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A homologue of the yeast *SHE4* gene is essential for the transition between the syncytial and cellular stages during sexual reproduction of the fungus *Podospora anserina*

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The *Podospora anserina cro1* gene was identified as a gene required for sexual sporulation. Crosses homozygous for the *cro1-1* mutation yield fruiting bodies which produce few asci due to the formation of giant plurinucleate cells instead of dikaryotic cells after fertilization. This defect does not impair karyogamy, but meioses of the resultant polyploid nuclei are most often abortive. Cytological studies suggest that the primary defect of the mutant is its inability to form septa between the daughter nuclei after each mitosis, a step specific for normal dikaryotic cell divisions. The *cro1-1* mutant would thus be unable to leave the syncytial vegetative state while abiding by the meiotic programme. *cro1-1* also shows defects in ascospore germination and growth rate. GFP-tagging of the CRO1 protein reveals that it is a cytosolic protein mainly expressed at the beginning of the dikaryotic stage and at the time of ascospore maturation. The CRO1 protein exhibits significant similarity to the SHE4 protein, which is required for asymmetric mating-type switching in budding yeast cells. Thus, a gene involved in asymmetric cell divisions in a unicellular organism plays a key role at the transition between the syncytial (vegetative) state and the cellular (sexual) state in a filamentous fungus.

Keywords: cellularization/evolutionary transition/ *Podospora anserina*/sexual development/SHE4–CRO1

Introduction

The transition from unicellular forms to pluricellular organization was certainly a key step in the evolutionary history of eukaryotes. At least two non-exclusive scenarios could result in this transition. In the first scenario, a pluricellular state was obtained through the aggregation of individual cells, as is observed in the life cycle of lower eukaryotes such as the slime mould *Dictyostelium discoideum* (reviewed in Gross, 1994). In the second scenario, pluricellular organization arose from a syncytial plurinucleate state. This type of transition is encountered in the early development of insects, the paradigm of which being *Drosophila* (see Schejter and Wieschaus, 1993 for a review). The genes which control this syncytial/cellular switch are of considerable interest from both the developmental and the evolutionary points of view. In addition to *Drosophila*, simpler organisms such as the filamentous ascomycetes *Neurospora crassa* and *Podospora anserina* also show a transition between a syncytial and a true cellular state. This switch is required for proper sexual development and can thus present an interesting complementary model system to understand such transitions.

In these fungi, as shown in Figure 1, fertilization does not alter the syncytial (vegetative) state since both reproductive nuclei (carrying different mating types) divide in a common cytoplasm, without undergoing cytokinesis. The true cells, which arise from this plurinucleate heterokaryotic syncytium, contain one nucleus of each mating type (*mat*+ and *mat*– in *P.anserina*). In these hook-shaped crozier cells, coordination between nuclear and cellular division ensures the formation of three cells: a dikaryotic upper cell which can differentiate into an ascus in which meiosis will take place, and two uninucleate cells which, after fusion, will give rise to a new dikaryotic cell (see Figure 1). It is noteworthy that this dikaryotic phase is the only part of the fungal life cycle in which a septum is formed between two daughter nuclei after each mitosis. This is, in fact, a prerequisite for a true cellular organization and its maintenance.

In our systematic investigation of the sexual cycle of *P.anserina*, we have shown previously that the mating-type genes control the proper assortment of one *mat*+ and one *mat*– nucleus in the dikaryotic cells. However, these genes are not involved in the syncytial/cellular switch (Zickler *et al.*, 1995). Among the mutants identified during a systematic search for impaired sexual development (Simonet and Zickler, 1978), the *cro1-1* mutant showed abnormal cellularization. The *cro1-1* mutation leads, when homozygous, to croziers that can contain up to several dozens of nuclei instead of the two normally seen in wild-type croziers. Cytological analyses of fruiting body development, meiotic nuclear divisions and cytoskeleton components, led to the hypothesis that the primary defect of the mutant is its inability to switch from the syncytial to the cellular state. The cloning and sequencing of the *cro1* gene revealed a significant similarity between the *P.anserina* CRO1 protein and the *Saccharomyces cerevisiae* SHE4 protein (Jansen *et al.*, 1996). In yeast, the SHE4 protein is required, at the time of cell division, for asymmetric mating-type switching. Contrary to the yeast *she4* mutants (Wendland *et al.*, 1996), the *P.anserina cro1-1* mutant does not show internalization defects as revealed by the FM4-64 endocytic probe (Vida and Emr, 1995). The CRO1 protein is localized in the cytosol and displays peaks of expression at developmental stages which are coincident with the mutant defects, i.e. at the beginning of the dikaryotic stage and during ascospore maturation.

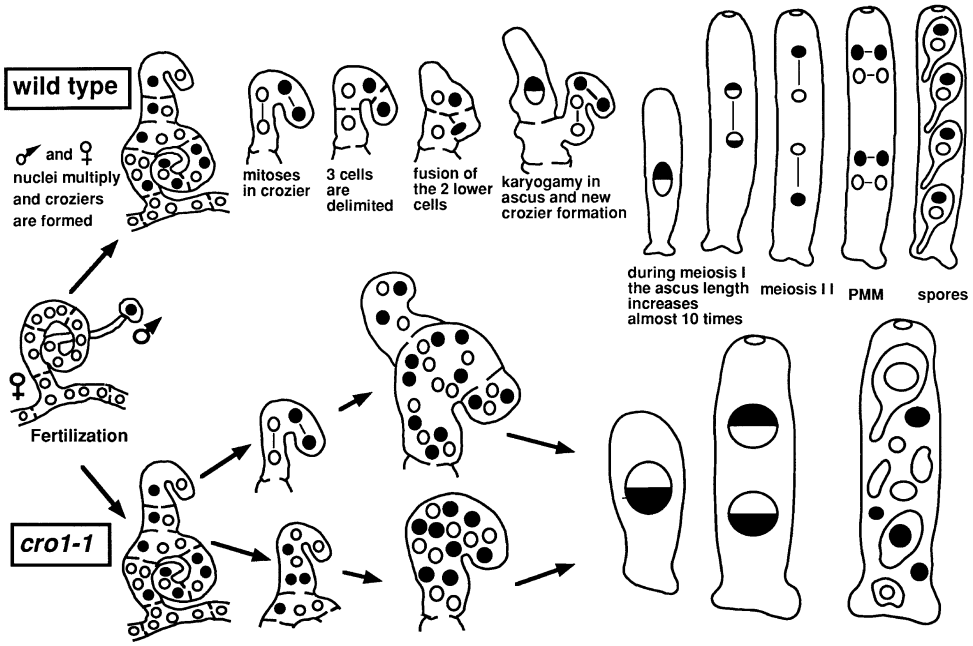

Fig. 1. Sexual cycle of wild-type *Podospora anserina* and homozygous *cro1-1* mutant with emphasis on crozier and ascus formation. Note that in the mutant strain, multinucleate croziers can form by two different mechanisms. Either two nuclei are isolated as in wild-type dikaryons but they divide without septum formation (top), or several nuclei migrate in the crozier (below).

Results

The cro1-1 mutation affects sexual development mainly during the dikaryotic phase

In *Podospora*, the sexual cycle is initiated when a female structure (the ascogonium) is fertilized by a male cell of opposite mating type. Fertilized female organs develop into fruiting bodies (perithecia), within which meiotic cells (asci) form from successively developing dikaryons. After meiosis and a postmeiotic mitosis, each ascus produces four dikaryotic ascospores (Figure 1). Fertilization occurs normally in a *cro1-1*×*cro1-1* cross, but perithecium development is defective at several stages.

First, compared with the 160–200 asci observed in each wild-type perithecium, only 3–30 asci are formed per perithecium (50 analysed) in the homozygous mutant crosses, and most ascospores are abnormal in shape and size.

Second, all perithecia are filled with giant, highly multinucleate cells which are more or less hook-shaped. This is never observed in wild-type, in which hook-shaped croziers always contain two nuclei of opposite mating type. After a coordinated mitosis, septa form on each side of the crook, resulting in three cells: an upper binucleate ascus-mother cell, flanked by a basal and a lateral uninucleate cell (Figure 2A). In *cro1-1* crosses, a few normal croziers are also observed, and the small number of wild-type asci and ascospores probably result from such croziers. All other croziers are plurinucleate and of varying morphology and size. Some are aseptate (Figure 2B); in the others, despite formation of septa, all three cells contain varying numbers of nuclei (Figure 2C). These multinucleate cells exhibit, however, the characteristics of crozier cells. Their nuclei are larger than the vegetative and ascogonal nuclei and, more strikingly, all mitoses remain synchronous whatever the number of nuclei (from three to over 100) contained in a cell (see below). In wild-type perithecia, several 'trees' of asci are formed from successively developing croziers. In the mutant perithecia, abnormal croziers also form 'trees', but although interconnected, they are not affected in the same way: croziers with few nuclei and normal septa are connected to aseptate and multinucleate giant croziers (Figure 2B); 'normal' binucleate croziers can also form on deformed cells containing over 100 nuclei (Figure 2D).

Third, karyogamy can take place—whatever the number of nuclei present—in the upper cell of the crozier or in the giant cells with mislocalized septa. Nuclear size being correlated with the number of nucleoli visible in these large nuclei, these latter presumably arise from nuclear fusion (Figure 2E). This assertion is based on the fact that young croziers often contain groups of nuclei which could correspond to the divisions of the four nuclei issued from the first mitosis while older croziers contain groups of nuclei with a different level of ploidy (Figure 2F). Although we cannot exclude that some polyploid nuclei result from re-replication events, those containing an uneven set of chromosomes (3n, 5n, . . .) support the karyogamy assumption. However, karyogamy does not occur in all croziers and most abnormal cells degenerate.

Abnormal croziers result in polyploid nuclei with large spindle pole bodies, but asci can proceed through meiosis and sporulation

The wild-type diploid nucleus isolated in the upper cell of the crozier enters meiosis as the ascus mother cell begins to elongate (Figure 1). Asci reach their full length by the end of the first meiotic division. This developmental pattern is similar to that of a *cro1-1* cross except that, probably due to polyploidy, asci are always larger than in wild-type crosses and are sometimes bifurcate (Figure

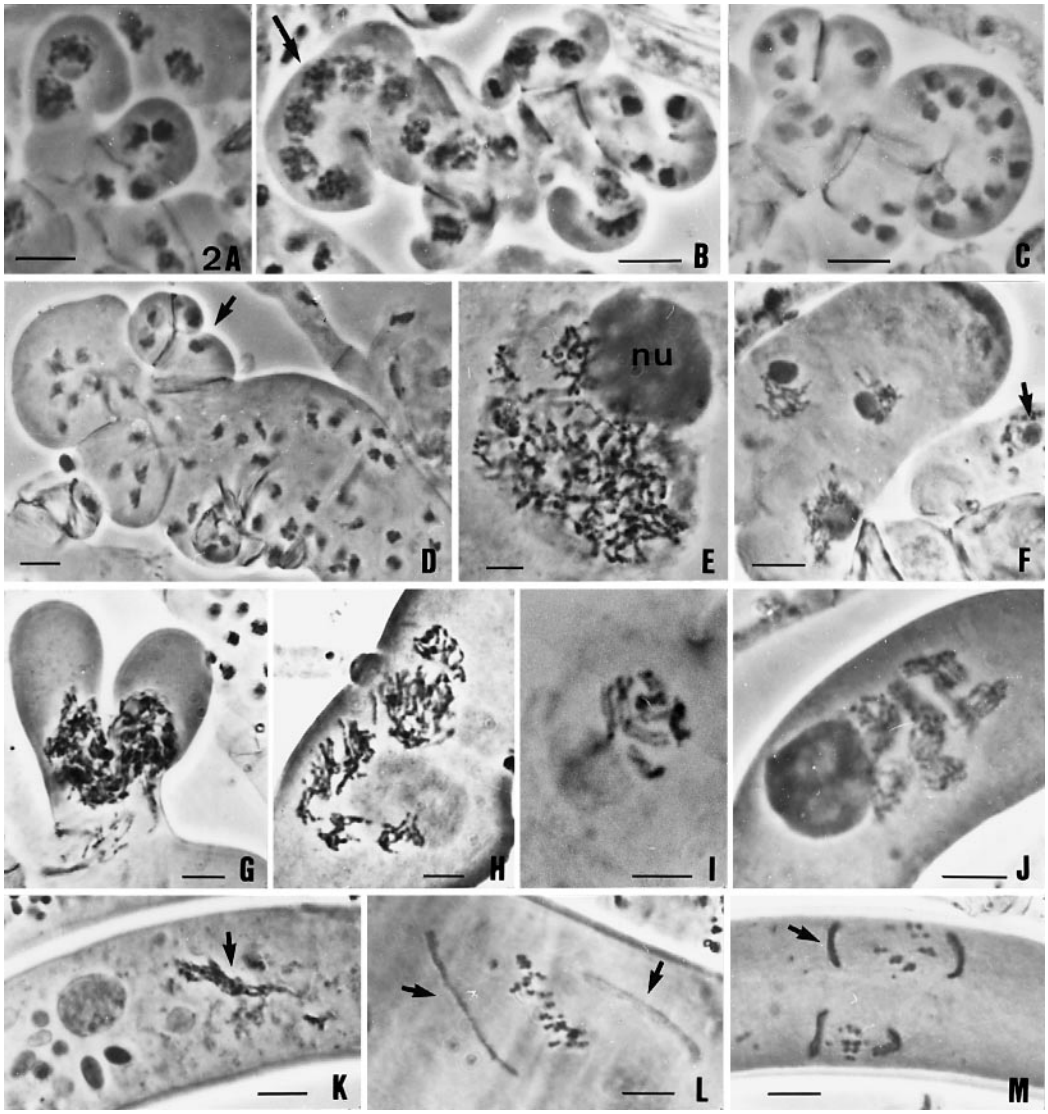

Fig. 2. Crozier and ascus phenotypes in a *cro1-1* mutant. (**A**) Wild-type binucleate croziers. Crozier on the right side contains two nuclei while crozier on the left side shows four nuclei separated by two septa. (**B**) A group of five croziers showing on the left side, a large plurinucleate crozier (arrow) in which no septa are formed, while the croziers at the right side show septa and fewer nuclei. (**C**) Croziers with septa, separating plurinucleate cells. (**D**) A giant plurinucleate crozier on which a normal crozier (arrow) has developed with an upper binucleate cell and a lateral plus a basal uninucleate cell. (**E**) Karyogamy of several haploid nuclei: compare the size of the nucleus and nucleolus (nu) with the size of the crozier nuclei seen in (A). (**F**) A giant crozier with three nuclei of intermediate sizes between haploid and polyploid nuclei. (**G**) Bifurcated ascus with degenerating giant polyploid nucleus. (**H**) *P.anserina* has seven chromosomes. This spread early meiotic prophase (leptotene) nucleus shows clearly that more than two nuclei were fused. (**I**) Wild-type pachytene with seven paired homologues. (**J**) Pachytene in a probable tetraploid nucleus (compare nucleolar size with the diploid nucleolus of (I). Note that the four homologues of each set of chromosomes are paired; (I) and (J) were taken and printed at the same magnification. (**K**) Abnormal spores with excluded nucleus (arrow). (**L**) In this triploid postmeiotic mitosis, both SPBs are large [compare with the haploid metaphase plates of (M)]. (**M**) Normal postmeiotic mitotic spindles with clear SPBs (arrow on one of the four SPBs); note that (L) and (M) were taken and printed with the same magnification. Scale bars represent 5 μm.

2G). In asci proceeding through both meiotic divisions, polyploidy ranges from triploid to octoploid, larger nuclei aborting at early prophase stages (Figure 2H). Nuclear evolution during prophase I (nucleolar increase from leptotene to diplotene and chromatin condensation) is normal when compared with wild-type (Figure 2I). In addition, at pachytene, all homologues (from three to eight) pair along their entire length, as clearly seen in Figure 2J. In the occasional postmeiotic mitoses, ploidy does not exceed 4n. Ascospore formation is mainly abnormal (Figure 2K) and, occasionally, all nuclei in an ascus are delimited within a single giant spore.

The spindle pole body (SPB) of filamentous ascomycetes is composed of a plaque structure apposed to the nuclear envelope (Zickler, 1970). As in yeast, where diploid SPBs' increase in size and nucleating capacity are compared with those of haploid SPBs (for a review see Rout and Kilmartin, 1990), SPB size appears to be a function of ploidy in the *cro1-1* mutant (compare Figure 2L and M). The SPBs of wild-type strains alter in size, nucleating capacity and orientation over the sexual cycle (Zickler, 1970; Thompson-Coffe and Zickler, 1994). Similar ultrastructural differentiation processes are observed in the giant SPBs (data not shown).

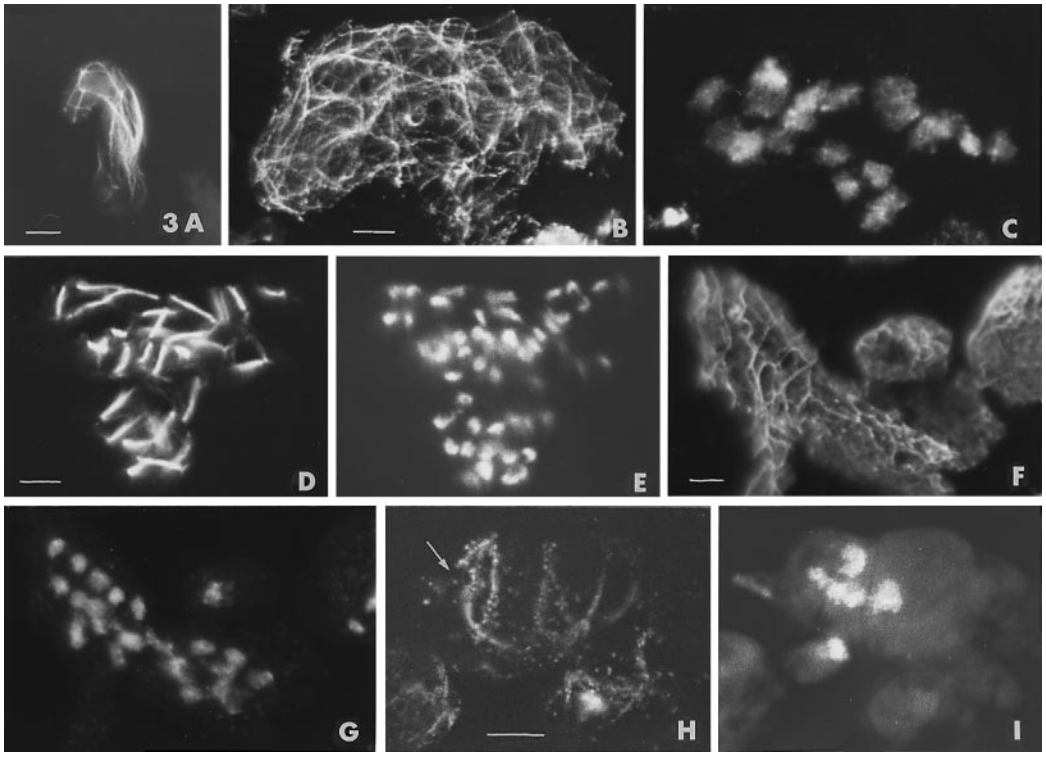

Fig. 3. Cytoskeleton organization in the mutant croziers. (**A**) Anti-tubulin of wild-type croziers. (**B**) Anti-tubulin of a *cro1-1* giant crozier with numerous microtubules spanning the gap between the nuclei [note that (A) and (B) were printed at the same magnification]. (**C**) DAPI of the same crozier. (**D**) Synchronous mitoses in one giant crozier. Note that the cortical microtubules seen in (B) are all gone and that microtubules are only in the spindles. (**E**) DAPI of the same crozier showing that the dividing nuclei were in anaphase. (**F**) Anti-actin of an aseptate giant crozier with broad cortical microfilaments. (**G**) DAPI of the same crozier. (**H**) Anti-actin of a septate giant crozier with actin-plaques and no cortical microfilaments. Microfilaments form belts (arrows), but contrary to what is observed in wild type, they do not correspond to the metaphase plates as shown by the disposition of the corresponding nuclei seen in (I) DAPI staining. Scale bars represent 5 μm.

Cytoskeleton organization in the cro1-1 sexual cycle

Analysis of the distribution of microtubules and actin microfilaments during crozier and ascus development was performed by anti-tubulin and anti-actin immunofluorescence. In wild-type croziers, cytoplasmic microtubules are relatively sparse, bundled, and appear to originate mostly on or near the nuclear envelope (Figure 3A). These microtubules are disassembled during the coordinate mitosis. Immediately after division, all four nuclei display large asters with open ends towards the septal sites (Thompson-Coffe and Zickler, 1994). The wild-type arrangement is observed in the few normal *cro1-1* croziers. However, in the giant multinucleate croziers, microtubule organization differs significantly from that of wild-type. Microtubules are abundant, curving, and appear mainly cortical (Figure 3B and C). As in wild-type, these non-nuclear microtubules are no longer visible during nuclear divisions and mitoses remain strictly coordinate (Figure 3D and E). However, the spindles are randomly oriented and few astral microtubules are visible, while in wild-type the astral microtubules are quite long and apposed to the plasma membrane.

Actin is present as both plaques and fibres in wild-type and mutant cells, but its distribution differs between the two. In premeiotic wild-type croziers, filaments are relatively sparse, mainly seen in the crook area and between the nuclei (Thompson-Coffe and Zickler, 1993). The microfilaments of giant *cro1-1* croziers are abundant and, like the microtubules, appear to be mostly cortical with little evident orientation respective to the nuclei (Figure 3F and G). Wild-type actin belts assemble as future septa sites in anaphase of crozier mitosis and later disassemble (Thompson-Coffe and Zickler, 1993). In *cro1-1* croziers, such belts may form between more or less properly oriented nuclei (Figure 3H and I) or may not be seen at all. After development of the ascus, actin is organized as a cortical array of longitudinal microfilaments with associated plaques, parallel to the cortical microtubule array, in both wild-type and mutant strains (not shown).

The cro1-1 mutant also displays a vegetative phenotype

The *cro1-1* mutant grows slowly and in waves: first flat, the mycelium becomes increasingly dense before initiating a new flat surface, the band size and period varying during radial growth. However, septa and nuclear distribution are similar to what is observed in wild-type mycelia (data not shown). The longevity of the *cro1-1* strain is just slightly shorter than the wild-type lifespan (Rossignol and Silar, 1996). The second vegetative defect of *cro1-1* concerns ascospore germination. This process, which takes a few hours for wild-type ascospores (at 27°C), can take several days or even several weeks for the mutant. This defect is less pronounced at 20°C. However, at both temperatures, the mycelium issued from the mutant ascospores is first very spindly and then displays a normal thickness. Both vegetative characteristics and the sporulation defect of the mutant co-segregate in crosses and appear recessive.

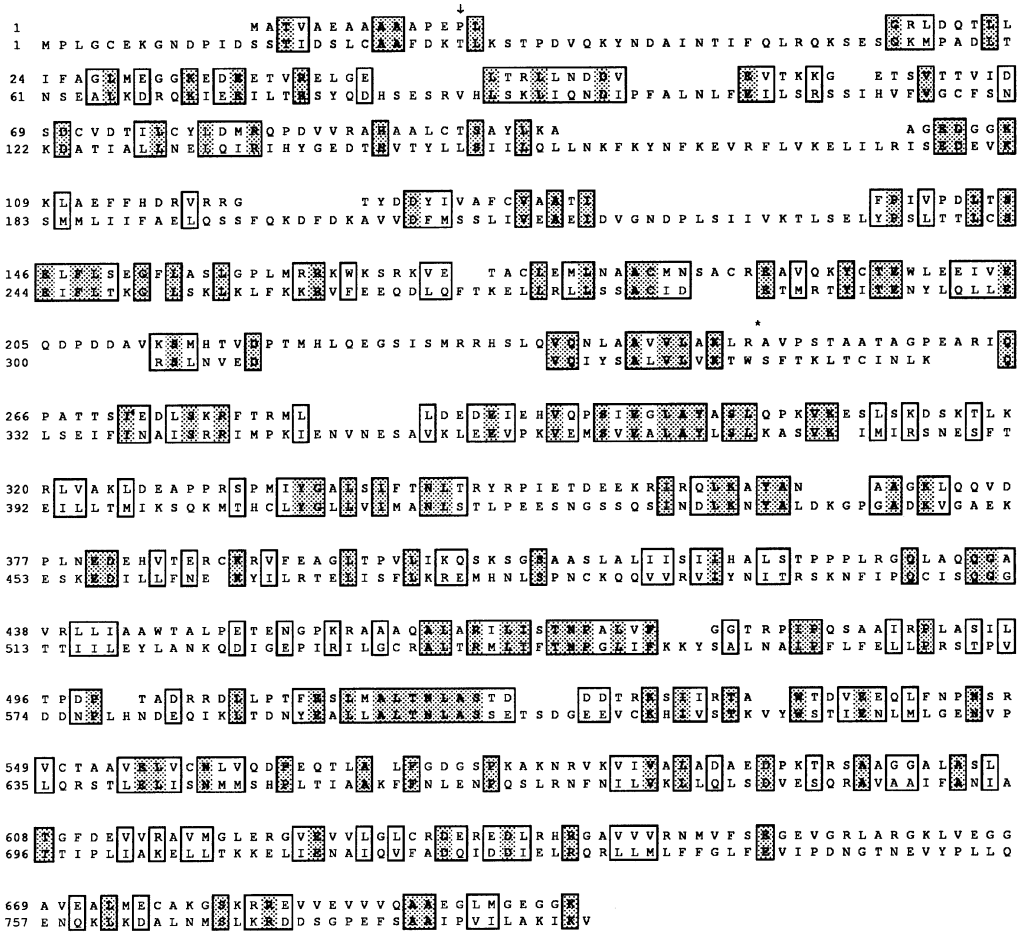

Fig. 4. Comparison of the amino acid sequences deduced from the *P.anserina cro1-1* gene and the *S.cerevisiae SHE4* gene. *P.anserina cro1-1* gene upper line; yeast *SHE4* gene, lower line. Similar and identical amino acids are boxed; identical amino acids are stippled. The localization of the frameshift in the *cro1-1* mutant is designated by an arrow. The star above the sequence shows the intron position. The alignment was obtained with the GAP algorithm, with a gap penality of 3.000 and a length penality of 0.100. The DDBJ/EMBL/GenBank accession number for the *cro1* nucleotide sequence is Y16261.

The CRO1 protein shows similarity with the yeast SHE4 protein

The *cro1* gene was cloned by complementation and SIB selection (Akins and Lambowitz, 1985) using the *cro1-1* strain as the recipient. The cosmid library used contained, as selectable marker, the bacterial hygromycin resistance gene (see Materials and methods). Hygromycin-resistant transformants displaying a wild-type growth phenotype were crossed with the *cro1-1* mutant to test their sporulation ability. Some showed a completely wild-type phenotype while others conserved a sporulation defect. One possible explanation is that, in some cases, the integration site of the cosmid carrying the *cro1* gene led to a low expression of the gene. This expression might be sufficient for growth, but not for restoration of the wild-type ability to sporulate. In any case, the relevant cosmid was subcloned and a 2.3 kb DNA fragment, which complemented all the phenotypic defects of the *cro1-1* mutant, was sequenced.

Analysis of the nucleotide sequence identified two open reading frames. A putative intron was found by consensus sequences for the 5′, 3′ splice sites and for the lariat formation site (Ballance, 1986). In addition, the cDNA corresponding to the *cro1* gene was obtained by reverse transcription and PCR amplification of the *cro1* mRNA. This experiment was performed on RNA extracted from wild-type mycelia and perithecia (see Materials and methods). The gene was found to be expressed in both vegetative and sexual stages of the *Podospora* life cycle. However, amplification of the *cro1* mRNA was always difficult to obtain and Northern blot analysis provided no signal despite positive controls (data not shown). Furthermore, the *cro1* gene displays a low codon usage bias (data not shown). Taken together, these results suggest a low expression of the *cro1* gene. The *cro1* cDNA analysis confirmed the presence of the intron (see star in Figure 4).

The gene encodes a putative protein of 702 amino acids. Analysis of the protein sequence reveals several interesting points. First, there are several regions rich in positively charged residues, for instance between amino acids 162 and 169 (RRKWKSRK) which might be nuclear localization signals (for a review see Garcia-Bustos *et al.*, 1991). Second, a putative zinc finger motif (Coleman, 1992) of the C2H2 family was found between amino acids 71 and 94 (CX5CX12HX3C). Third, several regions are rich enough in proline, negatively charged and hydroxylated amino acids, to resemble PEST motifs, especially since they are bordered by positively charged residues (for a review see Rogers *et al.*, 1986). Fourth, the carboxy-

terminal part of the protein is rich in valine residues (among 57 valines, 26 are contained within the last 160 amino acids of the protein). Furthermore, these valines are generally located in pairs or triplets. Finally, the CRO1 protein shows (between amino acids 231 and 235) the sequence RRHSL, which is one of the motifs for a phosphorylation site by the cAMP-dependent protein kinase, PKA (consensus: RRXS/TY with X being any residue and Y a hydrophobic residue; for review see Taylor and Radzio-Andzelm, 1994).

A search of the *Saccharomyces Genome Database* with the FASTA program revealed a similarity between the putative CRO1 protein and the budding yeast SHE4 protein required for asymmetric mating-type switching in haploid cell divisions (Jansen *et al.*, 1996). As shown in Figure 4, the yeast and the fungal proteins display 21% identity and 40% similarity in a 702 amino acid overlap. The yeast and *Podospora* sequences were compared by the BESTFIT program (data not shown). The parameter termed 'quality of the alignment' was compared with the average quality of 100 alignments of random permutations. The reduced deviation (Z parameter), calculated as (cognate quality–average quality/standard deviation of quality of random permutations), was 14. This value is highly significant (Slonimski and Brouillet, 1993). Another search was performed at the NCBI using the BLAST network service and revealed a low, but significant similarity of the CRO1 protein with a 993 amino acid protein of *Caenorhabditis elegans* encoded by a gene of chromosome III (EMBL accession number U29096): the two proteins show 24% identity and 48% similarity; the Z parameter is 10.2. A tentative alignment was performed between SHE4 and the *C.elegans* protein: the Z parameter being very low (4.8), the similarity found was statistically not significant.

To confirm that the *cro1* gene was actually cloned and to identify the nature of the mutation, the *cro1-1* mutant gene was sequenced. In the mutant allele a cytosine is missing 40 bases downstream of the ATG. This frameshift leads to a premature chain termination, 45 codons after the start codon (the localization of the frameshift is designated by an arrow in Figure 4). Thus, *cro1-1* is a null mutation of the *cro1* gene.

CRO1 is a cytosolic protein mainly expressed in young croziers and at the time of ascospore maturation

To elucidate the localization of the CRO1 protein, fusions were constructed between the *cro1* gene and the green fluorescent protein (EGFP) sequence (see Materials and methods). Fourteen *cro1-1* strains carrying the transgene encoding the CRO1–GFP fusion protein and exhibiting a wild-type phenotype, were first screened for GFP expression during vegetative growth. Green fluorescence was observed in the cytoplasm of the living mycelium whatever the age and size of the hyphae (Figure 5A). However, GFP expression varied among the 14 strains: three showed a bright uniform distribution of the green fluorescence in all compartments of the growing mycelium, the ascogonia but not the microconidia; five gave a weaker signal; and six showed no fluorescence (however, a weak emission signal cannot be excluded, the mycelium being observed with conventional fluorescence microscopy and not with a high-sensitivity video camera). In comparison, a transformant expressing GFP under the control of the strong *Aspergillus nidulans* GPD promoter (Punt *et al.*, 1987; see Materials and methods) used as a control, showed also GFP fluorescence in the cytoplasm, but with a much brighter intensity, including in the microconidia. Strains expressing GFP–CRO1 grew and sporulated at a rate indistinguishable from that of strains expressing wild-type CRO1.

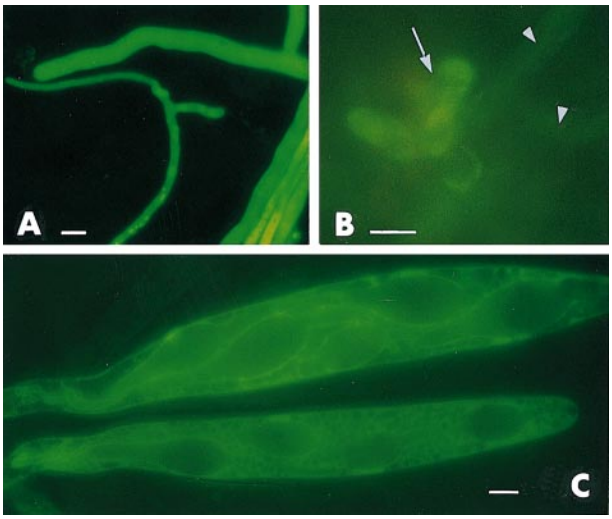

Fig. 5. Expression of the green fluorescent protein (EGFP) in *P.anserina* strains carrying the transgene encoding the CRO1–GFP fusion protein. (**A**) All hyphae, whatever their thickness, are labelled over their entire surface. (**B**) In this young fruiting body, only the croziers (arrow points to one) are brightly fluorescent. The surrounding asci (arrowheads) are hardly visible. (**C**) Two asci with four ascospores. The upper ascus is older and shows also a brighter fluorescence than the ascus below, which is younger (compare the size of the ascospores). Scale bars represent 5 μm.

In contrast to what is seen in the mycelium, there was no difference in GFP expression during the sexual cycle of different strains. In all crosses between *mat+* and *mat–* strains issued from the same primary transformant (see Materials and methods), the CRO1–GFP was highly expressed in the croziers and very young asci as well as in older asci with maturating ascospores (Figure 5B and C). During meiosis, postmeiotic mitosis and ascospore delimitation, the cytoplasm of the asci was only slightly green (Figure 5C). No fluorescence was observed in the hypha-like plurinucleate paraphyses which are formed between the asci. In contrast, the control GPD::GFP cross showed a strong GFP fluorescence only in the paraphyses and the ascospores.

Prior to sporulation, the SPB alters in size, nucleating capacity and orientation (Thompson-Coffe and Zickler, 1994). This SPB also incorporates GFP–CRO1 and could be identified as a bright fluorescent body, similar in size to what is observed in fixed cells by the mitotic phosphoprotein antibody MPM-2 (Davis *et al.*, 1983; Thompson-Coffe and Zickler, 1994), while the SPBs present on the vegetative and meiotic nuclei could not be visualized (data not shown).

Cro1-1 is able to internalize FM4-64 as efficiently as the wild-type strain

Identification of a mutant allele of the budding yeast *SHE4* gene by a screen for mutants defective in endocytosis

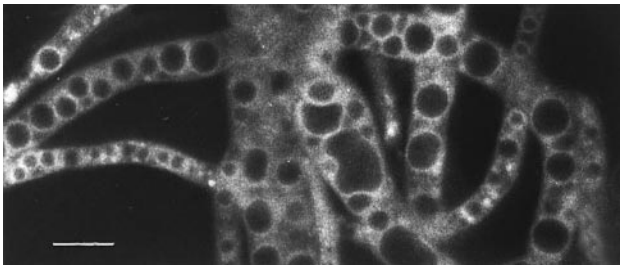

Fig. 6. FM4-64 labelling of *cro1-1* mutant mycelium at 34°C. Vacuoles are clearly visible due to their high fluorescent intensity. As in wild-type, they show differences in size and distribution along the different compartments of the mycelium: for example, compare the large vacuoles of the branch point seen in the middle of the picture to the smaller vacuoles of the thin young hypha visible on the right side. Scale bar represents 5 μm.

(Wendland *et al.*, 1996), prompted us to follow the bulk internalization of plasma membrane in both the wild-type and the *cro1-1* mutant strains with the endocytic tracer FM4-64 (Vida and Emr, 1995; Wendland *et al.*, 1996; see Materials and methods). As the internalization defect of the *she4* mutants was shown to be temperature-dependent, the experiments were performed at 27°C (normal growth temperature) and 34°C (highest temperature before cellular death). They led to three conclusions. First, the fluorescent dye FM4-64 is clearly a useful marker to observe vacuolar membranes in the filamentous fungus *P.anserina* (Figure 6). Second, the number, distribution and size of the vacuoles were similar in the wild-type and *cro1-1* (Figure 6) mycelia. Third, in both strains, the size and number of vacuoles were clearly increased at high temperature. Thus, in contrast to the *she4* mutants, the *cro1-1* mutant does not show internalization defects, at least when the process is investigated with FM4-64 as an endocytic tracer.

Discussion

The primary defect of the cro1-1 mutant stands at the transition to the cellular stage

The *cro1-1* mutation is pleiotropic, as are many other mutations which impair sexual reproduction (e.g. Zickler and Simonet, 1980; Raju, 1992; Berteaux-Lecellier *et al.*, 1995). Its most striking effect is observed after fertilization at the time of crozier formation. In the wild-type, these dikaryotic cells exhibit two remarkable features which are not observed during vegetative growth: the two nuclei, with precisely oriented spindles, divide synchronously and this mitosis is followed by formation of a septum (cross-wall) at the midline defined by the position of each division spindle. This latter event is indeed required to establish and to maintain a true cellular state. Rather than being binucleate, the crozier-like cells of *cro1-1* are plurinucleate: however, while mitoses are coordinate, spindle positioning is random. Both actin belts and septa may be seen, but frequently they are misplaced or absent.

From the cytoskeletal point of view, the most plausible explanation for the absence or misplacement of actin belts and septa following mitosis is the problem of lack of a common division midline. In animal cells, it is assumed from the available data that the plane of cell division is determined by factors originating at each pole of the spindle and travelling along astral microtubules toward the midline of the spindle (reviewed in Strome, 1993). In the wild-type croziers, precise positioning of the two nuclei and the spatial constraints of the narrow, hook-shaped cell ensure a single permitted orientation of the two spindles during mitosis; thus the division midlines are clearly established. In the large *cro1-1* cell with its chaotic spindle orientation, the signals designating the midline for future cellularization are dispersed and contradictory; thus, the concentration of factors necessary to define the septal site would seldom be attained, and only in cases where two or more properly oriented nuclei are in close proximity.

From a developmental viewpoint the primary defect of the *cro1-1* mutant could be either an initial failure in the establishment of the dikaryotic state or a loss of a proper dikaryotic state through divisions (Figure 1). In the first hypothesis, the default could be an initial failure in controlling the migration of nuclei. This would either result in plurinucleate cells which are misshapen and unable to properly manage nuclear orientation and thus septation, or in an initial default in cell shape which results in aberrant nuclear migration. In the second hypothesis, the initial dikaryotic state can be lost through divisions, due to the lack of coordination between mitoses and septum formation. In this case, the primary defect of the mutant would be the inability to form septa at the right place and at the right moment after cellularization. The sparse support for this last mechanism is limited to the observation of few croziers with four groups of nuclei: this suggests that the first mitosis which yielded four nuclei was not followed by septum formation. The data do not permit favour of one default over another. However, in either case, the result of the *cro1-1* defect is a failure in proper establishment and/or maintenance of true cell organization.

Septum formation in filamentous fungi

The *cro1-1* mutant is able to form septa during vegetative growth: the mycelial compartments of the mutant display normal lengths and show an average number of nuclei similar to that observed in a wild-type mycelium (data not shown). These data are not in contradiction to the hypothesis of a defect in septum formation in the mutant croziers. In fact, this process must be subject to different controls during the vegetative and sexual dikaryotic phases, due to the differences in septum localization and timing in the two stages.

The problem of septum formation in filamentous ascomycetes has been investigated in the vegetative cycle of *Aspergillus nidulans* with respect to germinating conidia (Harris *et al.*, 1994) and in *N.crassa* with respect to mycelial growth (Bruno *et al.*, 1996). Unfortunately, to our knowledge, the control of septum formation in the sexual dikaryotic phase of these fungi has not yet been addressed. It would also be of remarkable interest to identify the genes which control this process in basidiomycetes since their filaments are formed of monokaryotic or dikaryotic compartments.

Although the CRO1 protein displays interesting features, its function is still unknown. However, the finding of a target sequence for cAMP-dependent kinase (PKA) is noteworthy. The PKA pathway has been shown to be involved in vegetative growth of filamentous fungi, especially *N.crassa* (Bruno *et al.*, 1996 and references therein).

Interestingly, increased PKA activity results in the mislocalization of septa and in multiple rounds of septation at one site (Bruno *et al.*, 1996). However, the putative sexual defects of the *N.crassa* mutants remain unknown. Nevertheless, cAMP and the PKA signal pathway play a key role in the switch between the unicellular (yeast-like) and the filamentous modes of growth in the dimorphic fungus *Ustilago maydis* (Gold *et al.*, 1994). Further studies are required to know if this pathway also controls the sexual syncytial/cellular transition in the filamentous ascomycetes and if the CRO1 protein is a target for the PKA.

Plurinucleate croziers in N.crassa mutants

Four mutants showing plurinucleate croziers have been described in *N.crassa* (reviewed in Raju, 1992). The first two (*cwl-1* and *cwl-2*) are not relevant to this discussion since their primary defect is their inability to form septa even in the mycelium. The two others, *Banana* (*Ban*) and *Perforated* (*Prf*) display specific features but also share some properties with the *cro1-1* mutant. *Prf* and *Ban* are dominant and female sterile while *cro1-1* is recessive and female fertile. Both *Neurospora* mutants produce mainly a single giant ascospore per ascus. In the *Podospora* mutant, only a few asci contain a single giant ascospore. Most importantly, both *Ban* and *Prf* show plurinucleate croziers in which nuclei undergo synchronous mitosis and may fuse (Raju and Newmeyer, 1977; Raju, 1987). However, the timing of this defect is different from what is observed in the *cro1-1* mutant. In the latter, abnormal croziers are the rule all along the fruiting body development, while in the *Neurospora* mutants the early-formed croziers are normal. The plurinucleate croziers then appear, eventually followed by a second wave of normal croziers. In the *cro1-1* mutant the rare normal croziers do not appear in this way: they are always mixed and connected with abnormal cells. Thus, they can be formed by chance or due to the sporadic expression of an alternative, weakly efficient, pathway. In *N.crassa*, this pathway may be more efficient at the beginning and at the end of the fruiting body development. In spite of their differences, the *N.crassa* mutants along with *P.anserina cro1-1* focus on the same fundamental problem, i.e. the control of this switch from a syncytial to a cellular stage in sexual reproduction of filamentous ascomycetes.

The meiotic developmental programme does not require a true cellularization process

It has been assumed that the formation of dikaryotic (*mat*+/*mat*–) cells from the sexual syncytium, triggers the meiotic programme: karyogamy in specialized cells (the ascus mother cell) followed by meiosis and ascospore formation. The *cro1-1* mutant abides by this programme in spite of its inability to establish a true cellular state. This conclusion is supported by several observations. First, the 'false' cells which arise from the sexual syncytium take a crozier shape. Second, karyogamy occurs in some of these 'cells', even though they are plurinucleate and present no septation. Third, polyploid nuclei can enter meiosis. Fourth, when polyploidy is not too high (from 2n to 8n), meiosis proceeds quite normally. Clearly, defects in meiotic divisions are due to high polyploidy, and defects in the subsequent steps can be related to abnormal chromosome and/or nuclear segregation leading to abortive ascospore formation.

Independence between the meiotic programme and the establishment of a proper dikaryotic stage has been previously noticed for mating-type mutants. When one of the *P.anserina mat* genes is inactivated, the cellularization process still occurs but most cells contain one nucleus instead of two. These monokaryotic croziers are nevertheless able to undergo meiosis even though these haploid meioses are mostly abortive. Furthermore, dikaryotic cells can also form around two nuclei exhibiting the same (mutant) mating type: this homokaryotic state does not impair the meiotic programme since asci are produced which contain uniparental progeny (Zickler *et al.*, 1995; Arnaise *et al.*, 1997). These observations led to the conclusions that the *mat* genes were required for a proper biparental dikaryotic cellular state but that they were not required for the subsequent developmental programme. Data concerning the *cro1-1* mutant add a noteworthy conclusion. The meiotic programme is indeed induced at the time cells are emerging from the sexual syncytium. However, it is executed not only when the cells contain a single set of *mat* mutant information, but also when they are plurinucleate, i.e. when the syncytial state is maintained. Thus, the meiotic programme does not require a true cellularization process: while these two steps are normally coincident, they are uncoupled in the *cro1-1* mutant.

The Drosophila embryo, the Podospora sexual transition and the yeast SHE4 gene

The switch from a syncytial to a cellular state is a key step in insect embryogenesis. This transition has been extensively studied in *Drosophila* and requires a number of genes, some of which fulfil functions specific to the embryonic cellularization process (for review see Schejter and Wieschaus, 1993). Mutations in these genes result in the formation of plurinucleate cells. However, to our knowledge, there is no report of mutants which maintain the syncytial state. Nevertheless, the *maternal haploid* mutant is highly relevant to our present concern. In fact, this mutant—which has half the normal DNA content—undergoes an extra round of mitosis before cellularization (Edgar *et al.*, 1986). This observation has led to the assumption that the transition to cellularization is controlled by the nucleocytoplasmic ratio. Interestingly, the general activation of zygotic genes occurs at the normal mitotic cycle in the mutant (Edgar *et al.*, 1986). Thus two events, which are concomitant in wild-type embryos, are uncoupled in the *haploid maternal* mutant. It is tempting to use a similar rationale for the *Podospora* sexual transition. Two signals would be given at the end of the syncytial stage. The first would lead to the cellularization step (through a nucleocytoplasmic ratio achieved in the sexual syncytium?), the second triggering the meiotic developmental programme. The *cro1-1* mutant would 'understand' the second instruction while being unable to receive (or transduce) the first. In fact, such long-term timing mechanisms which initiate new developmental programmes, are found not only in *Drosophila* but also in many other systems (see Kirschner *et al.*, 1985 for a review in *Xenopus*).

The CRO1 protein shows significant similarity with the

S.cerevisiae SHE4 protein (Jansen *et al.*, 1996) which is involved, with four other SHE proteins, in the asymmetric expression of the *HO* gene. This latter, required for mating-type switching is expressed only in haploid mother cells, not in the buds (reviewed by Herskowitz, 1988; Nasmyth, 1993). A negative regulator of *HO* expression, which is bud specific, has been identified (Bobola *et al.*, 1996; Sil and Herskowitz, 1996). This protein (Ash1p) accumulates mostly in the daughter cell nucleus, due to the fact that the Ash1 mRNA is transported to and maintained in daughter cells. The *she4* mutants, along with mutants of three other *SHE* genes, are incapable of localizing Ash1 mRNA (Long *et al.*, 1997; Takizawa *et al.*, 1997). The *SHE4* gene was also identified with a screen for endocytosis-defective mutants (Wendland *et al.*, 1996). Strains deleted for *SHE4* are temperature-sensitive for growth and show stronger internalization defects at 38°C than at 27°C. As suggested by Wendland *et al.* (1996), the inability of the *she4* mutants to achieve mating-type switching (i.e. to localize Ash1 mRNA), may be an indirect consequence of a defect in actin polarization. However, She4p remains an uncharacterized protein whose cellular location is still unknown.

Whatever the precise role of *SHE4*, it is nevertheless involved in the asymmetric expression of gene(s) which is(are) responsible for the different fates of mother and daughter cells in *S.cerevisiae*. It is noteworthy that its *Podospora* homologue *cro1* controls a key transition in the life cycle of the fungus. In contrast to the *she4* mutants, the *cro1-1* mutant (which is a null mutant) does not show internalization defects, at least when tested with FM4-64 as an endocytic tracer. This suggests, along with the normal phenotype of the mutant hyphae at all temperatures, that actin cytoskeleton is not the main defect of *cro1-1*. Moreover, in the rare asci formed in the mutant strain, the organization of actin as a cortical array of longitudinal microfilaments with associated plaques (parallel to the cortical microtubule array) is normal.

GFP-tagging shows that CRO1 is a cytosolic protein. The difference in the GFP fluorescence intensity seen in the different transformants is probably due to different expression levels, according to the integration sites (position effect) and/or to the copy number of the transgene. In six among 14 transformants, no GFP signal was detected in the mycelium in spite of complete growth and sporulation complementation of the *cro1-1* mutation by the transgene. However, in all transformants, GFP labelling was observed in the fruiting bodies, showing that the *cro1* gene is better expressed during the sexual stage. Furthermore, fluorescence was maximum at the two steps which are impaired in the mutant: the beginning of the dikaryotic phase and the time of ascospore maturation. This developmental regulation of the *cro1* gene was not unexpected. However, the cellular location of the protein does not shed light on its function. A possible mislocalization of the fusion protein (due to an overexpression of the transgene) cannot be completely ruled out. This hypothesis is, however, weakened by the following data. First, all transformants showed the same pattern of expression and location of the CRO1–GFP protein during the sexual cycle, even though for half of them the protein was not detectable during the vegetative phase. Second, among six transformants expressing a fusion of CRO1 and the SGFP variant (Sheen *et al.*, 1995) instead of the EGFP variant (Yang *et al.*, 1996), four showed no fluorescence in the mycelium while GFP labelling was observed in the fruiting bodies of all six strains (data not shown). Moreover, the expression pattern and the protein location were similar in both types of GFP transgenes.

The precise role of the *SHE4/cro1* gene clearly requires further studies, not only in these two systems but also in the fission yeast in which the spatial and temporal controls of septum formation are extensively studied (for review see Chang and Nurse, 1996). Last but not least, the similarity of the CRO1 protein and a protein of the nematode *C.elegans* (EMBL accession number U29096) opens the way of finding this gene in *Drosophila* and should help to characterize its functions in these two true multicellular organisms. Additionally, the search for other *P.anserina* mutants displaying defects similar to those observed in *cro1-1* and for suppressors of this mutation will be fruitful. We are indeed convinced that these kinds of genes, which play a role in asymmetric divisions of a unicellular organism and in the syncytial/cellular transition of a filamentous fungus are of considerable interest both in terms of development and with respect to the evolutionary history of eukaryotes. In fact, as underlined by Denis and Mignot (1994), the syncytial state might have been an intermediary form in the transition between unicellular and pluricellular eukaryotes.

Materials and methods

Strains and media

All strains of *P.anserina* are derived from the wild-type *S* strain. The culture and germination media were recently reviewed by Berteaux-Lecellier *et al.* (1995). The library used for this study was constructed from the wild-type *s* strain which differs from the *S* strain only by two incompatibility genes (Bernet, 1965).

The *cro1-1* mutant was obtained after UV mutagenesis and the corresponding gene maps on the right arm (85% second division segregation) of linkage group III (Simonet and Zickler, 1978).

Cosmids, plasmids and bacterial strains

The genomic library used for these experiments was constructed from an *s mat*+ strain. The integrative cosmid vector carries, as selectable marker, the bacterial hygromycin resistance gene under the control of the *cpc1* promoter of *N.crassa* (Orbach *et al.*, 1991). The *cro1* gene was subcloned using either pUC18, pBluescript SK(+) or KS(+) (Stratagene). Cosmids and plasmids preparations were performed in *Escherichia coli* DH5α (Hanahan, 1983) or CM5α (Camonis *et al.*, 1990).

Cloning methodology

The *cro1* gene was cloned by complementation of the *cro1-1* mutant using the SIB selection method (Akins and Lambowitz, 1985). Recovery of a wild-type growth was expected. The library contained ~6000 cosmids from the whole genome, divided into 60 pools. In the 15th pool tested, among 325 hygromycin-resistant transformants, two presented a wild-type growth. Both also displayed a wild-type sporulation. Two successive rounds of SIB selection allowed isolation of the cosmid carrying the *cro1* gene. Localization of the gene was obtained according to the procedure developed by Turcq *et al.* (1990). Briefly, the cosmid was digested with one of several restriction enzymes. Each restriction mixture was used to transform the *cro1-1* strain using the pMOcosX reporter vector (Orbach, 1994). Hygromycin-resistant transformants were observed for their growth phenotype and then tested for their sporulation ability. This method demonstrated which enzymes inactivate or fail to inactivate the gene. Finally, a 2.3 kb *Pst*I–*Dra*I fragment able to complement the *cro1-1* mutant was recovered.

Transformation experiments were performed as previously described (Picard *et al.*, 1991), except that protoplasts were made with Glucanex (Novo Nordisk Ferment AG) instead of Novozym.

Sequencing

The *Pst*I–*Dra*I fragment was sequenced on both strands with an automatic sequencing machine (373A DNA sequencer, Applied Biosystems) by the method of DyeDeoxy Terminator, Cycle Sequencing Kit (Applied). Universal primer and synthetic oligonucleotides were used to start the reactions.

Small-scale RNA extraction from perithecia has already been described (Debuchy *et al.*, 1993). The same procedure was used for extraction from mycelia grown on cellophane membranes. After 3–4 days of growth, the mycelia were recovered by scraping with a spatula. Amplification of the cDNA was performed using two synthetic oligonucleotides encompassing the putative location of the intron. Probably due to a low expression of the gene, it was difficult to amplify the cDNA. For this reason, the amplified fragment (0.6 kb) was used for a second PCR round (using the same primers) and then prepared for direct sequencing according to Rosenthal *et al.* (1993). The sequence was performed on one strand, using a specific oligonucleotide deduced from the DNA sequence and chosen from inside the amplified fragment.

The mutated gene was obtained through amplification with PCR on genomic DNA of the *cro1-1* mutant. Total DNA was extracted by a miniprep method (Lecellier and Silar, 1994). Different oligonucleotides were used to amplify the whole gene or part of the gene. The amplified fragments were then prepared for direct sequencing as described above for cDNA sequencing. Most of the gene was sequenced on both strands, in particular, the mutation site.

CRO1 tagging

Plasmid pCBcroEGFP is derived from the pCBcroKCX which contains the *cro1* gene with a multiple cloning site at the 3′ end of the coding sequence. It was checked that pCBcroKCX was able to complement the *cro1-1* mutant. The multiple cloning site was introduced through several steps. First, a fragment encompassing the 5′ untranslated region of *cro1* starting at the *Kpn*I site and the major part of the *cro1* 5′ coding sequence truncated at the *Xho*I site, was cloned into pCB1004 (Carroll *et al.*, 1994) to give pCBcroKX. Second, the 3′ end of the *cro1* coding region was amplified with primers cro10 (5′-ctcgcctctacggacgatgac-3′) and orc NSH (5′-cccaagcttacccccgggccatggcctttccccctccccatcaaccc-3′) and digested with *Xho*I and *Hin*dIII. An additional 185 bp of the 3′ untranslated region were amplified with primers croHX (5′-gggaagcttctagagctgagggagcgggttgttttaaa-3′) and orc Nhe (5′-gggggctagcgggataccaccaccagaagagttggg-3′) and digested with *Hin*dIII and *Nhe*I. Then the two PCR products were cloned into pCBcroKX digested with *Xho*I and *Xba*I. The *cro1* sequence obtained from PCR was checked for the absence of mutation. The cloning site contains a *Nco*I site a few nucleotides upstream of the stop codon, while an *Xba*I site is localized a few nucleotides downstream of the stop codon. Finally, the *Nco*I–*Xba*I fragment of pEGFP-1 (Clontech Laboratories Inc.; Yang *et al.*, 1996) containing the EGFP coding sequence starting at the *Nco*I site and its stop codon was cloned into the corresponding sites of pCBcroKCX to obtain pCBcroEGFP.

Transformation with pCBcroEGFP was performed with a *cro1-1 mat*– strain as the recipient. Twenty-four hygromycin-resistant transformants were recovered of which 15 displayed a wild-type growth phenotype. They were then tested for their sporulation ability through crosses with a *cro1-1 mat*+ strain. All except one (EGFP13) displayed a wild-type sporulation phenotype. Analysis of the progeny permitted recovery of *mat*+ and *mat*– strains displaying the same integration event except for the EGFP15 transformant in which the transgene was integrated near the *mat* locus.

A *P.anserina* transformant in which the SGFP protein was expressed under the control of the *A.nidulans GPD* promoter (Punt *et al.*, 1987) was kindly provided by C.Clavé (Laboratoire de Génétique Moléculaire des champignons filamenteux, Université Bordeaux II). Crosses of this primary transformant to a wild-type strain led to the recovery of *mat*+ and *mat*– strains carrying the transgene. The SGFP clone was kindly provided by J.Sheen.

Cytology

Light and electron microscopy preparation: Light microscopy staining was done by the iron–haematoxylin procedure. Specimens were fixed in fresh Lu's fixative (butanol, propionic acid, and 10% aqueous chromic acid, 9:6:2 v/v). After 10 min of hydrolysis at 70°C, asci were stained in two drops of 2% haematoxylin mixed with one drop of ferric acetate solution. For electron microscopy, asci were fixed in 2% glutaraldehyde in phosphate buffer, pH 7.0, for 3 h, post-fixed in phosphate-buffered 2% osmium tetroxide for 1 h, and dehydrated through an alcohol series. Single asci were embedded in Epon 812 at 60°C for 24 h. Sections were stained in aqueous uranyl acetate for 30 min, followed by lead citrate for 10 min.

FM4-64 labelling: Staining with the lipophilic styryl dye *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl) pyridium dibromide (FM4-64; Molecular Probes), was performed according to Vida and Emr (1995) and to Wendland *et al.* (1996). Mycelia were incubated during 30 min in liquid medium containing FM4-64 (32 μM). Mycelia were then resuspended in fresh medium during 60 min before examination. The labelling and chase steps were performed at both 27°C and 34°C. In the second case, a 1 h preshift to 34°C was achieved before labelling.

Fluorescence microscopy: Microtubule and actin organization of wild-type and *cro1-1* strains were determined using immunofluorescence microscopy as described by Thomson-Coffe and Zickler (1993). A minor change was carried out to preserve microfilaments: pepstatin, antipain, chymostatin and leupeptin were added to the fixative at the concentration of 1.25 μg/ml. Primary antibodies used were: anti-actin (1:3000) and anti-β-tubulin (1:1600) (Amersham France). Secondary antibodies were FITC-conjugated polyvalent donkey anti-mouse (Jackson) used at 1:100. Controls included the use of primary or secondary antibodies alone. Asci were examined on a Zeiss Axioplan photomicroscope and photographed using T-Max 400 film.

GFP was visualized at room temperature using the Zeiss filter set for fluorescein isothiocyanate fluorescence and photographed using Fujicolor 400 film. Mycelia treated with FM4-64 were observed with the same filter set and photographed using T-Max 400 film.

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