functional receptor. This mutant has been shown to have similar displaceable cytokinin-binding activity as the wild type and therefore if this binding is due to a receptor, bar 1 must be blocked at a later stage in the initiation of the gametophore. When the pellet is diluted by a half, the number of displaceable counts in the pellet are also reduced by about a half. This indicates that the displaceable binding in the pellet is limiting. The dilution effect is also shown with ove 78, which appears to have more displaceable binding per gram of tissue than wild type. ove mutants could well have more cytokinin receptors than wild type as a bud needs cytokinin to be present for the first three days after initiation to prevent dedifferentiation, and so each cell of the abnormal buds of the ove mutants may contain cytokinin receptors. If this is the case, then ove mutants would be a good source for the

isolation of a cytokinin receptor.

Table 16 Effect of heat on binding activity.

Strain	wild type
Tissue/assay	4.9g filtered fresh weight
Extraction	glass bead
Fraction used	2,000-80,000g pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
unboiled				
0	1.5	5198	5263	1094
		5308		
		5283		
10000	10002	4060	4169	
		4140		
		4307		
boiled				
0	1.5	4685	4702	167
		4718		
10000	10002	4488	4535	
		4581		

Before being used for the binding assay, some of the particulate fraction was heated to 100°C for 2 minutes. For further details see Table 3.

Table 17 Binding activity after treatment with acetone.

Strain	wild type
Tissue/assay	4.7g filtered fresh weight
Extraction	glass bead
Fraction used	13,000-80,000 pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	equilibrium dialysis
membrane	Spectrapor 2
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM sample	DPM other	difference	displacement
supernatant					
0	1.5	3551	3479	72	180
10000	10002	4286	4394	-108	
pellet					
0	1.5	6333	2695	3638	362
10000	10002	6379	3103	3276	

One method used to attempt to solubilize the cytokinin-binding activity was to treat the resuspended particulate fraction with 20 times volume of acetone at 4^{o}C. The pellet was centrifuged at 38,000g and re-extracted in MES2 as described in Chapter II section 10 b (i). Both the acetone-treated supernatant and pellet were tested for cytokinin activity. For further details see table 3.

Table 18 Binding activity in a Triton solubilised fraction.

Strain	wild type
Tissue/assay	6.2g filtered fresh weight
Extraction	glass bead
Fraction used	13,000-80,000 pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	equilibrium dialysis
membrane	Spectrapor 2
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM sample	DPM other	difference	displacement
supernatant					
0	1.5	8211	1709	6502	953
10000	10002	7354	1805	5549	
pellet					
0	1.5	6766	2306	4460	1112
10000	10002	6216	2868	3348	

An alternative method to the one used in Table 17, of solubilizing the cytokinin-binding activity is to resuspend the pellet in 20% Triton. This method is described in Chapter II section 10 b (ii). The supernatant was obtained by treating the 13,000-80,000g particulate fraction with 20% Triton and then centrifuging at 80,000g. The pellet was the 13,000-80,000g pellet that was not treated with Triton. For further details see table 3.

Table 19 Binding activity in a phosphate-free grown culture.

Strain	phosphate-free grown wild type
Tissue/assay	1.2g filtered fresh weight
Extraction	glass bead
Fraction used	13,000-80,000 pellet
Radiolabel	i2Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	2457	2428	754
		2410		
		2417		
10000	10002	1671	1674	
		1728		
		1624		

A phosphate-free-grown culture was used for a cytokinin-binding assay, as such a culture consists mainly of caulonemata, the target cells for cytokinin action (see Chapter II section 10). The binding activity obtained with this tissue was by using only 1.2g fresh weight of tissue per assay. For further details see Table 3.

Table 20 Triton solubilisation of binding activity from a phosphate-free-grown culture.

Strain	phosphate-free grown wild type
Tissue/assay	3.3g filtered fresh weight
Extraction	glass bead
Fraction used	13,000-80,000 pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	equilibrium dialysis
membrane	Spectrapor 2
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM sample	DPM other	difference	displacement
supernatant					
0	1.5	7289	1986	5303	-31
0	1.5	7728	1896	5832	498
10000	10002	7545	2211	5334	
pellet					
0	1.5	8448	1698	6750	2202
10000	10002	7018	2470	4548	

An attempt was made to solubilize the binding activity obtained from the phosphate-free culture in Table 19. The supernatant and the pellet used in the assay were the ones obtained after treating the 13,000-80,000g pellet with Triton and centrifuging at 80,000g. For further details see Table 3.

Table 21 Binding activity in a culture grown on medium containing reduced amounts of phosphate and nitrate.

Strain	wild type
	low phosphate and nitrate
Tissue/assay	6.4g filtered fresh weight
Extraction	glass bead
Fraction used	13,000-80,000 pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	equilibrium dialysis
membrane	Spectrapor 2
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM sample	DPM other	difference	displacement
0	1.5	9946	2817	7129	1209
		9336	2415	6921	
		8479	2299	6180	
10000	10002	7583	2217	5366	
		7709	2008	5701	

Although the phosphate-free wild type cultures used in Tables 19 and 20 consisted almost entirely of caulonemata, the filaments did not have side branch initials that are present on a culture grown on solid medium without phosphate. Side branch initials could be induced to form without developing further by growing the culture in medium with a reduced amount of phosphate and nitrate. For further details see Table 3.

Table 22 Cytokinin-binding activity in the mutant bar 1.

Strain	bar 1
Tissue/assay	3.4g fresh weight
Extraction	glass bead
Fraction used	13,000-80,000 pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	4521	4359	1129
		4296		
		4259		
10000	10002	3592	3230	
		3046		
		3051		

bar 1 is a mutant that does produce some dark-grown filaments, bud does not produce buds, even with exogenous cytoknin. This mutant has the phenotype of a mutant that does not have a functional cytokinin receptor, although other mutants with this phenotype could have a functional receptor but are blocked at a later stage in the induction of the gametophore. For further details see Table 3.

Table 23 Cytokinin-binding activity in the mutant bar 1.

Strain	bar 1
Tissue/assay	6.8g fresh weight
Extraction	glass bead
Fraction used	13,000-80,000 pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	6165	5814	1501
		5633		
		5643		
10000	10002	4513	4313	
		4193		
		4233		
resuspended pellet diluted by 1/2				
0	1.5	4082	3883	900
		3683		
10000	10002	3325	2983	
		2640		

The above results were obtained using bar 1 as in Table 22. Some of the resuspended pellet was diluted by a 1/2 to see if the binding activity was limiting. For further details see Table 3.

Table 24 Cytokinin-binding activity in the mutant ove 78.

Strain	ove 78
Tissue/assay	7.3g filtered fresh weight
Extraction	glass bead
Fraction used	13,000-80,000 pellet
Radiolabel	12Ci/mmol ^{3}H-BAP
Amount/assay	0.08ng
Ligand	BAP
Assay method	centrifugation
Bleaching	oxidiser

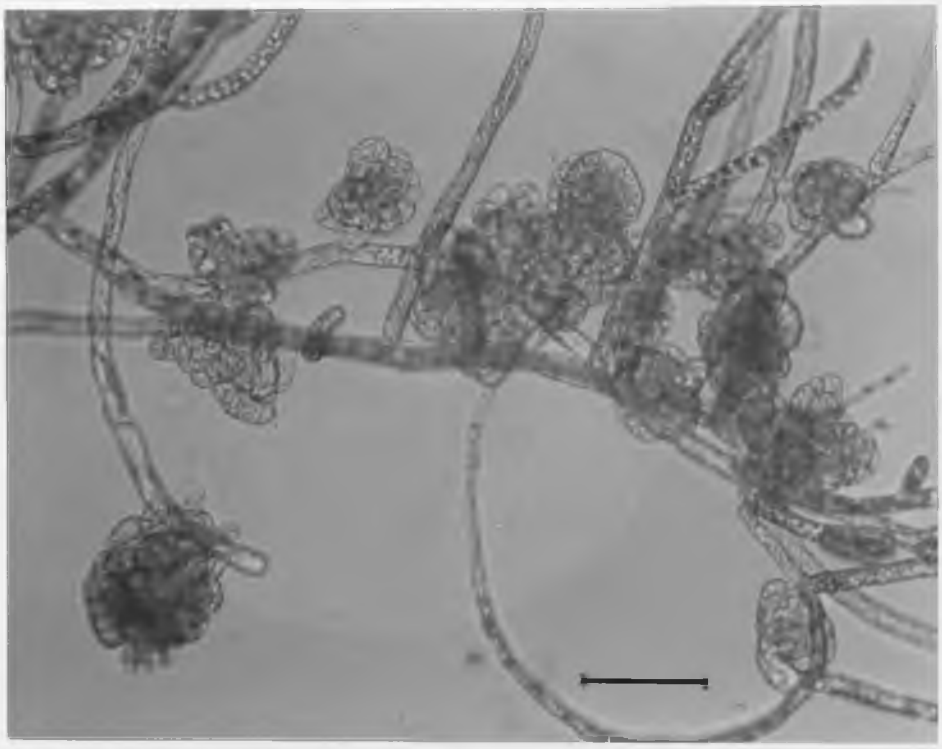
Fold excess BAP	^{1}H/^{3}H-BAP	DPM	mean	displacement
0	1.5	4927	4720	1855
		4775		
		4457		
10000	10002	2998	2865	
		2737		
		2860		
resuspended pellet diluted by 1/2				
0	1.5	2999	2919	781
		2839		
10000	10002	2520	2138	
		1755		

ove mutants are mutants that over-produce gametophores. The phenotype of ove 78 is due to it overproducing cytokinin. Such a mutant may be rich in cytokinin receptors. Some of the resuspended pellet was diluted by a 1/2 to see if the binding activity was limiting. For further details see Table 3.

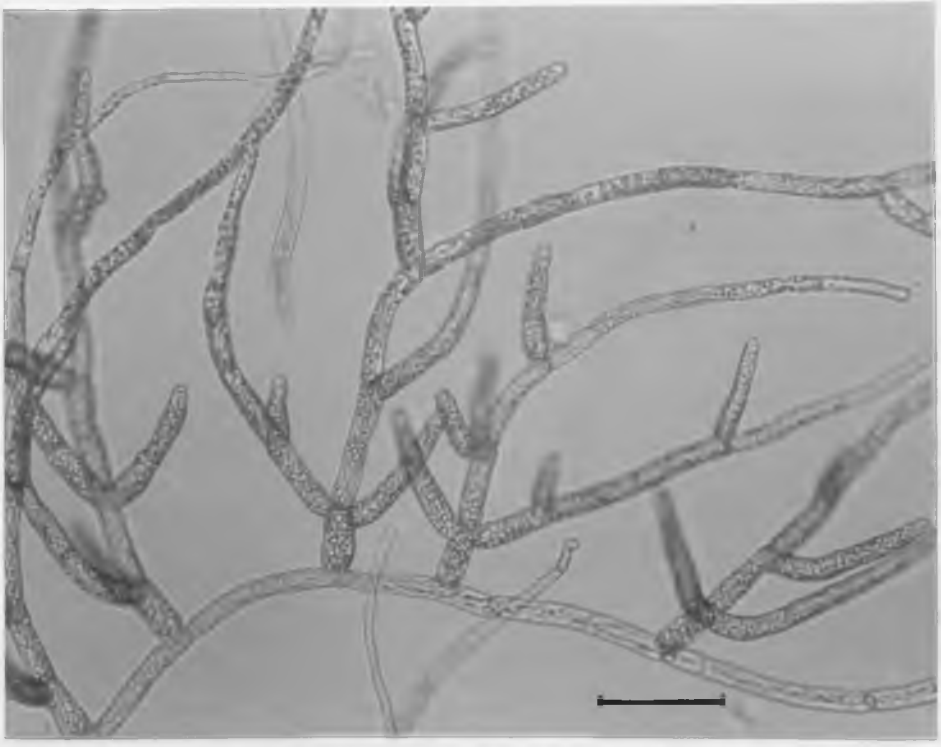
2. Biochemical initiation of the gametophore

The calcium ionophore A23187 (a gift from E. Lilly and Co., Indianapolis, U.S.A.) was used to see if increasing intracellular calcium induced bud induction in Physcomitrella patens as it does in Funaria hygrometrica (Saunders and Hepler, 1982). Wild type spores were germinated for 8 days on Knops medium overlaid with cellophane. Four germinated spores were added to each 50 ml flask containing 15 ml of a modified Knops medium (0.3 mM $Ca(NO_3)_2$ and 0.1 mM $MgSO_4$). A small volume of a 15 mM stock of A23187 in 100% methanol was added to each flask, after autoclaving, to give concentrations of 0, 10, 20, 40 and 60 μM. All the flasks, and a control flask with 1 μM BAP, each were inoculated with 4 plants, which had been germinated for 8 days. The plants were exposed to the ionophore for 2 weeks. Preliminary experiments were performed by transferring the plants into fresh medium every three days to prevent the build-up of endogenous cytokinin. All cultures from these experiments appeared to be mainly chloronemal, even the ones in 1 μM BAP. This could be due to damage to the plants in transferring them to fresh medium, causing the plants to undergo regeneration; thus experiments using A23187 were repeated without transferring to fresh medium. After 7 days, the flasks containig 1μM BAP had produced many abnormal buds (figure 19a). The flasks containing no BAP or A23187 had produced normal branching filaments (figure 19b). There was no visible effect of the ionophore below 20 μM. At 60 μM the ionophore was very inhibitory. At 40 μM the plants grew as a tight sphere of highly-branched chloronemata (figures 20a and 20b). Some caulonemata were present, but no signs of bud

Figure 19a and b. Effect of 1μM BAP on a wild type culture.

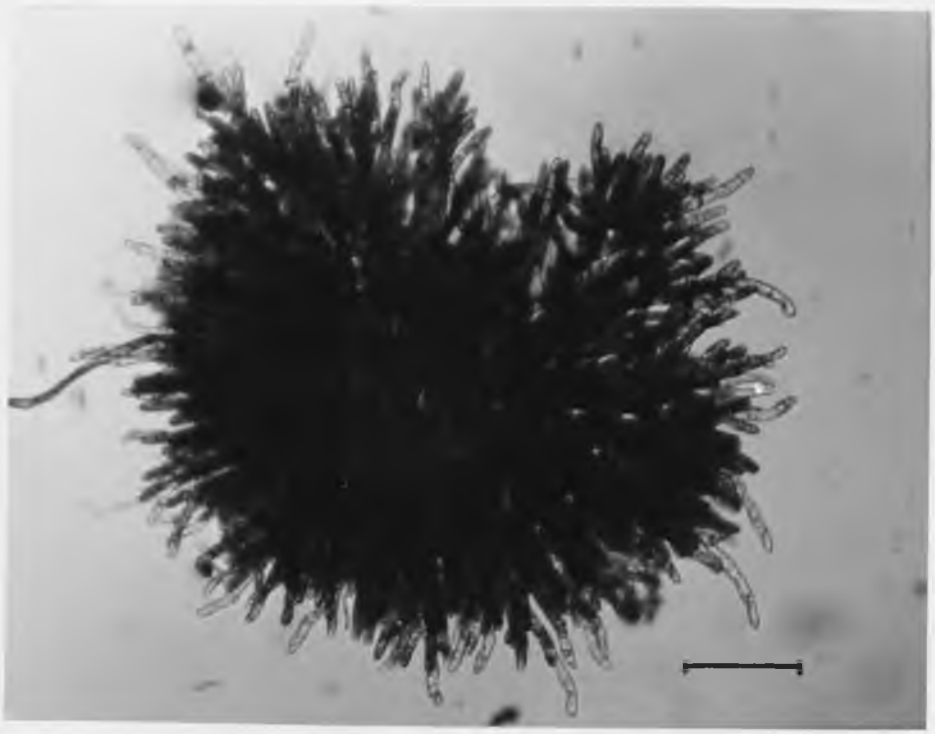


A culture grown for 8 days in minimal medium and 14 days in the presence of 1μM BAP. (Bar=100μm).



A culture grown from a spore in liquid medium for 22 days. (Bar=100μm).

Figure 20a and b. Effect of A23187 on a wild type culture.



A culture grown for 8 days in minimal medium and 14 days in the presence of 40μM A23187. (Figure 20a bar=250μm. Figure 20b bar=100μm).

induction. The plants resembled nar mutants with more primary chloronemata than a normal wild type culture.

The effect of the calcium ionophore A23187 on Physcomitrella patens is very different from the effect on Funaria hygrometrica reported by Saunders and Hepler (1982). The calcium ionophore appeared to induce side-branching but not the initiation of buds. In Physcomitrella patens increasing intracellular calcium appears to induce side branching but may not be a second messenger for cytokinin-induced bud production. Bud production can be prevented in Physcomitrella patens by concentrations of calcium ions above 10mM (McClelland, personal communication). These high levels of calcium produce mainly highly-branched primary chloronemata. This is very similar to the effect of low levels of calcium (0.3μM) in the presence of the calcium ionophore A23187. Therefore, increasing intracellular calcium by a calcium ionophore or by high levels of extracellular calcium appears to induce branching of primary chloronemata. The preventing of bud induction by calcium could be due to either a direct or an indirect effect by promoting branching of primary chloronemata. There would then be only a small amount of target tissue for bud induction and so the role of calcium in the action of cytokinin in Physcomitrella patens cannot be discounted. The results of the calcium ionophore on Physcomitrella patens support the observations of Bopp (1984) on Funaria hygrometrica, that although an increase in calcium may be an important step in the induction of the bud, it is not a replacement for cytokinin.

CHAPTER VII

General discussion

The results of this investigation have shown that the mode of action of cytokinin is difficult to elucidate, even in the simple model organism Physcomitrella patens, which has a clearly defined target tissue with a specific morphogenetic response to cytokinin. A useful method of showing that the binding of a particular hormone to a sub-cellular fraction is biologically meaningful is to demonstrate the lack of the same type of binding with a mutant that is unable to respond to endogenous or exogenous hormone. The isolation of a cytokinin non-responding mutant in Physcomitrella patens has been shown to be more difficult than would be expected if such a phenotype could arise by a single mutational event. Some of the possible reasons for cytokinin non-responding mutants not being isolated were suggested in Chapter III section 1. One possibility was that insufficient mutants had been screened. However, other mutants, such as cytokinin over-producing mutants, are obtained at a frequency of about 1 in every 1000 clones screened. Therefore it is unlikely that non-responding mutants can be obtained by a single mutational event. This could be due to there being duplicate genes coding for a cytokinin receptor. Alternatively, the same cytokinin receptor might be essential for some process such as cell division, or for the production of the filaments which develop in the dark and which were used as target cells for the screening procedure. If cytokinin non-responding mutants are not lethal, then screening for such mutants in ove revertants may be more successful. The

screening procedure could be done in two stages. The majority of mutants could be eliminated by their continued over-production of buds. Only a few mutants would have to be tested for a lack of bud production in the presence of supplied cytokinin. Screening ove revertants for cytokinin non-responding mutants would also enable the isolation of cytokinin non-responding mutants even if such mutants were unable to produce caulonemal filaments in the dark.

One mutant isolated in the mutation runs for cytokinin non-responding mutants, ove 409, could be useful in the isolation of ove revertants. As this mutant is temperature-sensitive, having an ove phenotype above 23°C and a wild type phenotype below 17°C, ove revertants could be selected at the non-permissive temperature. Some of the mutants that do not produce buds at the non-permissive temperature may not produce buds at the permissive temperature when supplied with cytokinin. And mutants that do not respond to supplied cytokinin may be defective in a cytokinin receptor. The non-responding phenotype could be studied at 17°C, in the absence of the ove phenotype. By the use of protoplast fusion, ove 409 has been shown to be in complementation group A and is recessive to wild type.

The phenotype of ove 409 has been shown to be due to the over-production of isopentenyladenine at the non-permissive temperature. The two other strains studied for cytokinin production at 24 and 15°C, ove 78 and wild type, also produced more isopentenyladenine per mg dry weight at the higher temperature. However, there was only about a 6-fold change in cytokinin production for these strains whereas ove 409 produced

14 times more isopentenyladenine at the higher temperature. The phenotype change of ove 409 with temperature is due to a change in cytokinin production, from similar levels as those found in wild type to higher levels, with a change from 15 to 24°C. This change in cytokinin production may be partially due to the same change in cytokinin production found in wild type and ove 78. The temperature-dependence of all three strains on cytokinin production may be due to slower growth, and hence delayed differentiation, at the lower temperature . The cultures grown at 15°C were more chloronemal than the cultures at 24°C, and so temperature may have a more direct effect on inhibiting differentiation. A similar temperature effect could be investigated with respect to auxin production. Slower or delayed auxin production or response would result in a culture that was more chloronemal at the lower temperature. Such a culture may produce less cytokinin particularly if cytokinin production occurs in the caulonemata. A temperature-sensitive ove mutant could be obtained if the level of over-production of cytokinin was at such a level that the six-fold decrease was sufficent to drop the level of cytokinin production to levels found in wild type. The change in production of 2iP by ove 409 between these two temperatures is larger than with ove 78 and wild type. Therefore ove 409 may have an additional mechanism resulting in its temperature-sensitive phenotype. If the temperature sensitivity is due to a protein losing activity at the non-permissive temperature, it is most likely to be a regulator of cytokinin synthesis or an enzyme involved in cytokinin metabolism.

The induction of gametophores in Physcomitrella patens, even

following treatment with high levels of cytokinin, does not occur in the dark. There appears to be a specific light requirement for the differentiation of the caulonemal side branch into a bud. When dark-grown caulonemal filaments are supplied with cytokinin and exposed to 556nM quanta $m^{-2}s^{-1}$ for three days, bud induction is strong with red light of around 665nm. Bud induction does not occur with far-red light or with light below about 600nm, apart from the small amount of bud induction with blue light of 442nm. This response to red light indicates that the photoreceptor involved could be phytochrome. Evidence to support the involvement of phytochrome has come from experiments where cultures were exposed to red light before the addition of cytokinin. Some plates were exposed to half-an-hour far-red light immediately after the red light. All cultures were then placed in the dark, and after different time intervals supplied with cytokinin and left for three days for buds to develop in the dark. The bud induction that occurred due to the red light exposure was reduced by exposure to far-red light. The amount of bud induction was also reduced if the red light exposure was separated from the addition of cytokinin by more than four hours. Both types of reduction of bud induction support the role of phytochrome, as the conversion of P_{fr} to P_r occurs rapidly under far-red light and slowly in the dark. Therefore the response to cytokinin to induce the gametophore may only occur when the equilibrium of phytochrome is towards P_{fr}. The involvement of phytochrome in bud induction could be investigated further by exposing cultures to alternating red and far-red light of different intensities and duration.

One unexpected feature of bud induction, when the cytokinin

is supplied after the exposure to red light, is that the amount of bud induction was greater if the light exposure was separated from the addition of cytokinin by a two-hour dark period than when the cytokinin was added immediately after the light period. This occurred when the length of the light period was 24, 30 or 48 hours. This phenomenon indicates that there is a dark reaction involved in the response to cytokinin. This reaction may be inhibited when cytokinin is applied. Bud induction with cultures where the cytokinin is applied after the dark period but before exposure to the red light was about 40%. This was similar to the bud induction of cultures where the red light exposure and the addition of cytokinin were separated by two hours. Therefore, it may be possible for this dark reaction to occur before or after the exposure to light. The induction of a gametophore on a target cell appears to require red light and a short dark period before the addition of cytokinin if bud induction is going to occur in the dark. Bud induction is not reduced by the absence of a dark period if the cytokinin and light are present at the same time. This phenomenon could be investigated further to find out what length of dark period between the light exposure and the addition of cytokinin gives the maximum bud induction. The light period could also be interspersed with dark periods to see if the amount of bud induction could be increased. The length and intensity of the red light exposure could also be investigated to see if they affect the requirement for a dark period before the addition of cytokinin. It may be interesting to see if speed of bud induction can be altered by putting a light-grown culture in the dark before the addition of cytokinin.

Light could be involved in the synthesis or sensitivity of a cytokinin receptor, or in a later process in the development of the bud. If red light was required for the synthesis of the receptor, far-red light would have to able to inactivate the receptor. If red light is involved in both the synthesis and sensitivity of a cytokinin receptor, the reactivation of the cytokinin response by red light after a far-red light exposure should be less than the 6 hours originally required for bud induction.

Studies on the effect of auxin and cytokinin on the regeneration of protoplasts were undertaken to see if isolated protoplasts had the ability to respond to the hormones. This would show that the cells contained the receptors during the process of regeneration. Protoplasts of Physcomitrella patens are unaffected by the presence of NAA and BAP during regeneration. With respect to cytokinin, the lack of response to the hormone could be due to the loss of ability to respond or due to the regenerating protoplasts being derived from chloronemal cells. It was not possible to investigate this further as all enzymes used to try to make protoplasts from dark-grown caulonemal filaments, which would initially contain receptors for cytokinin, had no observable effect. If protoplasts isolated from caulonemal filaments could be shown not to respond to cytokinin they might be a suitable material for showing the amount and type of cytokinin binding that was not due to a true cytokinin receptor.

In developing the assays for a cytokinin receptor, four main improvements have been made to the original assay. These were to

increase the amount of tissue used, the use of a tritiated BAP, the oxidation of the samples before counting and incubation of the samples for 1 hour before centrifugation. Attempts to detect cytokinin binding activity in both particulate and soluble fractions with ^{14}C-BAP were unsuccessful. m-iodo-BAP was therefore sythesised and sent to Amersham International to be tritiated. The ^{3}H-BAP thus produced had a much higher specific activity than the ^{14}C-BAP. By the use of glass bead homogenisers a larger amount of tissue could be prepared for each assay. Using the increased amount of tissue and the tritiated cytokinin, displaceable cytokinin binding activity was detected. More consistent results were obtained by the oxidation of the samples rather than bleaching them. With the centrifugation assay, maximum binding activity was found by incubating the samples on ice for 1 hour before isolating the particulate fraction by centrifugation. The binding activity was reduced with longer incubations. The assay may be improved further by a higher degree of purification or by incubating the assay mixture at different temperatures. The equilibrium dialysis binding assay could be performed for longer or shorter times than the 5 hours that have been used.

The centrifugation and equilibrium dialysis binding assays that have been developed here have been used to characterise the type of cytokinin binding that has been detected in P.patens to see if it has the characteristics expected for a cytokinin receptor. The cytokinin binding activity, the binding of the radioactive cytokinin that is prevented from binding in the presence of excess unlabelled cytokinin, has been shown to be

greatly reduced by boiling the sample before the assay. The cytokinin binding that is not displaced with excess unlabelled cytokinin is not reduced after heat treatment. This demonstrates that the binding activity is heat labile, as would be expected for a receptor if it was a protein. Heat treatment causes many proteins to be denatured and so any specific binding sites are likely to be lost. The background binding, the cytokinin binding that is not displaced by excess unlabelled cytokinin, was not reduced by heat treatment. This is the result expected if the background binding is due to non-specific binding to macromolecules and lipids.

The study of the binding fraction in the particulate fraction would be aided if the cytokinin-binding component could be isolated from the particulate fraction. This can be achieved by solubilising the binding component. The acetone treatment, which has been used to solubilise an auxin-binding particulate fraction in maize coleoptiles (Venis, 1977a), greatly reduced cytokinin binding activity in both the supernatant and the pellet. Acetone dissolves the lipids and so either the cytokinin-binding component is sensitive to acetone or has to be associated with membrane to remain active. This would be the case for an intrinsic protein. The Triton method, used to solubilise the cytokinin-binding activity, recovered about 85% of the displaceable cytokinin-binding activity. Triton appears to have solubilised the cytokinin-binding component. If the cytokinin-binding component that has been solubilised is an intrinsic protein, the Triton presumably has not denatured it due to solubilising it with some of the membrane or by protecting the hydrophobic domains. Gardner et al. (1978) reported that

treatment with Triton removed the cytokinin-binding activity in the particulate fraction. The loss of cytokinin-binding activity could be due to solubilisation or denaturation of the cytokinin-binding component. Gardner et al. were unable to distinguish between the two possibilities as the Triton appears to cause binding in the soluble fraction, using a gel filtration binding assay. The Triton interfering in the cytokinin binding, reported by Gardner et al. (1978), does not appear to be responsible for the cytokinin-binding activity obtained by equilibrium dialysis. This is demonstrated by the attempts to solubilise the binding activity from phosphate-starved cultures (see below). In such a culture almost all the cytokinin-binding activity remained in the Triton treated pellet.

Cytokinin binding assays have been performed using phosphate- and nitrate-starved tissue. Tissue grown under conditions of phosphate starvation consists mainly of caulonemata, the target tissue for cytokinin action. For a much smaller amount of tissue per assay, the phosphate-free-grown cultures gave almost as much cytokinin-binding activity with about a third the amount of background binding. This indicates that the phosphate-starved tissue, which may be richer in cytokinin receptors, is richer for displaceable cytokinin-binding activity. The binding-activity present in a culture grown in phosphate-free medium was not solubilised by treatment with Triton, but was still present in the Triton-treated pellet. The inability to solubilise the cytokinin binding component in the phosphate-starved culture may be due to other components in this type of tissue which prevent solubilisation of the cytokinin-binding component. The phosphate-starved caulonemal

filaments are tougher than tissue grown on complete medium, and so may not release membrane components so readily. These filaments are similar in appearance and toughness to dark-grown caulonemal filaments. The difficulty in releasing the cytokinin-binding component of phosphate-starved tissue may be related to the difficulty in isolating protoplasts from dark-grown caulonemal filaments.

As mentioned previously, in the identification of a hormone receptor it is useful to have mutants which are altered in their ability to bind the hormone. Mutants that are unable to bind the hormone are particularly useful in showing that a mutant that lacks the ability to respond to a particular hormone also lacks the specific hormone binding that is present in the wild type. Although no cytokinin non-responding mutants were isolated, a mutant that was originally isolated as a BAP resistant mutant, bar 1, does produce a few dark-grown caulonemal filaments, but does not produce buds when supplied with BAP. This mutant was used in a cytokinin-binding assay as it has the phenotype of a mutant that does not have a receptor. bar 1 showed similar cytokinin binding activity. If this binding activity is due to the same component as that in wild type, then this component is not the cytokinin receptor for bud induction, or bar 1 is blocked at a later stage in the production of the gametophore.

Other types of mutants that could be useful in the isolation of a cytokinin receptor are mutants that contain more receptors than wild type. As cytokinin has to be present for more than 48 hours to prevent dedifferentiation of the bud, a young bud may be rich in cytokinin receptors. Mutants that over-produce cytokinin

contain many abnormal buds and therefore may be a richer source of cytokinin receptors than wild type. ove 78 was found to contain similar displaceable cytokinin-binding activity to wild type. Although the assay cannot be used quantitatively, the results suggest that this mutant and bar 1 have almost twice the amount of displaceable cytokinin-binding activity per gram of tissue than wild type. Mutants should be useful in further studies on cytokinin binding. If there is more cytokinin-binding activity in ove mutants, as well as supporting that this binding activity is due to a receptor, such mutants may be a good source for the isolation and identification of the component responsible for the cytokinin-binding activity. ove mutants may also be useful in the isolation of cytokinin non-responding mutants. As mentioned earlier, the temperature-sensitive mutant, ove 409, may be particularly useful in this respect.

Other improvements to the isolation of a cytokinin-binding fraction as a putative receptor may be to check for and reduce any protease activity that may be present, as this could be destroying the cytokinin receptor before it can be assayed. Cytokinin-binding components may be purified on an affinity column to which has been linked a cytokinin such as BAP. If such a column could be used it might be a quick method to isolate cytokinin-binding components from a crude tissue homogenate and so reduce the possible reduction in activity due to the action of proteases. If the cytokinin-binding fraction could be purified further, it would the be worth assaying for protein and calculating the amount of cytokinin-binding activity per mg of protein. Further purification would also show whether the assay technique is consistent enough to use the data for kinetics

calculations. When consistent data are obtained, these can be used to calculate dissociation constants and the number of binding sites by the use of Scatchard plots (Scatchard, 1949).

Work so far on the possible receptor for cytokinin in the moss Physcomitrella patens, displaceable cytokinin-binding activity has been isolated in a particulate fraction which is denatured by heat, appears to be present at higher concentrations in phosphate-starved wild type and in an ove mutant than in wild type. It appears to be solubilised by Triton, but not acetone. This binding fraction therefore satisfies the criteria for a membrane protein which binds cytokinin and is richer in tissue which is expected to be rich in a cytokinin receptor. This fraction could contain the cytokinin receptor; however, a cytokinin carrier protein might also be expected to have similar characteristics.

Without further kinetic data, the results of cytokinin binding in the moss Physcomitrella patens cannot be compared with cytokinin binding associated with the ribosomes (Berridge et al.,1970; Fox and Erion,1975; Takegami and Yoshida, 1977). The cytokinin binding activity in the 13,000-80,000g particulate fraction of the moss Physcomitrella patens is similar to that reported by Gardner et al. (1978) for the moss Funaria hygrometrica. The cytokinin-binding activity in both mosses has only been detected using a cytokinin with a high specific activity, is in a 13,000-80,000g particulate fraction, is heat labile and is removed from the pellet by Triton. The cytokinin-binding activity in Funaria hygrometrica was only reduced by a half by heating to 100oC for 5 minutes whereas that

in Physcomitrella patens was reduced by over 6-fold after heating to 100°C for 2 minutes. This binding activity also appears to be solubilised by treatment with Triton. Gardner et al. were unable to determine whether the loss of cytokinin-binding activity in the particulate fraction of Funaria hygrometrica was due to denaturation or solubilisation of the cytokinin-binding component by the detergent. For the cytokinin-binding component to be isolated and identified it is useful to be able to solubilise it from the membrane. One other useful feature of the Physcomitrella patens system is that there are developmental mutants in the cytokinin response available which may be useful in showing that the cytokinin binding activity is biologically meaningful.

Once a cytokinin-binding component has been isolated and identified, it then has to be linked to the response before it can be called the receptor. Saunders and Hepler (1982) have reported that calcium ions may act as a second messenger in the cytokinin-induced bud induction in the moss Funaria hygrometrica. The claim by Saunders and Hepler is based on the initial swelling of a side branch in the presence of calcium ions and the calcium ionophore A23187. The effect of this ionophore and calcium ions on Physcomitrella patens is very different. Under these conditions highly-branched chloronemal filaments are produced, causing the plants to grow as tight spheres of protonemata. So if calcium ions have a role in the response to cytokinin, it appears also to have a role in chloronemal branching. Calcium ions could be involved in the branching of protonemata and so are increased in the response to cytokinin, but there may also be other factors which are responsible for the side branch to

develop into a gametophore. There is still a large amount of work to be done on the mechanism of cytokinin action and this may prove to be more difficult to elucidate than the identification of possible receptors for cytokinin. However, once identified in relatively simple organisms as mosses, the elucidation of hormone mechanisms may be simplified in higher plants.

Appendix I

Synthesis of [^{3}H]-benzyl aminopurine

The tritiated benzylaminopurine was synthesised as described by Fox et al. (1979). Meta-iodo benzylaminopurine was synthesised by refluxing 500mg of 6-chloropurine with 500mg m-iodo benzylamine-HCl(97%) (both from Aldrich Chemical Co.) for 6 hours in 25ml of sodium phosphate buffer, pH7.8. The precipitated crystals produced were collected by centrifugation, washed 3 times with water at 4^{o}C and purified by two recrystallizations in 50% ethanol. A sample of the purified product was identified by mass spectrometry. The results shown in figure 21 confirm that the product was m-iodo benzylaminopurine. A sample of the m-iodo benzylaminopurine was then sent to Amersham International plc, (Amersham, Buckinghamshire) to be catalytically tritiated using process TR.3. Attempts were made to separate the product from the precursor by thin layer chromatography. As none of the solvents tried separated the m-iodo BAP from BAP, they were separated on reverse phase high performance liquid chromatography isocratically using 35% acetonitrile (pH 7 with triethylammonium bicarbonate) as elutant (figure 22). By separating known aliquot on HPLC the final specific activity was calculated to be 12 Ci/mmol.

Figure 21. Mass spectra of m-iodo BAP.

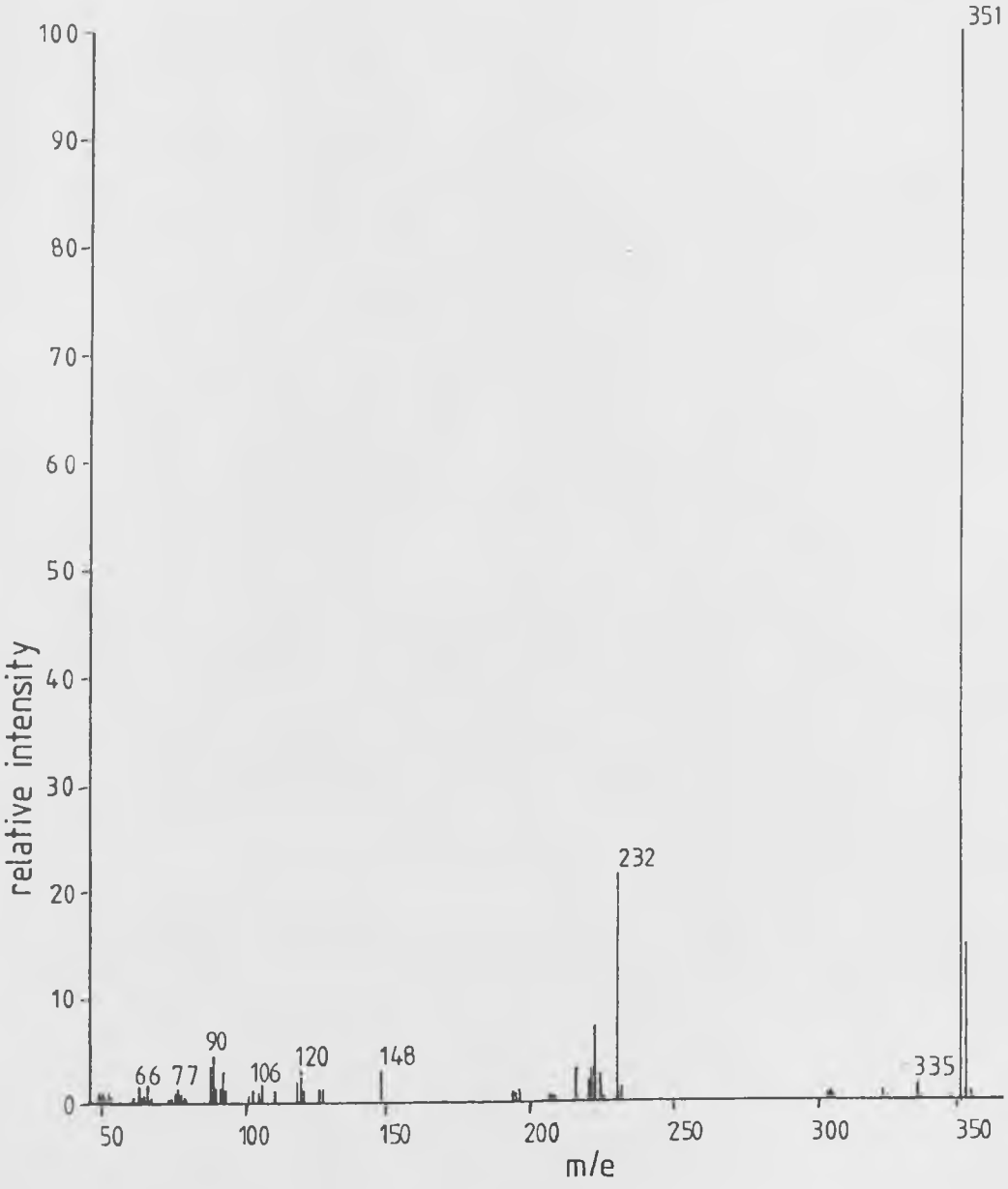
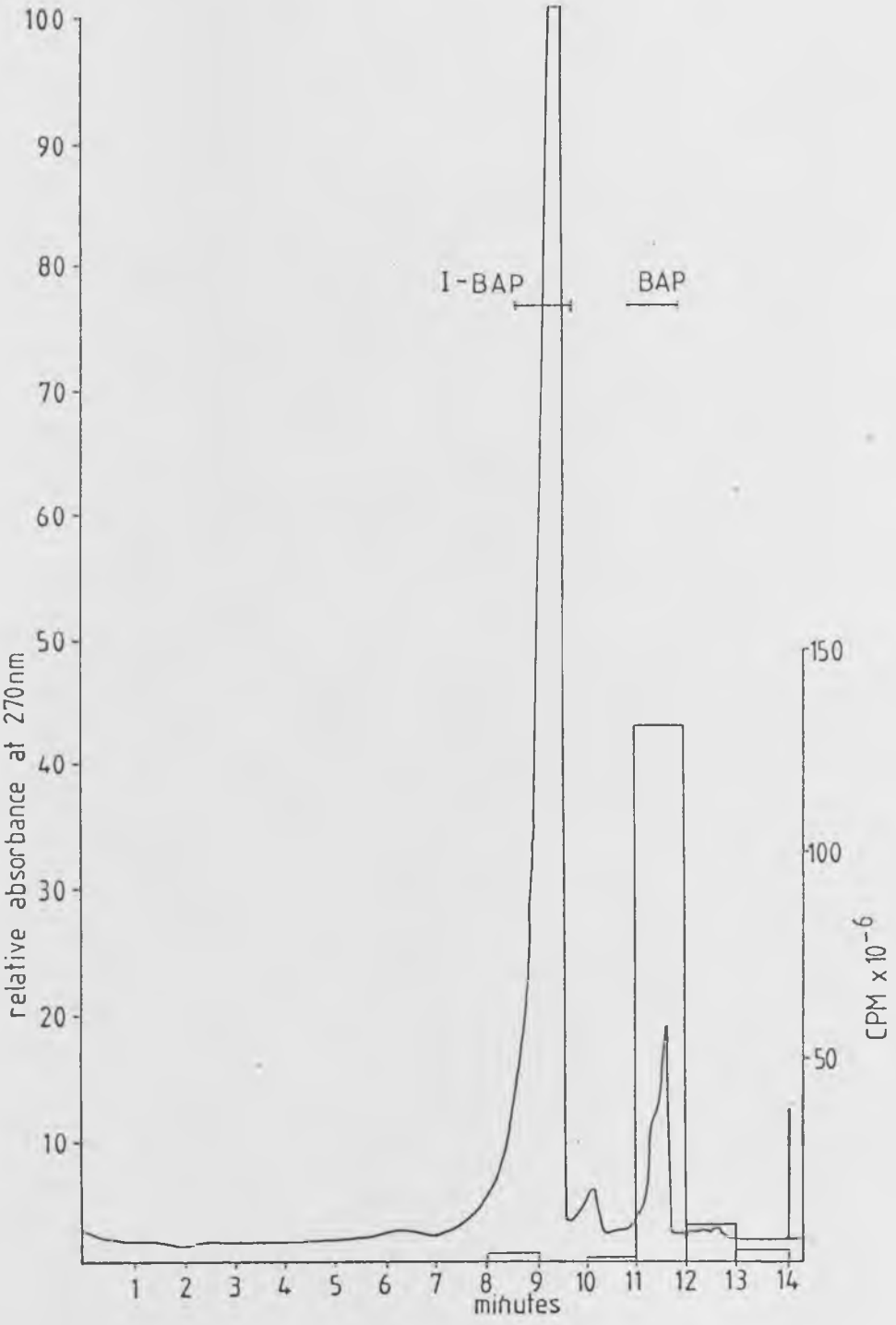


Figure 22. HPLC trace of the separation of ^{3}H-BAP from iodo-BAP.



The continuous trace is the absobance at 270 nm of the ellutant from the column. The histogram shows the counts per minute (CPM) in the fractions. For further details see text.

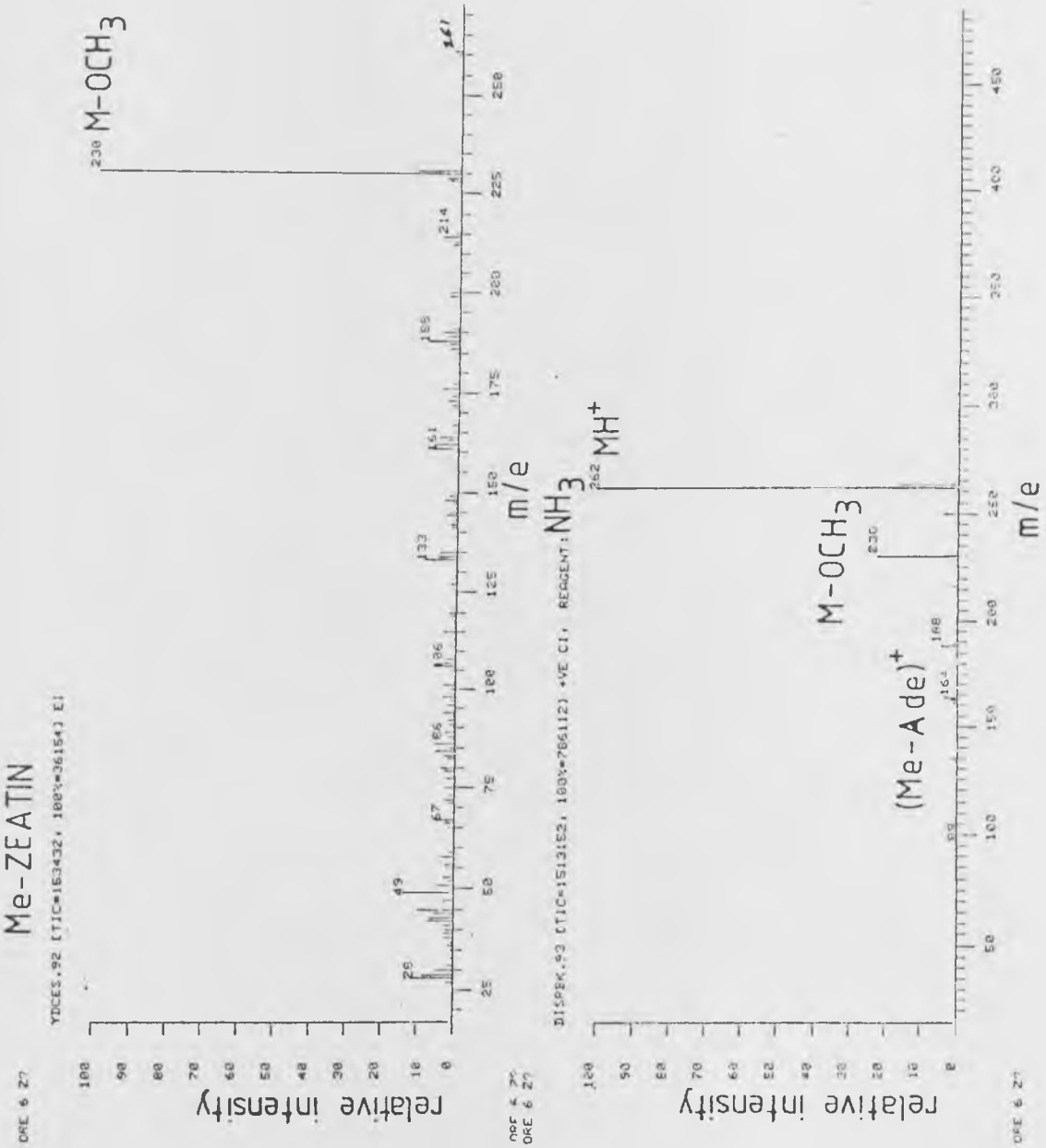
Appendix II

Mass spectrometer analysis of cytokinins

1. Analysis of cytokinins from ore 6

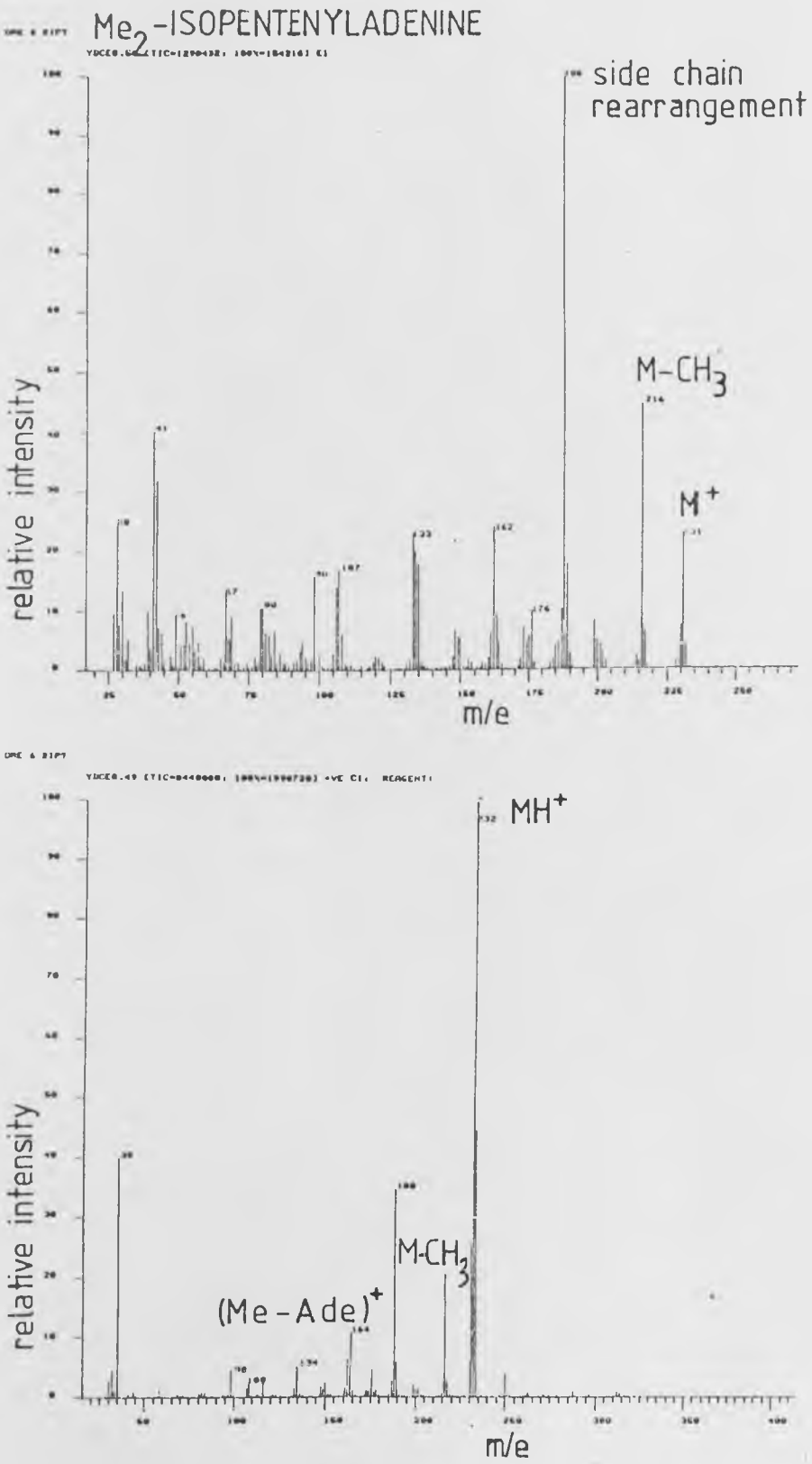
The pooled fractions of ore 6 medium from the Sephadex LH20 column described in Chapter III section 5 were analysed by combined gas chromatography-mass spectrometry. Fractions 19-24 and 28-34 were analysed as the permethyl derivatives. Derivatisation was performed as previously described (Wang et al. 1981b) and the samples chromatographed on a 12m capillary column of OV-1 (Phase Separations, Queensferry, Clwyd, UK) using helium as the carrier gas. The chromatograph (Carlo Erba, Milan, Italy) was linked directly to a Kratos MS80 mass spectrometer using direct probe insertion. The probe was heated to 300°C and spectra recorded using a DS55 data system at 1 second per mass decade under alternate chemical ionisation (CI) and electron impact (EI) ionisation (ACE). For chemical ionisation, NH_3 was used as the reagent gas. Source pressures were 4×10^{-6} torr (EI) and 7×10^{-5} torr (CI). Figure 23 and figure 24 show the spectra obtained for fractions 19-24 and 28-34 and were the same as authentic 2iP and zeatin respectively.

Figure 23. ACE mass spectra of ore 6 Sephadex LH 20 fractions 19-24.



Alternate chemical and electron impact ionisation (ACE) mass spectra of fractions 19-24 from a Sephadex LH20 column of a cytokinin extraction from the culture medium of ore 6. For further details see section 1.

Figure 24. ACE mass spectra of ore 6 Sephadex LH 20 fractions 28-34.

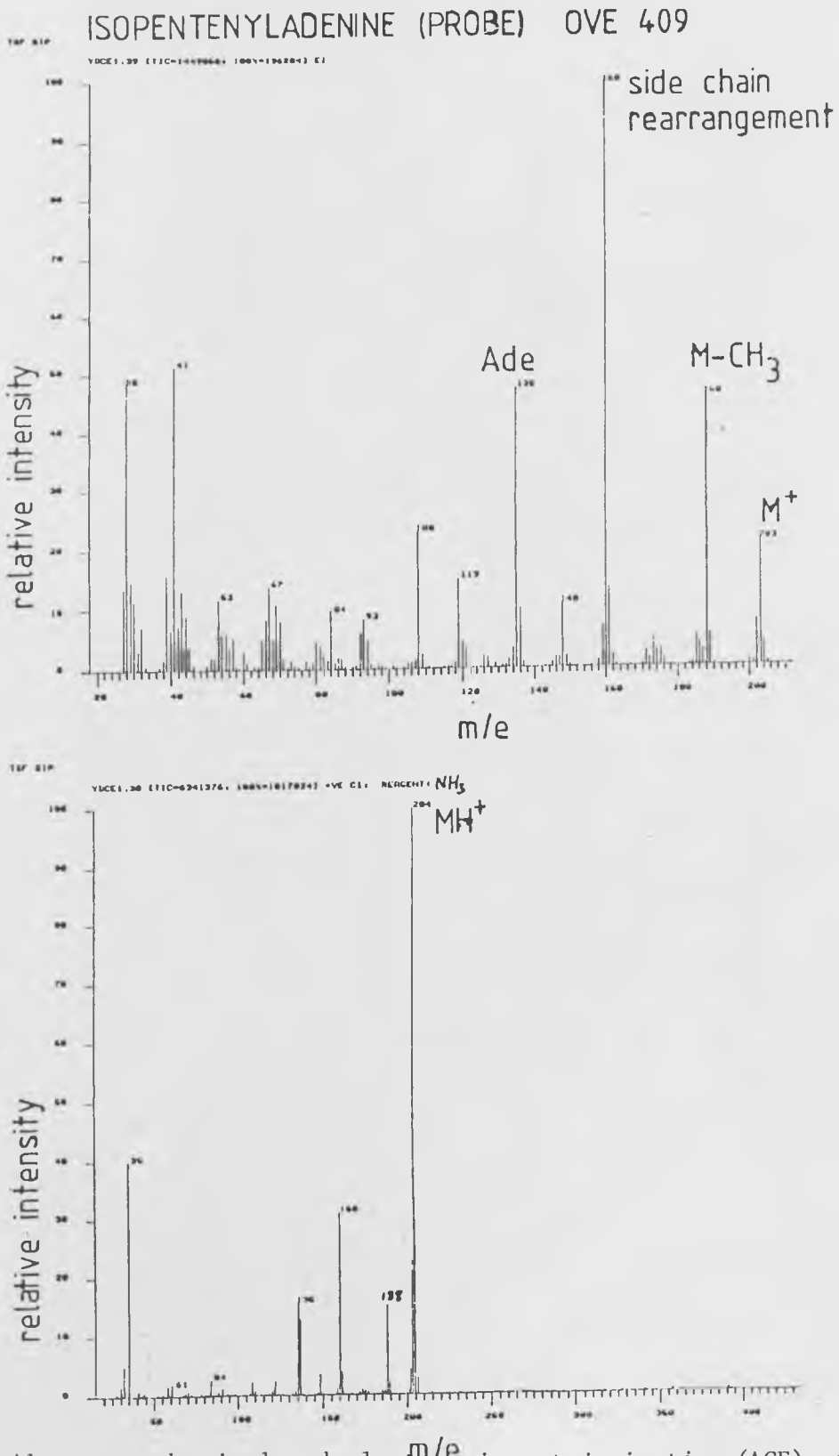


Alternate chemical and electron impact ionisation (ACE) mass spectra of fractions 28-34 from a Sephadex LH20 column of a cytokinin extraction from the culture medium of ore 6. For further details see section 1.

2. Analysis of cytokinins from ove 409

The putative 2iP sample from the medium of ove 409 described in Chapter III section 5 was analysed by using direct probe insertion mass spectrometry (Kratos MS80) as described in section 1 above. Figure 25 shows the spectra obtained which contain the same ions as authentic 2iP under the same conditions.

Figure 25. ACE mass spectra of ove 409.



Alternate chemical and electron impact ionisation (ACE) mass spectra of fractions 32-33 from a Sephadex LH20 column of a cytokinin extraction from the culture medium of ove 409. For further details see section 2.

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