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Chapter 7
Control of growth and patterning in the fungal fruiting structure. A case for the involvement of hormones

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Summary

Development and tissue differentiation in animal and plant multicellular organisms results from communication between cells via hormones and growth factors. The patterns associated with tissue differentiation and growth in fungal fruiting structures have been observed and described at the macroscopic, cellular, ultrastructural and even genetic/molecular levels. Although the formation of a fully differentiated mushroom, composed of distinct, organised tissues, from an initial mass of uncoordinated hyphae, provides the *prima facie* evidence for fungal hormones or morphogens coordinating differentiation and growth in developing fruit bodies, this has not been substantiated since few chemical candidates have been identified. This is in stark contrast to the animal and plant kingdoms where hormones and growth factors have been discovered, identified, purified and are commercially available. Fungal hormones are known to occur in lower fungi (oomycetes, chytridiomycetes, zygomycetes) where they are essential for mating, sexual development and differentiation and there is some evidence for their presence during differentiation of sexual fruiting structures in higher fungi (ascomycetes, basidiomycetes) but there is little information about hormonal coordination of growth during the development of fruit bodies or vegetative, multihyphal structures. The possibility that hormones or growth factors may be involved in the development of sexual and vegetative fruiting structures and in differential growth during tropic responses to external stimuli is discussed.

Introduction

Development in multicellular organisms is complex and depends on a multitude of morphological, physiological and genetic changes regulated in part by morphogens, hormones and growth factors. In animals, morphogens are important in setting up patterns in the mass of unspecialised cells comprising the embryo, resulting in the development of a mature, fully differentiated organism. Hormones are important in regulating the physiology of the organism, from the embryonic to the mature adult stage, and growth factors regulate directed and differential growth. Peptide and steroid hormones have long been known to regulate the physiology of organisms including man (Nicola, 1994) while morphogens, such as retinoic acid, active in the development of limb buds in chick embryos (Brickell & Tickle, 1989; Tickle, 1991) and the recently discovered protein products of the *bicoid*, *dorsal* and *hedgehog* genes in *Drosophila* (Ip, Levine & Small, 1992; St Johnston & Nüsslein-Volhard, 1992; Lawrence, 1992), are important in establishing the positional information required for cell differentiation at the embryonic stage.

While retinoic acid could be considered a ‘classical’ morphogen in that it controls gene expression indirectly by binding to plasmalemma and nuclear membrane receptors (Tickle, 1991) via a concentration gradient, the *bicoid*, *dorsal* and *hedgehog* gene products, transcription factors which bind

to specific DNA sequences and either stimulate or repress gene expression directly, also function in a concentration-dependent manner (Ip *et al*., 1992) and thus can also be considered morphogens. Similarly, in plants, auxins are responsible for controlling cell division and differentiation at all stages of plant development (Sachs, 1991), cytokinins and gibberellins promote overall growth while abscissic acid and other compounds inhibit growth (Salisbury & Ross, 1985). Auxins and other plant growth regulators have also been found in fungi but these compounds have never been shown to have a hormonal role in the fungi that produce them or in other fungi (reviewed by Gruen, 1982). Their occurrence in phytopathogenic fungi is thought to be a consequence of secondary metabolism or a result of uptake from the host (Gooday, 1994). Remembering that gibberellins were named for the fungus from which these plant hormones were isolated, the prime role of any fungus-produced plant hormone is most likely to be to modify the growth of plant tissues which the fungus parasitises.

Several mating hormones have been characterised in a few fungal species and their role in sexual reproduction has been reviewed recently (Dyer, Ingram & Johnstone, 1992; Gooday & Adams, 1992; Bölker & Kahmann, 1993; Duntze, Betz & Nientiedt, 1994; Gooday, 1994; Mullins, 1994; Staben, 1995). On the other hand, evidence of hormonal involvement in coordinating other developmental processes, such as primordium induction and fruit body differentiation and maturation, is sparse, while belief in their involvement in tropic responses and even in the formation of multihyphal, vegetative structures is based on indirect and mainly circumstantial evidence.

The underlying rationale for investigating the presence and possible roles of morphogens, hormones and growth factors in fungi is based on the perceived level of evolutionary conservation in fundamental processes among diverse organisms. For example, transcription factors show a remarkable diversity in sequence variation and yet the homology of DNA-binding sites among taxonomically unlike species is striking as in the cases described above: the *bicoid* and *hedgehog* proteins contain homeobox motifs while the *dorsal* protein contains a REL domain related to that found in mammalian regulatory factor NF-kB and in the vertebrate oncoprotein *rel* (Ip *et al*., 1992). Tymon *et al.* (1992) determined that the *A* factor mating type in *Coprinus cinereus* encoded a transcription factor, responsible for regulating both sexual and asexual development, which contains a POU domain similar in part to the bipartite DNA binding domain in certain animal transcription factors. The evidence that transcription factors, with DNA-binding sites sharing homology with those found in animals and plants, are also present in fungi (Kelly *et al.*, 1988; Kües *et al.*, 1992) begs the question of how widespread their presence and function is in multihyphal, fungal structures.

In this chapter, the possibility of morphogen, hormone and growth factor control at all stages of multihyphal development, both sexual and vegetative, is discussed on the basis of information derived from morphological, physiological and genetic/molecular biological analyses of development in a variety of species, predominantly filamentous basidiomycetes. Special emphasis is placed on *Coprinus cinereus*, which was the model organism used in experiments to be discussed in the final section (and see Chapters 1 and 6).

Fungal sex hormones, mating type gene products and fruiting inducers

The evidence for the presence and function of hormones (specifically, pheromones) is well-established in lower fungi (chytridiomycetes, oomycetes, zygomycetes) where they are involved in coordinating sexual differentiation and mating (see reviews by Gooday & Adams, 1992; Gooday, 1994; Mullins, 1994). In higher fungi, mating through the activity of hormones is well known in ascomycetous and basidiomycetous yeasts (Kelly *et al.*, 1988; Dyer *et al*., 1992; Bölker & Kahmann, 1993; Duntze *et al*., 1994) and there is some evidence that diffusible factors also have a role in inducing ascogonial and trichogyne formation in some filamentous ascomycetes (reviewed in Dyer *et al*., 1992). In contrast,

anastomosis between monokaryons (even incompatible ones) occurs freely in filamentous basidiomycetes and cell fusion between compatible monokaryons is thought to occur without the need for specific mating hormones (Bölker & Kahmann, 1993). But recent analysis of the mating type factor B in *Schizophyllum commune* has shown that its sequence shares homology with the pheromones and pheromone receptors of lower fungi (Kües & Casselton, 1992), thus a cell signalling mechanism seems to be required for effective mating even in this filamentous basidiomycete.

Dikaryon formation in filamentous basidiomycetes is under the control of mating type loci and the decision to undergo sexual development after cell fusion is initiated and regulated by interactions of regulatory proteins (putative transcription factors) related to the homeodomain proteins of higher eukaryotes (Kelly *et al.*, 1988; Banuett, 1992; Kües & Casselton, 1992, 1994; Kües *et al.*, 1992, 1994; Wessels, 1993a; Casselton & Kües, 1994; Glass & Nelson, 1994; Kämper, Bölker & Kahmann, 1994; Wessels, 1992 1994; Staben, 1995). These mating type loci are usually thought of, and described, as the master regulatory genes for sexual development but present knowledge limits their activity to the initial steps in the process (Kües & Casselton, 1992), in particular mating resulting in dikaryon formation and regulation of nuclear segregation through formation of clamp connections. Whether products of these gene complexes control any steps in fruit body formation or maturation is unknown.

The dikaryotic phase in filamentous ascomycetes is limited to the ascogenous hyphae, any fruiting structures produced are constructed from the heterokaryotic hyphae. In most basidiomycetes (but see Chapter 5) the mating process forms a dikaryon which can grow vegetatively for an indeterminate period before sexual reproduction is induced by external factors, such as light, aeration and nutrient depletion (Wessels, 1993a; 1994). These external stimuli presumably activate expression of fruiting genes which eventually enable formation of a fruit body primordium. The basidiomycete primordium is normally comprised of heterokaryotic hyphae but only the basidia embark on karyogamy, meiosis and sporulation (see Chapters 5 & 6). Cap and stem tissues differentiate early in the initial stages of primordium formation (Chapter 1), grow, expand and mature into a fruit body (Fig. 1).

Probably the most fundamental change which occurs after sensitisation by the external factors mentioned above is the mechanism by which heterokaryotic hyphae, growing in an outwardly, diffuse, vegetative manner, and yet primed for sexual development, are stimulated to grow together, to branch, and to co-operate in formation of the fruit body initial as a hyphal knot which is the building block for more intricate tissues within the fruit body (Moore, 1995). Unfortunately, we are completely ignorant of how this morphogenetic change is brought about. Light is the primary trigger for fruiting induction in many basidiomycetes (Ross, 1985; Kozak & Ross, 1991; Wessels, 1993a; 1994) and acts on plasma membrane receptors to sensitise hyphal tips for sexual development. How hyphal tips find each other and then have their branching pattern changed in a co-ordinated way is unknown.

Homing reactions have been described (e.g. Kemp, 1977; and see discussion in Moore, 1984b) in which hyphal tips tend to grow towards germinating spores of the same species. However, this is, by definition, a reaction between different individuals of the same species; its mechanism is unknown, as is its relevance to co-operation between hyphae of the same individual to produce a fruiting structure. Bringing hyphal tips together and co-ordination of branching patterns are obvious targets for hormonal control but very few chemicals which may function in these ways during the inductive process have been identified (reviewed by Uno & Ishikawa (1982) and Wessels (1993a)): cAMP and AMP in *C. cinereus* (Uno & Ishikawa, 1971, 1973a & b, 1982), cerebrosides in *Schizophyllum commune* (Kawai & Ikeda, 1982), an unidentified low molecular weight compound from *Agaricus bisporus* causing fruiting induction in *S. commune* (Rusmin & Leonard, 1978) and an unidentified, diffusible factor(s) in *Phellinus contiguus* (Butler, 1995) have been found to induce mono- and dikaryotic fruiting but the

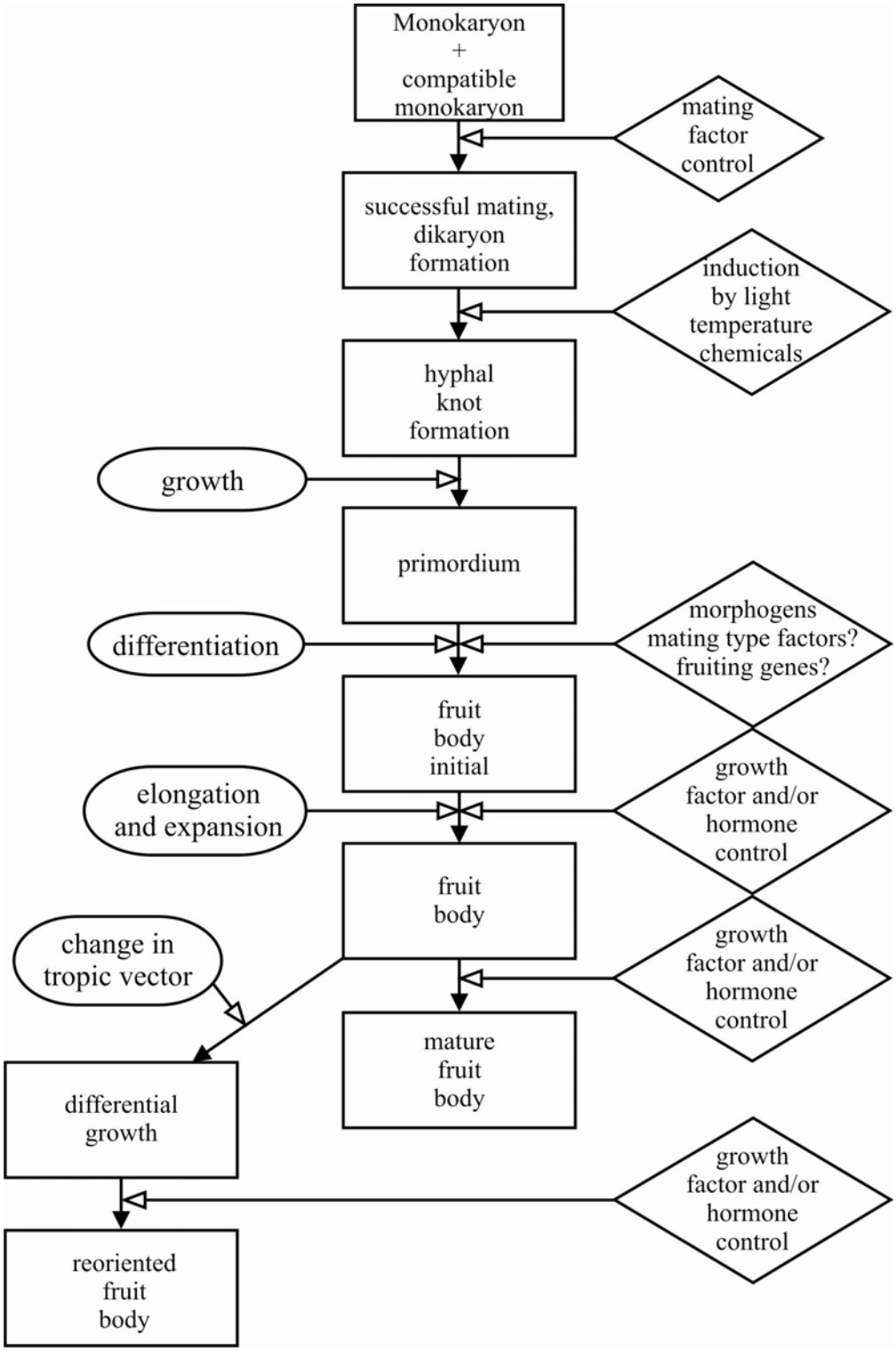

Fig 1. Potential target sites for growth factors and/or hormones during mating, dikaryon formation, primordium induction, fruit body differentiation and maturation.

manner in which these substances interact with the fungus to elicit fruiting is unknown. The low molecular weight fruiting inducing substance extracted from *A. bisporus* is interesting because its cross-reactivity on the taxonomically unrelated *Schizophyllum commune* and its general chemical similarity to substances with growth factor-like properties extracted from *Flammulina velutipes* (Table 1) and *C. cinereus* (see below) imply that there may be a family of chemically related factors or hormones responsible for mediating primordium initiation and fruit body elongation. The recent discovery of hydrophobins, hydrophobic proteins abundantly expressed and deposited in the cell walls during emergent growth in *Schizophyllum commune* (Wessels, 1993a, 1994), reveals another class of molecules which may be involved in hyphal aggregation by manipulating the biophysics of the surface of the aggregate.

Detection of fruit body inducing substances is difficult as their production is, presumably, limited to very low concentrations as they are likely to be secreted into the medium in order to attract tips together over a very restricted area. The complex media which are usually needed to induce fruiting also create difficulties, so it is not surprising that so few chemicals have been identified as putative inducers. Molecular techniques, as those used previously by Yashar & Pukkila (1985) for *C. cinereus* and by Mulder & Wessels (1986) for *S. commune*, that is, analysing mRNA expression (but at low levels) at different but specific stages should be more productive in identifying hormones than trying to extract them from media.

Differentiation within the primordium

The initial hyphal knot comprises more or less similar, intertwining hyphae (prosenchyma). There are many studies describing the morphological, physiological and even genetic and molecular changes which are correlated with fruit body formation (reviews by Gooday, 1982; Moore, 1984a & b, 1995; Manachère, 1988; Reijnders & Moore, 1985; Rosin, Horner & Moore, 1985; Watling & Moore, 1994; Wessels, 1993a, 1994) but few studies indicate how these various processes might be coordinated between these two stages.

There is some evidence for the involvement of particular chemicals during sexual differentiation in filamentous ascomycetes (e.g. production of linoleic acid in *Ceratocystis* spp., *Neurospora crassa*, *Nectria haematococca* and mycosporines in *Pyronema omphalodes*, *Morchella esculenta*, *Nectria galligena* (reviewed by Dyer *et al*., 1992)) but whether these compounds act as morphogens or metabolites is not established. The theories and experimental evidence correlating morphological, physiological and genetic changes with differentiation in sexual fruit bodies are covered in more detail in Chapter 1. Since there is no direct experimental evidence for the existence of morphogens in the differentiating primordium it is useful to draw from other biological systems where more information exists about the coordination of developmental processes. An animal analogy of the early primordial stage is the chick limb bud, a multicellular but undifferentiated structure, while a useful plant analogy is vascular differentiation in plant embryos.

In the chick limb bud, retinoic acid, a derivative of vitamin A, is an endogenous signalling substance which specifies the position of mesenchymal cells within the embryo based on the gradient of retinoic acid in the interstitial space around cells (Tickle, 1989, 1991). The activity of retinoic acid is dose- and position-dependent and signals the formation of digits in the wing. Retinoic acid also regulates the expression of several homeobox genes, which specify for the anterior position of digits in the limb bud, by binding to nuclear retinoic acid receptors (Tickle, 1991). Thus, retinoic acid acts as a morphogen (dose-dependent specification of position of cells) and regulates gene expression (homeobox genes); its action via a concentration gradient in the interstitial environment establishes the cell-to-cell communication required for the formation of digits in the correct orientation.

Table 1. Growth factor-type substances, extracted from basidiomycetous fruit bodies, with fruiting inducing, stem elongation and/or cap expansion promoting properties

Source organism	Activity	Active substance	Stability	Solubility	Authors
Agaricus bisporus *Flammulina velutipes* *Lentinus edodes* *Pleurotus ostreatus*	mycelial growth; fruiting induction	whole fruitbody extract, active principle unknown, but ninhydrin +ve, reducing sugar +ve,organic acid -ve, phosphate -ve, fatty acid -ve	heat, acid, base stable	water, aqueous methanol (insoluble in absolute methanol, chloroform, petroleum, benzene)	Urayama (1969)
Coprinus cinereus	fruiting induction	cAMP, 3'-AMP, theophylline			Uno & Ishikawa (1971, 1973a & b)
Coprinus cinereus	fruiting induction	whole culture extract, active principle unknown		water	Uno & Ishikawa (1971, 1973a & b)
Coprinus cinereus	hyphal aggregates (fruiting induction)	cAMP			Matthews & Niederpruem (1972)
Agaricus bisporus	fruiting induction (in *Schizophyllum commune*)	fruitbody tissue extract, active principle unknown but <12,000 molecular weight	heat, acid, base stable	water, 50% ethanol,50% acetone	Rusmin & Leonard (1978)
Schizophyllum commune	fruiting induction	cerebrosides (glycosphingolipid)	unknown	acetone	Kawai & Ikeda (1982)
Phellinus contiguus	fruiting induction	mycelial extract, active principle unknown	heat stable	water	Butler (1995)
Agaricus bisporus	stem elongation, cap expansion	gills, active principle unknown	unknown	none	Hagimoto & Konishi (1959)
Agaricus bisporus *Coprinus macrorhizus = cinereus*?) *Armillaria matsutake* *Hypholoma fasciculare*	stem elongation, cap expansion	gills, active principle unknown. *(contains IAA, but inactive)	heat, acid, base stable	ether, acetone, ethanol,water (insoluble in petroleum ether, benzene)	Hagimoto & Konishi (1960)
Agaricus bisporus	stem elongation; converts tryptophan to IAA	gills, active principle unknown but <12,000 molecular weight. *(contains IAA, but inactive)	unknown		Konishi & Hagimoto (1961)
Agaricus bisporus	stem elongation	gills, active principle unknown. Glutamic acid, leucine, cysteine, glycine, serine, asparagine, glutamine, threonine, tyrosine, valine, proline, arginine, $(NH_4)_2SO_4$ & NH_4Cl all tested +ve in the bioassay	unknown	ether, acetone, ethanol (pet. ether, benzene insoluble)	Konishi (1967)

Agaricus bisporus	stem elongation	gills, active principle unknown.	unknown	none	Gruen (1963)
Flammulina velutipes	stem elongation	gills, active principle unknown.	unknown	none	Gruen (1969)
Coprinus radiatus	stem elongation	gills, active principle unknown.	unknown	none	Eilers (1974)
Flammulina velutipes	stem elongation	gills, active principle unknown.	unknown	none	Gruen (1976)
Flammulina velutipes	stem elongation	gills, active principle unknown, but <12,000 molecular weight	heat stable	none (diffused into agar blocks)	Gruen (1982)
Coprinus congregatus	stem elongation	cap inhibitor, active principle unknown, but <12,000 molecular weight	unknown	none	Robert & Bret (1987)
Coprinus congregatus	stem elongation	cap inhibitor and stimulator, active principle unknown	unknown	unknown	Robert (1990)
Agaricus bisporus	mycelial growth, stem elongation	wound hormone, ODA (=10-oxo-*trans*-8-decenoic acid), enzymatic degradation product of linoleic acid	unknown	extraction patented	Mau *et al.* (1992)
Coprinus micaceous	unknown	*cytokinin-like activity in fruit bodies	unknown	unknown	Szabo *et al*. (1970)(cited by Gruen, 1982)
Lentinus tigrinus	unknown	*auxin-like, gibberellin-like, cytokinin-like activities in stems and caps	unknown	unknown	Rypacek & Sladky (1972, 1973)(cited by Gruen, 1982)
Agaricus bisporus *Boletus elegans* *Grifola frondosa* *Phallus impudicus* *Phellinus pomaceous*	unknown	*gibberellin-like activity in caps	unknown	unknown	Pegg (1973)

* indicates the presence of plant growth regulators which have no stem elongation or cap expansion properties on the fungal fruit bodies tested (see Gruen, 1982).

In the case of vascular differentiation in plant embryos, auxin transport is instrumental in establishing polarised differentiation of vessels and cell shape in plant embryos via a concentration gradient and even acts during later stages when regenerative processes are required, for example during wound repair (Sachs, 1991). Exogenously added auxin alters gene expression by enhancing rapid transcription of specific mRNAs in plant tissues undergoing either cell elongation or cell division (Key, 1989; Guilfoyle *et al.*, 1993) and there is also evidence that auxin is bound to nuclear receptors (Löbler & Klämbt, 1985; Guilfoyle *et al.*, 1993; Ulmasov *et al.*, 1995 and references therein). Auxin-regulated mRNAs show distinct patterns of organ-specific, tissue-specific and development-specific expression (Guilfoyle *et al.*, 1993).

In *Dictyostelium discoideum* (which produces an asexual fruiting structure), starved amoebae are triggered to aggregate together into a mound by chemotaxis relayed by cAMP signals (Kay, Berks & Traynor, 1989). Cells of the mound differentiate further, into stalk or spore cells, via a gradient of diffusible differentiation inducing factors or DIFs (a family of chlorinated alkyl phenones) (Kay, Berks & Traynor, 1989; Berks *et al*., 1991).

As in the previous examples, DIFs induce specific gene expression via a cytosolic and nuclear binding protein (receptor) (Williams *et al.*, 1987; Insall & Kay, 1990). An important criterion in all three systems cited here as examples is that morphogenesis is polarised, that is, it proceeds in a certain direction. Polarisation involves controlling gene expression in a graded manner yet gene activity is not known to be directional (it is on/off) so polarised development must be expressed as a coordination of events of many cells (Sachs, 1991), i.e. external factors control gene expression within cells. Also, in the case of retinoic acid and auxin, the response to the morphogen is dependent on the tissue it acts upon and not specific to the morphogen molecule. In contrast, DIFs in *D. discoideum* cause the specific differentiation of immobilised amoebal cells into prestalk, and then stalk cells (Kay *et al*., 1989).

Are there parallels between these examples and primordium differentiation? Although there are light microscopic observations of early primordium structure (Reijnders & Moore, 1985 and references therein), no studies have explored whether there is hormonal specification of, for example, cap and stem regions in the developing primordium. The early primordial stages of *S. commune* and *C. cinereus* were described by van der Valk & Marchant (1978) as consisting of randomly oriented hyphae; at a later stage, hyphae were enveloped in a mucilage and clear demarcations of an apical centre in *S. commune* and of distinct cap and stem zones in *C. cinereus* were observed. Reijnders & Moore (1985) described that the early organisation of a primordium consists of two types of tissue, a bundle of nearly parallel hyphae and interwoven hyphae which form the plectenchyma. Microscopic studies describing morphogenesis in *C. cinereus* indicate that differentiation into cap and stem regions occurs very early in the development of a fruit body initial (Moore, Elhiti & Butler, 1979; and see Chapter 1). Similarly, in *A. bisporus*, 2 mm tall primordia consist of a disoriented mass of hyphae (Flegg & Wood, 1985) and yet by the time it reaches 6-10 mm in diameter, the primordium has differentiated into the tissue zones present in mature mushrooms. Clearly, even at this early stage, there is a polarity within the primordium, one of the requirements for establishing an actual/active morphogen gradient.

It is difficult to envisage that the primary differentiation of a primordium into distinct zones of differentiated tissues is attributable solely to the initial mating event. The process cannot represent the playing out of a sequence initiated by the first interaction of the mating type genes. It is far easier to believe that these fundamental differentiation events are directed by morphogens as in the case of retinoic acid regulation of digit formation in the chick limb bud (Tickle, 1991) or auxin-regulated vascular differentiation in plant stems (Sach, 1991). However, if the mating type genes are the master regulators of development, potential candidates for the control could be the proteins encoded by these

genes or genes under their control. But first it would have to be demonstrated that the putative protein products of these genes did have transcription factor function which was active in a concentration gradient-dependent manner. Neither of these conditions have yet been met.

An important feature of fungal development which is different from animal and, to a degree, plant systems is that most differentiated fungal tissue is not determined or irreversibly committed to a specific fate. Consequently, the differentiation which occurs is not terminal and all the tissues of a fruit body, other than the probasidia which are determined after prophase I (Chiu & Moore, 1988, 1990), can dedifferentiate and revert to vegetative growth upon transplantation to a new medium (see Chapter 6). Thus it is extremely important that a differentiating 'environment' is maintained within the intact tissue, possibly via morphogens, to ensure fulfilment of development. Primordia are often enveloped in a mucilage (van der Valk & Marchant, 1978) which could serve as the medium through which morphogens could maintain the differentiated state. Investigations with developing primordia, i.e. dissection and exogenous application of morphogens as was done to determine the presence and action of morphogens in the chick limb bud system (Brickell & Tickle, 1989), are difficult due to their small size and the unpredictability of the location of their formation; but the problems are not insurmountable (Chapter 6) and molecular approaches could also be useful. Although there are no reports in the literature of studies investigating the genetic changes correlated specifically with the formation of the cap or stem zones as yet, this field is worth further examination.

Growth and maturation of the fruit body

Fruit body growth involves cell expansion in species such as *C. cinereus* (Gooday, 1985; Hammad *et al.*, 1993; Hammad, Watling & Moore, 1993), cell expansion as well as division in other species, such as *Agaricus bisporus* (Craig, Gull & Wood, 1977) and only cell division in *S. commune* (Wessels, 1992). Different aspects of stem elongation during fruit body maturation have been reviewed by Gooday (1985) and recently by Kamada (1994). In plants and animals hormones and growth factors involved in morphogenesis as well as cell division and expansion are the subjects of active study. Sadly, this is not true for the fungi. With the one exception of 10-oxo-*trans*-8-decenoic acid (ODA, an enzymatic breakdown product of linoleic acid) (Tressl, Bahri & Engel, 1982), a hormone produced in *A. bisporus* as a result of wounding (Mau, Beelman & Ziegler, 1992), astonishingly little attention has been given to the compounds with hormone or growth factor-like activity described in earlier studies of extracts from *A. bisporus* (Hagimoto & Konishi, 1959, 1960; Konishi & Hagimoto, 1961; Gruen, 1963; Konishi, 1967), *F. velutipes* (Gruen, 1969, 1976) and other basidiomycetes (Hagimoto & Konishi, 1960; Eilers, 1974; Robert & Bret, 1987; Robert, 1990). The last extensive review of this topic appeared fourteen years ago (Gruen, 1982)!

Previous attempts to purify and identify fungal substances which might regulate fruit body growth are shown in Table 1. In most cases, the objectives of these studies was simply to determine whether hormone-like or growth factor-like compounds existed in fungi. Presence or absence was the essential criterion and any active ingredients found in fruit body extracts were not chemically identified; only their capacity to promote stem elongation and/or cap expansion was described. Konishi (1967) was the first to partially purify a substance from *A. bisporus* caps which enhanced stem elongation in the *Agaricus* test and determine that the growth factor was comprised of various amino acids, which he tested (in pure solution) for their individual effects on stem elongation. Whether these amino acids were functioning as individual growth factors or as nutrients (at a concentration of 10^{-4} M) is not known and there seems to have been no effort to purify the active, fungal ingredient further.

Considering that the evidence for the presence of fungal growth factors is so fragmentary and derives from experiments involving very different species and diverse extraction methods, it is surprising that

the various extracts (including those analyzed for their ability to elicit fruit body induction/formation) have exhibited similar activities and chemical properties. Extracts from *A. bisporus*, *Coprinus macrorhizus*, *Hypholoma fasciculare*, *Armillaria matsutake* (Hagimoto & Konishi, 1960; Konishi, 1967; Urayama, 1969), *Lentinus edodes*, *F. velutipes*, and *Pleurotus ostreatus* (Urayama, 1969; Gruen, 1982) all cause stem elongation and cap expansion, are <12,000 molecular weight, heat stable, acid/base stable and mostly soluble in polar solvents including water. These similarities in characteristics may suggest that the active compounds comprise a family of hormones or growth factors of slightly different chemical structure in each species, but with enough similarities to be cross-reactive, as in the case of the *A. bisporus* extracts which induce fruiting in *S. commune* (Rusmin & Leonard, 1978), and broadly similar in gross chemical character. This is not unlike the situation in plants where auxin is actually a family of related compounds based on indole-3-acetic acid (Salisbury & Ross, 1985) and active on a very wide variety of plants. While the majority of the substances in Table 1 stimulate extension, those isolated by Robert & Bret (1987) and Robert (1990) from *C. congregatus* have inhibitory activities and were extracted from fruit bodies at earlier stages of development (primordium). This is evidence that both inhibitory and stimulatory substances are produced in fruit bodies during growth and that there may be temporal control of growth factor activity or production which may be important at different stages of development. In contrast, the fruiting induction substance extracted by Rusmin & Leonard (1978) from different developmental stages of *A. bisporus* fruit bodies showed no differences in activities (that is, all stages produced equivalent fruiting inducing activity). Technically, these two cases are not totally comparable since the first pertains to a factor(s) which causes enhanced or inhibited stem extension while the second deals with fruiting induction.

Clearly, there is not yet enough evidence of the activity and identity of these substances to understand the manner in which such growth factor-like compounds regulate fruit body development. Purification is the key. Purification and determination of the complete chemical structure of the active ingredient. Many of the difficulties in purification attempts have been compounded by the use of complex agar media to isolate growth factor-type activity. Indiscriminate extraction processes, for example from whole fruit bodies, from damaged fruit bodies or using other than the gentlest extraction techniques, may have released cytosolic components able to degrade the activity of the very compounds the experimenters were trying to isolate. It would also be difficult to isolate a large enough quantity of growth factor if only small numbers of fruit bodies were used in the extraction processes, especially if the factors occur at concentrations lower than 10^{-6} M.

Probably the greatest area for error is the bioassay. Without a sensitive and appropriate bioassay, activity of the compounds in question may be missed altogether or conversely, the activity observed may not be solely due to hormonal effects but also due to nutrient metabolism. Most of the studies reported in the literature were performed over a very long time (24-72 h). Such lengthy assays immediately pose the question of whether stems, detached from the parent mycelium, were still viable especially in the case of *A. bisporus* and *F. velutipes* where full or normal extension of the fruit body is dependent on the parent mycelium (Gruen, 1982). An even more relevant question is whether the putative growth factor is likely to be active over the sort of time scale used for published bioassays. By definition, growth factors (especially those controlling small morphogenetic fields) must be unstable, either intrinsically or through active destruction, as one of the ways to establish the concentration gradient. Improvements to the bioassay techniques might include using species which react rapidly and/or assay criteria which can be judged more quickly so that results can be obtained in a few hours rather than days and by limiting the potential number of compounds being bioassayed by performing simple separation techniques, such as dialysis (Rusmin & Leonard, 1978; Gruen, 1982; Robert & Bret, 1987), gel filtration (Rusmin & Leonard, 1978) or thin-layer chromatography.

Vegetative structures
Multihyphal vegetative organs (mycelial strands, coremia, rhizomorphs and sclerotia) may also develop under the coordinating signal(s) of growth factors. These multihyphal structures are composed of distinct tissue zones, such as the rind, cortex and medulla of many sclerotia (Willetts & Bullock, 1992) and they perform an important role in the survival of the organism, be it nutrient retrieval in the case of coremia and rhizomorphs (Watkinson, 1979) or over-wintering in the case of sclerotia (Willetts & Bullock, 1992; Moore, 1995). Such structures are formed from vegetative, monokaryotic hyphae and so, mating type genes do not operate in this situation. Thus the same question needs to be posed about how the change comes about from a diffusely-growing, vegetative pattern of hyphal growth to one in which hyphae grow together in harmony as an aggregate, but without the involvement of any of the mechanisms which might be involved in finding and attracting mates.

The repulsion normally encountered by hyphae in the same colony must be replaced by attraction or, in the very least, by a neutral or no response between adjacent hyphae (Willetts & Bullock, 1992). These structures are highly differentiated; for example, sclerotia are composed of distinct tissues, each comprised of hyphae which differ in structure and chemical properties from the vegetative hyphae from which they originated (Willetts & Bullock, 1992). The hyphae comprising rhizomorphs, which are specialised for efficient translocation of nutrients and are therefore scavenging organs of phytopathogenic species such as *Armillaria mellea* (Watkinson, 1979), are physiologically different from the vegetative hyphae from which they developed. Thus, there is a need to determine whether chemical factors may be involved in coordinating the initial formation of these organised structures and once they are formed, whether there is a need for growth factor control between the different tissues comprising them. Many of these structures produce mucilage (Watkinson, 1979) or a layer of reactive quinone compounds (as a result of phenoloxidase activity which is elevated in vegetative, multihyphal structures (Willetts & Bullock, 1992)) and these extracellular matrices may facilitate intercellular communication via growth factors which can direct morphogenesis of hyphae in tissue zones in sclerotia and rhizomorphs. Again, hydrophobins may also be involved, perhaps in an adhesive role, since hydrophobins specific to monokaryons are produced (reviewed in Wessels, 1993b) and hydrophobic interactions between hyphae emerging from a medium may be sufficient to bind the hyphae together loosely.

Tropisms as morphogenetic changes
Fungi have evolved strategies for different tissues to develop and grow in different directions with respect to gravity, light and other external stimuli. For example, in mushrooms the cap expands revealing gills, pores, tubes or teeth on the underside, which must be oriented vertically downwards to allow the basidiospores to escape from the fruit body. The positive gravitropism of the gills and the negative gravitropism of the stem are the mechanisms which achieve the vertical orientation. In *Coprinus* spp. the gills are agravitropic so the vertical position of the cap is solely dependent on the responses of the stem (Moore, 1991). The tropic response involves the tissue somehow sensing the orientation stimulus (direction of ‘down’ for gravitropism, direction of ‘brightest’ for phototropism, etc.) and then differentially regulating the growth of its constituent cells so that the organ is repositioned. Thus a tropic response is a convenient tool for the study of morphogenesis since the application of the stimulus is in the hands of the experimenter and the response to the stimulus involves differential regulation of cell differentiation. We have done most work on gravitropism in *C. cinereus.*

When a mushroom is laid horizontally it is able to reorient its hymenium to the correct position by the stem growing differentially, raising the cap to the correct orientation (reviewed in Moore, 1991; Moore *et al*., 1996). Thus a change in the gravity vector induces a simple, developmental pattern-forming

process, that is a morphogenetic change, whereby the perceived external signal (change in the gravity vector) is transduced into a biological/cellular response resulting in regeneration of the hymenium or controlled differential growth. The gravitropism of *C. cinereus* has been used as a model to generate this morphogenetic change on demand and to look for hormonal or growth factor control of elongation during the differential growth generated for the gravitropic response.

The kinetics of the gravitropic response in the two most studied basidiomycetes, *C. cinereus* and *F. velutipes*, have recently been compared and reviewed (Moore *et al.*, 1996) and reveal evidence for the presence of growth factors controlling differential growth. In *C. cinereus*, the initial response is due to the lower hyphae (meaning those hyphae in the lower half of a horizontal stem) elongating faster than the upper hyphae (meaning those hyphae in the upper half of the same horizontal stem). Light microscopic studies reveal that lower hyphae increase in length by 4-5 fold without increase in girth, and growth studies indicate that the lower surface of the stem elongates at a faster rate than the upper surface to generate the gravitropic bend (Greening & Moore, 1996). Differential extension is achieved without an increase in the number of lower hyphae or inflation of narrow hyphae in the lower half or conversely, a decrease in the number of hyphae or deflation of inflated hyphae in the upper half (Greening & Moore, 1996). Clearly, the fact that different regions of the same gravitropically responding stem extend at different rates implies not only that the relative position of hyphae in the stem is recognised but that there must be a mechanism by which differential growth is coordinated.

These results, along with those presented below, implicate growth factor control in one of three ways: (i) a gradient of a stimulatory substance is established which induces extension of the lower hyphae preferentially; (ii) a gradient of an inhibitory substance is established which inhibits extension of the upper hyphae preferentially; or (iii) both types of substance are present/produced but their distribution in the stem results in the extension pattern described above. Curvature begins at the apex of the stem and proceeds in a basipetal direction (Kher *et al.*, 1992); it does not proceed in an apical direction nor remain at the location in which the bend originates. This implies that the transport of growth factor(s) is basipetal. Once the responding stem has reached a certain curvature (estimated to be approximately 35^{o} (Moore *et al.*, 1994)), the apex begins to straighten out or unbend so that it does not overshoot the vertical. This phenomenon is a separate process called curvature compensation (Kher *et al.*, 1992) and it implies a change in the transport or in the balance of growth signals present or active in the responding stem.

When a horizontal stem is attached by the apex rather than the base it initially responds normally, by lifting the basal portion of the stem until it reaches the vertical but it then continues to bend and curls over, often into a circle (Kher *et al.*, 1992). These observations indicate that the apex must be free to move for curvature compensation to occur, imply that there is polar transport of the compensation signal (from the apex to the base) and that there is interaction of growth signals during the normal response controlling the different extension rates of lower and upper portions of the stem. Preliminary experiments have been performed to determine whether stem hyphae react individually or in a concerted manner during the gravitropic response. Stems were split longitudinally (with the base left intact) and placed horizontally in a moist chamber (as described in Kher *et al.*, 1992); each piece exhibited negative gravitropism. Even though some pieces curled back in opposite directions, because of the release of tissue tensions when first cut, each portion always returned to the vertical.

Intact gravitropically stimulated stems still respond and grow normally under water (Kher *et al.*, 1992), but when longitudinally split stems were placed under water, they did not uncurl and did not return to the vertical even though extension was unaffected. This indicates that water immersion dissipated the polar transport or gradient of growth factors (Novak Frazer, unpublished results) and also shows that

the growth factors were active in the extracellular matrix of the stem. Experiments have also demonstrated that the stem is polarised with regard to the distribution of gravireceptive cells. There are fewer perceptive cells at the base than at the apex, so a faster response is realised at the apex (Moore *et al.*, 1994). The gradient of receptive cells (many at the apex, fewer at the base) has been shown by sequential removal of sections of stem and monitoring response of the remaining part (Greening, Holden & Moore, 1993): stem response was delayed in direct relationship to the amount of apex is removed. These results, although circumstantial, indicate the potential for growth factor involvement during gravimorphogenesis in *C. cinereus*. The ingredients for growth factor/hormonal control are present: the stem is a polar system and different regions within the stem (that is, the lower and upper portions) respond in a different manner, both physiologically and temporally.

Potential growth factors have been extracted from pre- and post-meiotic fruit bodies and two different activities found, possibly corresponding to two different substances (Moore & Novak Frazer, 1993, 1994). The bioassay used was similar to that described by Michalenko (1971) and Gruen (1982) although lanolin was not used as a carrier. One substance (Fungiflex 1) was produced by both immature and mature fruit bodies of *Coprinus cinereus* but the other (Fungiflex 2) was only produced by mature fruit bodies. Fungiflex 2 was found to be between 10-100 times more abundant in the cap than in the stem. Both substances were of low molecular weight (<12,000 molecular weight), as determined by dialysis, gel filtration and mass spectroscopy, and were heat stable. In these two respects, molecular weight and heat stability, the substances extracted are similar to those described in earlier studies (Table 1). Fungiflex 1 has a faster action (results seen after 1 h exposure) and has inhibitory properties, similar to substances extracted from *C. congregatus* (Bret & Robert, 1987). Fungiflex 2 has a slower action (results seen after 6 h exposure) and has stimulatory properties, similar to substances extracted from various basidiomycetes (see Gruen, 1982 for review).

Notwithstanding the previous similarities, Fungiflex 1 and 2 function in a much shorter time frame than those already described (bioassay results visible over 1-18 h rather than the 24-72 h time frame of other studies). This may be partially explained by the fact that *C. cinereus* is a relatively fast-growing and probably fast-reacting species compared to *Agaricus* and *Flammulina*. Fungiflex 1 and 2 are similar in their solubility characteristics (water and methanol soluble) to those of other extracted growth factors although generally soluble in fewer solvents (Table 1). The identity of Fungiflex 1 and 2 is unknown as they have not yet been purified.

Although these substances may function during the gravitropic response to generate differential growth and were originally extracted from gravireacting stems, it is unlikely that they are specifically gravitropic hormones or growth factors, whose synthesis is induced by the external stimulus. The fact that Fungiflex 1 and 2 (or substances with the same activities as those extracted from gravitropically reacting stems) can also be extracted from pre- and post-meiotic and vertically growing fruit bodies indicates that they may have a more general role in coordinating growth. Their similarity to other fungal growth factors (Table 1) may reflect a general role in controlling extension. During rapid elongation, which occurs soon after meiosis in *C. cinereus* (Hammad *et al.*, 1993), the growth of the expanding cap and elongating stem must be coordinated. The expansion of the whole fruit body must also proceed accurately in a vertical direction so that the gill surface is properly oriented when sporulation occurs a few hours later. Thus these growth factors are probably involved in continually correcting the direction of growth of the mushroom as well as coordinating the expansion of the cap and elongation of the stem during the later stages of fruit body development.

Future studies

Bioassays are cumbersome to perform, and if performed with impure extracts, which by their nature are

variable, are sometimes impossible to interpret. This is especially inconvenient when active substances are in short supply. Clearly, the growth factor-like substances extracted from *C. cinereus* (and from the fungi of previous studies) must be purified, identified and ideally, chemically synthesised so that their activity can be verified. Once the structure of DIF was determined (Morris *et al.*, 1987), its exact function during the differentiation process in *Dictyostelium discoideum* was verified (Williams *et al.*, 1987) and great strides were made in elucidating the binding proteins/receptors with which it interacted (Insall & Kay, 1990) to effect a developmental change in amoebae. Purified Fungiflex would provide a tool to establish which cells produce growth factors, their target(s), means of transport, mode of action, and cross-reactivity.

We are at the infancy in our knowledge of how mushrooms differentiate but the facilities and techniques for dissecting this question are at hand and we should adapt as many as are appropriate to the fungal system from the other embryological studies that are currently ongoing. The study of fungal growth factors and hormones promises to reveal a whole new world in mycology: understanding the interactions between neighbouring hyphae within a multihyphal structure, be it a sexual fruit body or a vegetative structure; understanding how the processes in the 'black box' between dikaryon formation and the development of a mature fruit body are coordinated; and opening up the possibilities in using these substances in commercial applications.

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