Cytokinin induces the developmentally restricted synthesis of an extracellular protein in Physcomitrella patens

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Summary
Early development of the moss Physcomitrella patens follows a simple course leading to the formation of a filamentous protonema containing only two cell-types, chloronema and caulonema. The addition of the hormone cytokinin leads to the induction of multicellular buds from such protonema. The spectrum of extracellular proteins (ECPs) synthesized by P. patens has been investigated at defined stages of development and under defined hormone treatments. It is found that in contrast to the limited changes in intracellular protein synthesis detectable, in the extracellular environment major and specific changes in the patterns of proteins synthesized occur. For example, the presence of caulonema cells is characterized by the synthesis of a 25 kDa ECP whereas early chloronema differentiation is distinguished by the presence of a 38 kDa ECP. The analysis of the pattern of ECPs synthesized by developmental mutants altered in bud formation, and in response to cytokinin in tunicamycin treated protonema (in which bud induction is blocked) indicate that the synthesis of a 14 kDa ECP is specifically induced by cytokinin. This protein represents a novel cytokinin-induced ECP.

These data show that the differentiation of particular cell types in plants is associated with the synthesis of particular ECPs, and suggest that hormones which induce specific morphogenic events may do so via the synthesis of specific ECPs.

Introduction
Extracellular proteins (ECPs) play an important role in the definition of cell wall structure (Varner and Lin, 1989), as well as in the defense of plants against pathogen attack (Dixon and Lamb, 1990). More recent evidence has suggested that a number of ECPs affect and perhaps regulate plant development (De Jong et al., 1992; De Vries et al., 1988; Gavish et al., 1992). Thus in carrot tissue culture, the formation of somatic embryos from proembryogenic masses has been found to depend upon the presence of glycosylated ECPs (De Vries et al., 1988). For example, inhibition of embryo formation by tunicamycin (an inhibitor of N-glycosylation) could be alleviated by the addition of a glycosylated cationic peroxidase purified from the medium of embryogenic cultures. This peroxidase has been proposed to prevent tunicamycin-induced cell expansion by the crosslinking of cell wall compounds (Cordewener et al., 1991; Van Engelen and De Vries, 1992). Recently, another ECP from embryogenic cultures was shown to rescue arrested embryogenesis in cultures of the temperature sensitive mutant of carrot, ts11. This ECP has been found to have chitinase activity (De Jong et al., 1992). In cell cultures of citrus, on the other hand, inhibition of the formation of somatic embryos by ECPs derived from non-embryogenic cultures has been demonstrated (Gavish et al., 1992). The mechanisms of action of these ECPs in affecting somatic embryogenesis is not known, although an effect on cell wall structure seems probable (Van Engelen and De Vries, 1992).

Analysis of the developmental role of ECPs in intact higher plants is complicated by both the invasive techniques required to identify such ECPs and technical problems in precisely manipulating temporal and spatial patterns of ECP-expression. Consequently, all the experiments described above were performed using in vitro cell culture systems.

In order to investigate the role of ECPs in plant development we have chosen to use the bryophyte Physcomitrella patens, a plant that can be grown in vivo in the laboratory under controlled conditions, independent of exogenous plant hormones. In P. patens haploid spores give rise to a network of branched filaments (termed protonemata) which initially contain only one cell type (chloronemata), but from which a second cell type (caulonemata) later arises. Multicellular buds develop from caulonema branch points (termed side-branch initial cells) and give rise to leaf-like gametophores upon which the diploid sporophyte generation develops. From these sporophytes new haploid spores are formed (Cove et al., 1991). Studies with a large variety of developmental mutants from P. patens have established a role for auxin...

In this study *P. patens* was chosen as the experimental system to analyse developmental and hormonal regulation of ECPs for three main reasons. Firstly, the intact plant can be grown in large quantities in liquid under laboratory conditions whilst going through a normal life cycle (Cove *et al.*, 1991). Soluble ECPs may therefore be easily isolated from the growth medium. Secondly, it is possible to observe developmental switches at a cellular level non-invasively under the microscope. Thirdly, because of the ability to grow the intact plant in liquid, radioactively labelled methionine is rapidly taken up and incorporated into newly synthesized proteins within the cell. Released ECPs can thus be easily visualized by SDS-PAGE fluorography. Moreover, although exogenous hormones are not required for tissue growth, supplying hormones in the liquid culture provides a means of controlling developmental switches such as cytokinin-induced bud formation (Cove, 1984).

Relatively little is known about the control of gene expression by cytokinins during normal plant growth and development (Lazarus, 1991), let alone whether cytokinins can affect the ECPs made by a cell. In this study, we have analysed the pattern of newly synthesized proteins from various developmental stages of *P. patens*, and in particular the role of cytokinin in bud induction. Whereas the pattern of intracellular proteins appears to be constant, the pattern of ECP changes dramatically during development in general, and in particular during cytokinin-induced bud formation. Taking advantage of mutants altered in development, we describe the in vivo developmental regulation and cytokinin induction of extracellular proteins from *P. patens*.

**Results**

To create a developmental framework for the analysis of ECP-synthesis, we examined the kinetics of moss development. Starting from spores, a network of branched filaments composed only of chloronema cells appeared during the next 4–5 days. This was defined as stage 1 tissue (Figure 1a). The tip-cells of chloronema filaments divide approximately every 20 h (Ashton and Cove, 1990). Over the next 2–3 days the second cell type, caulonema cells, become apparent (stage 2). Caulonema cells characteristically possess fewer chloroplasts and the division time of tip-cells of a caulonema filament is typically 5–6 h (Ashton and Cove, 1990). Three days later, at developmental stage 3, most of the now dominating caulonema cells had developed side-branch initials along the filaments (Figure 1b). After a total of approximately 15 days some of these side-branch initials had given rise to multicellular buds (Figure 1c; stage 4 tissue).

Previous work has shown that the cytokinin 6-benzylaminopurine (BAP) induces bud formation in *P. patens* in a developmentally restricted manner (Cove, 1984). Under the culture conditions chosen for this study we have found BAP to induce bud formation in stage 3 tissue and to increase the number of buds formed by stage 4 tissue at concentrations as low as 50 nM. Tissue at stages 1 and 2 remained insensitive to BAP in terms of bud formation even at concentrations of BAP greater than 1 µM. The data summarizing the different developmental stages and their phenotypic response to BAP are shown in Table 1.

To allow for variations in the growth rate of different cultures all the data presented in this study have been related to the observed developmental stage of the tissue being examined.

**Extracellular proteins are developmentally regulated**

To determine the synthesis of ECPs during development of *P. patens*, tissue was incubated in the presence of [35S]methionine for 3 days. At the end of the incubation period the developmental stage of the tissue was monitored and the ECPs were then precipitated from the medium, separated by SDS-PAGE and visualized by fluorography. Incubation of medium containing 35S-labelled ECPs at room temperature and pulse-chase experiments showed that labelled extracellular proteins are stable for at least 10 days both in the presence and absence of BAP (data not shown).

At the developmental stage 1 a protein of 38 kDa predominates in the newly synthesized ECPs (Figure 2, lane 1). With the appearance of caulonema cells, at stage 2 of development, a protein of 25 kDa becomes apparent, while the 38 kDa band decreases in intensity (Figure 2, lane 2). In mature protonema of stage 3 tissue, ECPs of low molecular weights become apparent (Figure 2, lane

<table>
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<th>Developmental stage</th>
<th>Cell type</th>
<th>Bud formation&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>4</td>
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<sup>a</sup>Chl, chloronema cells; Cau, caulonema cells.

<sup>b</sup>+, approximately one bud per filament. ++++, most caulonema side-branch initial cells gave rise to buds.
At this transition from stage 2 to stage 3 the tissue becomes competent for bud induction via cytokinin, although in the absence of exogenous cytokinin no buds are apparent. When buds are formed at stage 4 (Figure 2, lane 4), a doublet of newly synthesized ECPs of 12 and 14 kDa predominates.

In contrast to the drastic developmental changes in the pattern of ECPs synthesized, no major differences are detectable in the spectrum of intracellular proteins (Figure 2, lanes 5–8). This result is consistent with the observations from other studies (Cove et al., 1991; Reski and Abel, 1985).

Plant hormones induce changes in the pattern of ECPs

The formation of different cell types in Funariaceae such as P. patens has been shown to be hormone-induced both by studies of developmental mutants and by the
Figure 2. Developmental regulation of extracellular proteins. *P. patens* was incubated in the presence of [35S]methionine for 3 days. Cytoplasmic proteins were extracted and both cytoplasmic and extracellular proteins were precipitated by ethanol, separated by SDS-PAGE (Laemmli, 1970) on a 15% gel and visualized on a preflashed film by fluorography (Laskey and Mills, 1975).

Fig. 2.1-4, extracellular proteins (ECP) from tissue of the developmental stages 1-4; lanes 5-8, cytoplasmic proteins (ICP) from tissue of the developmental stages 1-4. Protein markers are shown with their molecular masses given in kilodaltons. Arrows, extracellular proteins of 38, 25, 14 and 12 kDa.

Cytokinin induces the synthesis of an extracellular protein of 14 kDa

The data from Figure 2 and Figure 3 suggested to us that the 14 kDa band seen in tissue at stage 4 was not simply a by-product of the buds formed at this stage of development, but rather that its synthesis might be linked to cytokinin levels. We therefore decided to further characterize the role of cytokinin in the induction of the 14 kDa protein. The results of this analysis are presented in Figure 4. BAP was found to induce the synthesis of an ECP of 14 kDa (Figure 4, lanes 4, 6 and 8) but this induction was

Figure 3. Analysis of hormonal regulation of extracellular proteins. Visualization of extracellular protein from tissue of developmental stages 1 and 2 as described in legend to Figure 2. Incubation in the presence or absence of 1 μM 6-benzylaminopurine (BAP) and/or 1 μM 1-naphthaleneacetic acid (NAA) for 3 days (together with [35S]methionine) as indicated. Protein markers with their molecular masses given in kilodaltons. Arrows, extracellular proteins of 14 and 12 kDa.
dependent on the cell type treated. The induction was first observed with stage 2 tissue (caulonema present, compare lanes 3 and 4), stage 1 tissue (chloronema) being non-responsive with respect to this protein. At neither of these two stages does BAP induce bud formation.

By final stage 3 the addition of BAP does lead to the formation of some buds and the synthesis of the 14 kDa ECP (Figure 4, lane 6). By stage 4 (when the tissue already contains a number of buds) the 14 kDa protein is synthesized in large amounts in non-BAP treated tissue (lane 7). The addition of BAP to this tissue leads to the formation of a very large number of buds and the 14 kDa protein continues to be synthesized in large amounts (lane 8). These results suggest that the synthesis of the 14 kDa protein precedes bud formation and may be associated with the presence of side-branch initial cells, upon which bud formation is induced by increased levels of cytokinin (Ashton and Cove, 1990). Further evidence that the 14 kDa ECP is cytokinin-induced came from experiments using the inhibitor of N-glycosylation, tunicamycin.

In carrot tissue culture tunicamycin has been shown to inhibit somatic embryogenesis (De Vries et al., 1988). Here we have examined the effect of tunicamycin on bud formation and induction of the 14 kDa ECP. We have found that tunicamycin at a concentration of 2.5 μg ml⁻¹ inhibited the induction of buds by BAP (compare Figure 5a and b). This effect was not due to cell death, as indicated by viability staining of tunicamycin-treated tissue (compare the green fluorescing tunicamycin-treated tissue in Figure 5b with the red autofluorescing cadmium-treated tissue in Figure 5c), by the fact that the tissue continued to grow in the presence of tunicamycin at this concentration, and also the fact that the spectrum of intracellular proteins synthesized in the presence of this concentration of tunicamycin was indistinguishable from that seen in untreated tissue (data not shown). Although BAP did not induce bud formation in the presence of tunicamycin, induction of the synthesis of the 14 kDa ECP still occurred, as is shown in Figure 5d, lane 3. Taken together, these results strongly indicate that the 14 kDa ECP is induced by cytokinin. To our knowledge this is the first description of a cytokinin-induced ECP in plants.

With increasing concentration of tunicamycin changes occurred in the pattern of ECPs synthesized, as expected for an inhibitor of N-glycosylation of proteins. Most notable are the decreased intensities of the bands at 12 and 25 kDa. The decreased intensity of the band at 25 kDa correlates with an increased band intensity at 21 kDa as the concentration of tunicamycin is increased (Figure 5d, lanes 1–4). The pattern of ionically bound cell wall proteins was clearly different from the pattern of soluble ECP (Figure 5d, compare lanes 1 and 5) confirming the extracellular origin of the medium proteins investigated.

ECPs correlate with mutant phenotypes

A large number of EMS-induced mutants of P. patens have been described (Ashton and Cove, 1990; Ashton et al., 1979). We therefore investigated whether the pattern of ECPs synthesized by these mutants correlated with their observed phenotype. The results from the analysis of three mutants (described in Table 2) are presented in Figure 6. Gam87 (labelled cytokinin requiring in Figure 6) has been described as a BAP requiring mutant in which cytokinin synthesis is strongly reduced in comparison to that found in the wild-type (Ashton et al., 1979). Under the culture conditions used in this study we have found that gam87 develops a few small, intensively dark green buds in the absence of exogenous cytokinin. Addition of 1 μM BAP caused bud formation similar to that observed in the wild-type. The pattern of ECPs isolated from the mutant shows a pronounced inducibility of the 14 kDa ECP by BAP (Figure 6, lanes 1 and 4). This result is consistent with expectations for a cytokinin underproducer.

Gam1 (labelled non-bud producer in Figure 6) develops normally to produce chloronema and caulonema cells but does not produce buds even in the presence of cytokinin (Figure 1d). Analysis of the ECPs synthesized by this mutant at the developmental stage 3 most notably reveals the loss of an ECP at 25 kDa visible in wild-type tissue (compare Figure 2, lane 3 and Figure 6, lane 2). The addition of BAP to this mutant did not induce the synthesis of the 14 kDa ECP observed in BAP-treated wild-type tissue (Figure 6, lanes 2 and 5).

The mutant ove99 (labelled over-producer in Figure 6) has the phenotype of a bud overproducer (Figure 1e). A phenotype similar to the one of ove99 was observed after incubation of wild-type tissue at stage 4 in the presence of 1 μM BAP for 8 days. During this prolonged growth in the presence of exogenous BAP callus-like buds are formed from almost all side-branch initials (Figure 1f), and analysis of the ECP pattern reveals the loss of the 25 kDa ECP and a strong increase in synthesis of the 12 kDa ECP in the mutant ove99 as well as in the wild-type.

Table 2. List of strains

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<thead>
<tr>
<th>Designation</th>
<th>Previous designations</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Gransden wild-type</td>
<td></td>
<td>Engel (1968)</td>
</tr>
<tr>
<td>Leman wild-type</td>
<td></td>
<td>Schaefer et al. (1991)</td>
</tr>
<tr>
<td>gam1/thiA1</td>
<td>bar1/thiA1</td>
<td>Ashton and Cove (1979)</td>
</tr>
<tr>
<td>gam87/thiA1</td>
<td>nar87/thiA1</td>
<td>Ashton and Cove (1979)</td>
</tr>
<tr>
<td>ove99/pabA3</td>
<td></td>
<td>unpublished data</td>
</tr>
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1Nomenclature introduced by D.J. Cove at the Conference 'Mosses 91' in Heidelberg, Germany.
2Nomenclature used in reference.
Figure 5. Inhibition of cytokinin induced bud formation by tunicamycin.
(a) Moss protonema in the presence of BAP (1 μM). Scale bar, 100 μm.
(b) Moss protonema in the presence of BAP (1 μM) + tunicamycin (2.5 μg ml⁻¹). Scale bar, 100 μm.
(c) Moss protonema in the presence of cadmium (200 μM). Scale bar, 100 μm.
Viability of moss protonema of the developmental stage 3 was examined using 0.0003% (w/v) fluorescein diacetate (Zimmermann et al., 1989) after incubating the tissue for 3 days in the presence of 1 μM BAP and in the absence or presence of tunicamycin (2.5 μg ml⁻¹). Viable cells appear green, dead cells red.
(d) Pattern of extracellular protein and ionically bound cell wall protein in the presence of cytokinin and tunicamycin.
Visualization of extracellular protein (ECP) from tissue of the developmental stage 3 as described in the legend to Figure 2. Incubation was in the presence of 1 μM 6-benzylaminopurine (BAP), [³⁵S]methionine and of the concentration (μg ml⁻¹) of tunicamycin (Tun) indicated. Ionically bound cell wall proteins (CWP) were eluted in the presence of 1 M NaCl and 20% sucrose (Faye and Chrispeels, 1989) and visualized as described for extracellular proteins. Protein markers with their molecular masses are given in kilodaltons.
+, approximately 1 bud per filament.

(Figure 6, lanes 3 and 7). Since synthesis of the ECP of 12 kDa may be observed in the absence of the 25 kDa ECP (Figure 3, lane 4) we propose this ECP not to be a degradation product of the 25 kDa ECP. Addition of BAP to the mutant did lead to a slight increase in the 14 kDa ECP, as expected for an ECP formed by side-branch initials (compare Figure 6, lanes 3 and 6). Analysis of the pattern of intracellular proteins synthesized by these three mutants under the same conditions of growth revealed no significant differences in comparison to the spectrum of proteins observed in the wild-type (data not shown).
Extracellular proteins from P. patens

Figure 6. Synthesis of extracellular proteins in developmental mutants. Mutants were cultivated in parallel to wild-type cultures until wild-type reached the developmental stage 3. BAP (1 μM) was added to wild-type, 5 days later [35S]methionine was added to all cultures and 1 μM BAP to mutant cultures as indicated. Extracellular proteins were collected after 3 more days and visualized as described in the legend to Figure 2. Protein markers with their molecular masses given in kilodaltons. Wild-type was incubated for 8 days in the presence of 1 μM BAP. wt, wildtype; cr, cytokinin requiring mutant gam87; np, non-bud producing mutant gaml; op, bud-overproducing mutant cve99; arrows, extracellular proteins of 14 and 12 kDa.

Quantitative and qualitative changes in ECPs in the presence of cytokinin

Figure 7 presents a comparison of the kinetics of ECP accumulation in the medium of moss cultures grown in the presence or absence of BAP. In this experiment a number of cultures were started with the same fresh weight of inoculum at the same developmental stage. The medium was replaced with fresh medium at intervals of 24 h and the fresh weight of the tissue was kept constant throughout the experiment. ECPs were quantified in each batch of medium removed. In Figure 7, ECPs quantified in the presence and absence of BAP are presented as a bar chart in which the amount of ECP in non-BAP treated cultures after 24 h has been set to a value of 1 (note the log scale).

The data indicate that following the addition of BAP a quantitative increase in ECPs occurs, leading to a rate of synthesis approximately 500-fold that observed in untreated tissue over a period of 4 days. By this time, under the culture conditions used in this experiment, the BAP-treated tissue has produced a large number of callus-like buds, while the untreated tissue consists mainly of chlorenchyma and caulonema cells. The maximum yield of ECP from BAP-treated tissue was of the order of 300 μg l⁻¹ day⁻¹.

To determine the pattern of medium protein, moss was cultivated in liquid medium as described above. Medium proteins were concentrated by ultrafiltration and visualized by silver staining after separation by SDS–PAGE (Figure 8). In line with the results from the feeding experiments and the determination of total medium proteins, a dramatic increase in the amount and spectrum of ECP was detected in the presence of BAP.

Substantiation of the extracellular source of ECPs

The interpretation of our data is based on the assumption that the labelled proteins observed by SDS–PAGE fluorography are derived from the extracellular (or apoplastic)
environment and are not due to cell leakage. Two lines of evidence suggest that this assumption is correct. Firstly, the patterns of ECPs observed are strikingly different from those detected intracellularly, both in spectrum and intensity. Secondly, during development we have observed striking differences in the pattern of ECPs synthesized, yet no obvious changes in the patterns of proteins located intracellularly. This altered pattern of ECPs can be mimicked by the addition of various hormones, again with no overt changes in the intracellular pattern. This finding is in line with previous investigations where attempts to demonstrate the presence of auxin or cytokinin-induced intracellular proteins have proven unsuccessful (Cove et al., 1991; Reski and Abel, 1985).

Similarly, the inhibitor of N-glycosylation tunicamycin induced obvious changes only in the pattern of ECPs but not in that of intracellular proteins. Taken together, these observations strongly suggest an extracellular nature of the newly synthesized medium proteins.

**Discussion**

A large and accumulating body of evidence from the animal field indicates that protein components of the extracellular matrix not only play a structural but also a regulatory role in development (Adams and Watt, 1993; Edelman, 1986; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). In the area of plant research, recent experiments with in vitro systems have provided strong evidence for a regulatory role of extracellular (or apoplastic) proteins in development (De Jong et al., 1992; De Vries et al., 1988; Gavish et al., 1992).

We have approached this problem using an in vivo plant system, *P. patens*, which grows independently of exogenously added hormones. Despite the developmental peculiarities specific to bryophytes such as *P. patens*, the basis of morphogenesis (i.e. orientation of cell division and cell elongation; Bopp, 1984; Hernández et al., 1991) and its apparent regulation by hormones such as auxins and cytokinins (Cove, 1984; Kaufmann and Song, 1987) seems analogous to that observed in higher plants. Taking advantage of this apparent universality of the basic mechanisms of plant development, combined with the possibility of non-invasive analysis and manipulation of the developmental stage of the moss, we have analysed here the hormonal regulation of ECP synthesis during development of *P. patens*.

**Moss development is characterized by the synthesis of specific ECPs**

Our data show, firstly, that particular stages of moss development, and thus particular cell phenotypes, are characterized by the synthesis of particular ECPs. Thus, for example, stage 1 tissue (chloronema) is characterized by the predominant synthesis of a 38 kDa ECP, whereas the formation of caulonema cells is distinguished by the synthesis of a 25 kDa ECP. Synthesis of the 25 kDa ECP was found to be restricted to the presence of the caulonema cell type. The ECP of 25 kDa is therefore a putative caulonema-specific protein. Work on glycine-rich proteins and proline-rich proteins in higher plants has also indicated that some tissue types are characterized by specific ECPs (Hong et al., 1989; Keller and Lamb, 1989) and immunocytochemical work using antibodies against arabinogalactan proteins has shown that specific regions of organs, and indeed whole organs, may be specified by particular ECP epitopes (Knox et al., 1991; Penell and Roberts, 1990). The results from this analysis thus contribute to a growing consensus that cell and tissue differentiation may be intimately linked to the synthesis of specific ECPs. In *P. patens* the induction of new ECPs by the exogenous application of hormones does not lead to the differentiation of the cell phenotype normally associated with that spectrum of ECPs. Thus, for example, incubation of tissue of final developmental stage 2 in the presence of both auxin and cytokinin leads to the synthesis of an ECP pattern very similar to that observed in stage 4 tissue. Stage 4 tissue is characterized by side-branch initial cells competent for bud formation and the presence of some buds, yet stage 2 tissue never produces this phenotype, even after treatment with the above two hormones. If ECPs were necessary and sufficient for induction of this developmental transition, one would have to propose that either the relative amounts or the timing of synthesis of the ECPs involved was crucial for the developmental switch. We did not find these conditions to be fulfilled in the development of *Physcomitrella*. In line with this observation, inhibition of cytokinin-induced bud formation by tunicamycin was not overruled by the addition of ECPs from bud-containing cultures. Moreover, co-cultivation of *gam1* with *ove99* did not rescue the wild-type phenotype in *gam1* (data not shown).

In *Physcomitrella*, bud induction represents a developmental transition analogous to the formation of an apical meristem in higher plants. This developmental switch in moss is induced by increased levels of cytokinin, and is characterized by rapid cell divisions and drastically increased levels of extracellular proteins. High levels of ECPs have also been found in proembryogenic masses in the carrot tissue culture system, another tissue where rapid cell divisions occur (De Vries et al., 1988). Clarifying the possible function of ECPs in mitosis is a tempting challenge for future research.

**Cytokinin induces a specific ECP**

Cytokinins have been identified as hormones that can induce cell divisions in plants (Miller et al., 1956). How-
however, the molecular mechanism of cytokinin action remains unknown. Even at the level of gene expression our knowledge of the effects of cytokinin remains rather limited compared with what is known of the mode of action of other hormones such as auxin, abscisic acid or ethylene (Lazarus, 1991). There are only a few reports on cytokinin-induced genes. For example, cytokinin-induced mRNAs have been found in cultured cells of soybean in the presence of cycloheximide (Cowell et al., 1990) and cytokinin stress has been described to change the developmental regulation of defense-related genes in ethylene (Lazarus, 1991). There are only a few reports on action of other hormones such as auxin, abscisic acid or cytokinin-induced protein. The protein is secreted and its synthesis is closely associated with a particular stage of development, characterized by the competence of the tissue for bud induction. Induction of this protein by cytokinin took place in the absence of accompanying bud formation. This observation clearly indicates that the 14 kDa ECP is not simply associated with the increased cell divisions that occur during bud formation, nor does it appear to be a structural part of the bud. This is the first description of a cytokinin-induced ECP. Moreover, the occurrence of cytokinin-induced synthesis of the 14 kDa ECP at developmental stages not yet competent for bud induction indicates that in caulonemal cells competence of the tissue to detect changes in the concentration of cytokinin is not the limiting factor for bud induction. Other changes, such as increased levels of endogenous calcium in side-branch initials (Saunders and Hepler, 1982), have been shown to precede the developmental switch. It therefore seems likely that bud induction requires the presence of several different factors, of which the cytokinin-induced 14 kDa protein is a major novel component in the extracellular matrix.

In conclusion, these results show that the synthesis of extracellular proteins in Physcomitrella is developmentally regulated and that specific developmental stages are characterized by the synthesis of specific patterns of ECPs. More detailed dissection of this process, made possible by the plant chosen, led to the identification of a cytokinin-induced ECP. These data are consistent with the hypothesis that the treatment of tissue of the requisite developmental competence with cytokinin induces the synthesis of a 14 kDa ECP whose presence is necessary, but not sufficient, for induction of bud formation.

The relative ease of liquid culture of P. patens will facilitate the future isolation and characterization of ECPs. Together with the possibility of supplying exogenous substances to the plant and the facility of transformation (Sawahel et al., 1992; Schaefer et al., 1991), this system will enable us to examine the functional role of extracellular proteins in the intact plant under controlled conditions.

### Experimental procedures

#### Growth of plants

Protoplasts from Physcomitrella patens were isolated by treating 6-day-old protonemata with 2% (w/v) D1ase (Sigma) in the presence of 0.4 M mannitol for 20 min (Schaefer et al., 1991). Cultures were started by spreading spores or protoplasts with regenerated cell walls on callophane overlaid plates (Bopp et al., 1984) containing modified Knops medium (Knight et al., 1988). For cultivation of mutants, medium contained 1.5 μM thiamin-HCl (gam87, gam1) or 1.8 μM p-aminobenzoic acid (ove99). Petri dishes were incubated at 25°C in a plexi-glas chamber, aerated with sterile-filtered (bacterial airvent No. 4210, Gelman Sciences) air in a day/night rhythm of 16 h light (100 μE m⁻² sec⁻¹) and 8 h dark.

Cultivation in liquid medium, started with protomenal fragments from tissue stocks (Jenkins and Cove, 1983), was done in 2-l Erlenmeyer flasks containing 1 l of modified Knops medium with 10 mM CaNO₃ as the source of nitrogen. Cultures were placed on a gyrotory shaker (100 r.p.m.), incubated at 25°C under intense continuous white light (700 μE m⁻² sec⁻¹) aerated using charcoal-filtered, sterile air enriched with 1% CO₂ at a rate of 10 l h⁻¹.

During kinetic experiments medium was replaced and fresh weight of tissue kept constant daily.

#### Determination of medium protein

Medium from liquid cultures was passed through 0.80 μm and 0.20 μm membranes (Schleicher + Schuell, ST68, OE66). Medium proteins were precipitated overnight after addition of 2.5 volumes of ethanol at 4°C. Supernatant was decanted and the proteins were collected by centrifugation for 30 min at 7500 g. The protein content was measured according to Bradford (1976) with BSA as a standard.

The patterns of medium proteins were visualized by silver staining (Blum et al., 1987), following concentration by pressure dialysis using Amicon YM3 membranes at 3 kDa cut-off and separation by SDS-PAGE.

#### Labelling of extracellular proteins

Tissue of defined developmental stages was collected from callophane overlaid plates, equally distributed to sterile multicell plates (Delta SI, Intermed) and incubated for 3 days in 250 μl of sterile liquid medium containing 3–30 μCi of [³⁵S]methionine (Nordion), 1-naphthylacetic acid (Sigma), 6-benzylaminopurine (Sigma) and tunicamycin (Calbiochem) at the concentrations indicated.

#### Preparation of labelled extracellular proteins

After cultivation of moss in the presence of [³⁵S]methionine, medium was separated from the tissue by filtration through 0.20 μm filters (Schleicher + Schuell, FP 003/3). Extracellular proteins were precipitated in the presence of unlabelled BSA (10 μg ml⁻¹) by the addition of 2.5 volumes of ethanol overnight at 4°C (De Vries et al., 1988), collected by centrifugation for 30 min at 12,000 g, washed twice with 80% (v/v) ethanol and resuspended in SDS-PAGE sample buffer. Incorporation was determined as hot TCA-insoluble material. Electrophoresis was performed on 12.5% or 15% SDS-PAGE, labelled proteins were visualized by
Preparation of ionically bound cell wall and cytoplasmic proteins

Labelled proteins ionically bound to the cell wall were eluted in the presence of 1 M NaCl and 20% sucrose (Faye and Chrispeels, 1989) overnight at 4°C, sterile filtration and further treatment were as described for extracellular proteins. Labelled cytoplasmic proteins were obtained after rinsing of salt-washed tissue with water (Faye and Chrispeels, 1989) by grinding at 4°C in extraction buffer containing 50 mM Tris pH 7.5, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM NaCl, 2% (w/v) polyvinyl-polypyrrolidon and 50 mM 2-mercaptoethanol. The homogenate was made cell-free by filtration through one layer of 100% salt-washed tissue with water (Faye and Chrispeels, 1989) by treatment were as described for extracellular proteins.

Preparation of ionically bound cell wall and cytoplasmic proteins

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