DISCOVERING GENES INVOLVED IN BRANCHING DECISIONS
IN Neurospora crassa

by

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ABSTRACT

The main objective of this study is to contribute to the understanding of branching in *Neurospora crassa* Shear & Dodge. Actin and actin-related proteins are present at hyphal tips and at sites where new branches emerge (Heath, 1990). Factors like cytochalasins (Allen *et al.*, 1980) and cold conditions (Uyeda and Furuya, 1986) disrupt their distribution and the branching pattern and frequency. To reveal the role of actin and actin-related proteins in branch initiation and formation, mutants resistant to cytochalasin A (*ccr*) and sensitive to cold (*cs*) were obtained. Purified strains with single gene mutations were morphologically characterized. The *ccr-100* strain is different from the wild type strain in several properties. It has different colony morphology, is predominantly dichotomously branched, has an increased branching frequency through development, and is cold sensitive. This mutant did not restore the wild type phenotype with the addition of calcium or cAMP to the medium, indicating that the mutation did not cause a deficiency in the calcium or cAMP content in hyphae. The cold sensitive mutant *cs-43* has a decreased distance between branching sites, and the mutants *cs-245*, *cs-466* and *cs-611* have an increased distance between branching sites when compared to the wild type strain. The linkage tester strain *alcoy* was crossed to the mutant strains to map the mutated loci. The mutations of the cold sensitive strains were located throughout the genome, and the mutation of the selected cytochalasin A resistant strain *ccr-100* was mapped to linkage group V. Further characterization of the obtained mutant strains will help to elucidate the role of specific gene products in branching events.
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CHAPTER I
GENERAL INTRODUCTION

Although branching events in all living systems have similarities in that they result in the formation of a branch, they are also different in many ways among themselves. In plants and animals, branching is precisely controlled by a combination of intercellular and intracellular interactions. It occurs in a multi-cellular setup, and the branch site can generally be predicted under defined conditions. Fungi are unique in this manner because they perform cellular branching and they have a certain degree of seemingly random events integrated in the developmental programs determining branch initiation. Having in mind the profound influence that fungi have on our lives, it is of great interest to reveal the details of this aspect of their development. This can eventually lead to a better control of their activity.

There are two ways in which fungi can grow: linear extension of a hypha and branching that can occur at the apex of a hypha or at a certain distance from the apex. In both cases hyphal growth is polarized and represents a dynamic process that coordinates production and incorporation of plasma membrane and cell wall constituents, arrangement and migration of cytoplasmic elements, generation and maintenance of turgor pressure and a number of other events. It is necessary to understand each of these processes and their interactions for completing our knowledge of hyphal tip growth and branching.

1.1. Cell components involved in hyphal growth and branching. In the past processes related to cell wall synthesis and turgor generation were accepted as the main factors involved in the regulation of tip growth and branching. The reason behind this
was that it is necessary to have an extensible cell wall at the hyphal tip, and a force from within a hypha to create an outward projection resulting in polarized growth. According to one model, the extensibility of the cell wall is a result of a balance between cell wall synthesis and cell wall lysis (Bartnicki-Garcia and Lippman, 1972). During subapical branching events the extensibility of the mature cell wall is regained by supplying adequate "softening" enzymes to the branching site. Another model attributes the extensibility of the cell wall to the absence of cross-links between components of the newly synthesized cell wall (Wessels, 1990). In this case additional components, such as the mentioned cell wall lysing enzymes, have to be introduced when considering branching events distant from the apex.

The turgor pressure exerted on the differentially extensible cell wall was considered to result in the cell wall expansion and tip growth in earlier models. However, subsequent studies showed that hyphal extension and branching also occur in the absence of turgor pressure (Harold et al., 1996). The lack of turgor pressure did not impair the cytoplasmic calcium gradient at the hyphal tip, or the reaction of hyphae to agents that increase the branching frequency. The authors of this study accepted Wessels’s model for the generation of cell wall extensibility (Wessels, 1990), and suggested two possible ways in which polarized growth can happen. One way is that low turgor conditions induce the production of enzymes that increase the extensibility of the cell wall, while the other possibility is that the rate of cell wall rigidification and turgor pressure are coupled, implying that low turgor pressure would automatically result in slower cell wall rigidification. Evidence that would unambiguously support either of these possibilities is still lacking, so both the explanations for cell wall expansion are not generally accepted.
The involvement of other cell components was subsequently demonstrated, indicating that the cell wall and turgor are not the only players in this game. The Oomycete *Saprolegnia ferax* (Gruithuysen) Thuret was the model organism in a number of studies investigating tip growth. Saprolegniaceae differ from Eumycota in the cell wall composition, presence of biflagellate zoospores and other characteristics. However, as the Eumycota, they have highly polarized growth with cellular branching, and it is likely that the same mechanisms of tip growth and branching are in action.

Picton and Steer (1982) were the first to propose that an actin-based microfilament network beneath the plasma membrane supported the newly synthesized cell wall against turgor pressure in pollen tubes. Pollen tubes, like hyphae, grow in the form of highly polarized tubular cells. Picton and Steer proposed that a microfilament network underlies the plasma membrane, and that the strength of this network is regulated by calcium. Specifically, the levels of calcium ions entering the tip of the pollen tube are reduced by the sequestering action of organelles situated at the hyphal tip, resulting in a subsequent weakening of the microfilament meshwork that facilitates cell wall expansion. An actin peripheral network is also present in growing hyphal tips of various fungi (Hoch, 1983; Butt and Heath, 1988; Roberson, 1992; Jackson and Heath, 1993b; Torralba et al., 1998) as well as at sites of branch formation, and disperses when growth ceases (Heath, 1987). Actin can be distributed in three main types of patterns in fungal hyphal tips: in the form of a filamentous cap, as plaques, or as a diffuse network in the apical region of leading and lateral hyphae (Heath, 1990).

Disruption of the function of F-actin revealed its participation in specific cell activities. When the apical actin meshwork was disturbed with UV microirradiation or
cytochalasin in *Saprolegnia ferax*, the hyphal tips burst, suggesting that F-actin functions in resisting the turgor pressure in expanding regions of the hypha where the cell wall is weak (Jackson and Heath, 1990; Jackson and Heath, 1993a). Disruption of the actin filament network under various turgor conditions showed that actin not only restrained extensive tip expansion in standard growth conditions, but also represented a force-creating factor that facilitated the protrusion of hyphal tips under conditions of low turgor pressure (Gupta and Heath, 1997). The obtained data provided an alternative explanation for hyphal growth in the absence of detectable turgor pressure in which actin is an active participant in the polarized apical extension of the cell wall. A function in turgor resistance and hyphal morphogenesis also requires apical microfilaments to be attached to the plasma membrane. Recently spectrin (Degousee et al., 1997) and integrin (Kaminskyj and Heath, 1995) were found to co-localize with actin at the hyphal apex, satisfying this requirement. Changes in the hyphal and colony morphologies as a result of treatment with cytochalasins further supported that the apical microfilament network is involved in tip morphogenesis (Betina et al., 1972; Allen et al., 1980).

Mutational analysis was also used to elucidate the function of actin cell function. In fungi G-actin is encoded by either one gene (Ng and Abelson, 1980; Fidel et al., 1988), or a gene family (Unkles et al., 1991). The actin protein is very conserved in fungi, with well established binding sites for other actin subunits, divalent cations, nucleotides and a number of actin-binding proteins (Hambly et al., 1986). Mutational analyses in the yeast ACT 1 gene revealed lethal, conditional-lethal and phenotypes that do not differ from the wild type (Wertman et al., 1992). The lethal alleles caused changes in residues that interacted with the divalent cation, the bound nucleotide, residues that
interacted with each other, actin filament assembly or proper folding of the actin protein (Wertman et al., 1992). This confirmed the conserved nature of the actin protein. Two different yeast actin mutants sensitive to high temperature were phenotypically analyzed and showed an array of effects (Novick and Botstein, 1985). At the non-permissive temperature the mutants showed an altered pattern of actin distribution, delocalized deposition of chitin into the cell wall, an accumulation of secretory vesicles and osmotic sensitivity. These altered characteristics of the actin mutants show that actin is involved in organization of the hyphal tip and polarized growth. In *Neurospora crassa* only one actin gene is identified and characterized (Tinsley et al., 1998). Actin was isolated and purified (Sikora and Marzluf, 1982), and three actin isoforms have been identified (Barja et al., 1991). Mutations in the actin gene in *N. crassa* have not been obtained so far.

The precursors of the plasma membrane and the cell wall are produced in the endoplasmic reticulum, packed into vesicles and processed in the dictyosome cisternae throughout the protoplasm of hyphae (Grove et al., 1970). The vesicles are then transported to growth sites where they fuse with the plasma membrane, and release their contents to the new cell wall (Grove et al., 1970). When observed by light microscopy, an apical body, or Spitzenkörper, is present in the apical cytoplasm of growing hyphal tips in most fungi (Grove and Bracker, 1970). The position of this apical body determines the direction in which the hypha will grow (Grove and Bracker, 1970), and is therefore important in morphogenesis. In early studies in this field analysis of various fungal species by electron microscopy revealed that the Spitzenkörper is composed of microvesicles, tubules and a small amount of ribosomes, and that it is surrounded with a cloud of vesicles (Grove and Bracker, 1970). Improvements in fixation procedures
allowed the discovery of microfilaments and microtubules in this region (Howard, 1981). The results of the study suggested that microfilaments might regulate the integrity of the Spitzenkörper, and enable its movement as a single unit. An advance in light microscopy facilitated a detailed investigation of the Spitzenkörper in various groups of fungi by computer-enhanced video microscopy and phase contrast optics (López-Franco and Bracker, 1996). The study showed that the Spitzenkörper of most species contained a cluster of apical vesicles, a core area and small cell components in both these parts, e.g. microfilaments, microtubules, microvesicles, or ribosomes. The core area was proposed to be the organizational center for the vesicle cluster. The Spitzenkörper of *Neurospora crassa* contains a dark vesicle cluster that varies in shape and a light core situated opposite from the apical side of the cluster, and microfilaments emanating peripherally and subapically from this complex (López-Franco and Bracker, 1996). In this study the core was proposed to be the component that organizes the vesicle cluster. Some models for branching assign the Spitzenkörper a crucial role by assigning it the function of distributing vesicles to their site of incorporation into the plasma membrane (see section 1.2). Because of the necessity of vesicles containing the plasma membrane and cell wall components for hyphal growth, a central role is attributed to these vesicles in the regulation of tip growth and branching (Trinci, 1974).

One of the factors involved in the control of tip growth and branching events is calcium. The presence of high concentrations of calcium in the cytoplasm is undesirable for the hypha because it binds phosphate groups (Gadd, 1994). For this reason the free calcium content is kept at a low level in the cytoplasm. However, a gradient of cytoplasmic free calcium exists at hyphal tips, with maximum concentrations at the
extreme apex (Garrill et al., 1993) or at a very small (Levina et al., 1995) distance from the apex. Tips of hyphae that do not grow lack this gradient (Garrill et al., 1993; Hyde and Heath, 1997), indicating the necessity of a cytoplasmic calcium gradient for hyphal tip growth.

Studies in *Saprolegnia ferax* suggest that one of the components involved in generation and maintenance of the gradient are stretch-activated channels that show a tip-high distribution (Garrill et al., 1992; Garrill et al., 1993). These studies showed that blocking of calcium channels with Gadolinium disrupted the gradient and stopped hyphal growth in *S. ferax*. The free calcium gradient was proposed to be generated by the combination of calcium import through the stretch-activated channels, and the sequestration of excessive calcium into mitochondria and/or endoplasmic reticulum situated close to the hyphal tip (Garrill et al., 1993). Large vacuoles containing the bulk of sequestered calcium are not present at the extreme tip of hyphae. The connection between calcium storage in these large vacuoles and calcium sequestering events at the hyphal tip was made by the discovery of a vacuolar reticulum at the hyphal tip of *S. ferax* (Allaway et al., 1997). This vacuolar reticulum appears to be continual with large vacuoles distant from the tip.

In *Achlya bisexualis* Coker & Couch ionophores elicited branching even in the presence of very low concentrations of calcium and magnesium ions in the medium (Harold and Harold, 1986). This supports the possibility that internal calcium stores release calcium into the cytoplasm creating these gradients. In *Neurospora crassa* the distribution of the stretch-activated channels is approximately uniform along the hypha, and the blockage of calcium channels with Gadolinium does not disrupt the calcium
gradient which peaks 3μm from the tip, or change the growth rates and hyphal morphology (Levina et al., 1995). This observation questions the involvement of stretch-activated channels in the generation or maintenance of the free calcium gradient in the cytoplasm of Neurospora crassa. It also indicates that although stretch-activated calcium channels may be important for the cytoplasmic calcium gradient in Oomycetes, their involvement in the generation and maintenance of the cytoplasmic calcium gradient may not be required in all fungi. On the other hand the fact that the cytoplasmic calcium gradient can exist even when calcium channels are blocked reinforces the view that the gradient is somehow implicated in intrinsic tip growth processes, and that it may be produced by the release of calcium from internal stores. Calcineurin, a protein involved in signal transduction with binding sites for calcium ions, and calmodulin (Means, 1994) were also shown to participate in the maintenance of the cytoplasmic calcium gradient (Prokisch et al., 1997). The study demonstrated that external calcium does not restore the calcium gradient in hyphae that lacked functional cna-1, a gene that encodes the catalytic subunit of the calcineurin heterotrimer.

The presence of a calcium gradient at the hyphal tip gave rise to assumptions that the disruption or generation of a calcium gradient is involved in branch initiation. Disturbance of the cytoplasmic calcium gradient at the hyphal tip by introducing an ionophore into the hypha caused an increased frequency of branching in Neurospora crassa (Reissig and Kinney, 1983) and Achlya bisexualis (Harold and Harold, 1986). In N. crassa cAMP was found to reduce the branching response caused by the presence of the ionophore, and a hypothesis explaining a signaling pathway that could be involved in branching evolved (Reissig and Kinney, 1983). According to this hypothesis a localized
elevated cytoplasmic calcium concentration would lead to a calcium-dependent increase in the activity of cyclic nucleotide phosphodiesterase, and a subsequent decrease in the cAMP concentration resulting in an elevated branching frequency. However, the way in which cAMP regulates the distance between adjacent branches was not discussed. The observation that in the presence of an ionophore the increase in biomass during hyphal growth stays approximately the same, while polarized extension changes, gave rise to another hypothesis (Schmid and Harold, 1988). According to this hypothesis the relatively high concentration of cytoplasmic calcium at the hyphal apex is responsible for preventing new branching events at the hyphal tip. A lower concentration in regions distant from the tip allows new branches to form. Another possibility is that proteins involved in cell signaling pathways, such as calcineurin affect hyphal growth and branch initiation by maintaining the cytoplasmic calcium gradient (Prokisch et al., 1997). The structure and function of some actin binding proteins are sensitive to intracellular calcium concentrations (Yin et al., 1981). Therefore another option is that the affect of calcium is expressed through its action on such actin binding proteins. A closer connection between branch initiation and a local increase in cytoplasmic calcium at sites of branch initiation was demonstrated by studies in *Saprolegnia ferax*. In this organism, irradiation of hyphae with UV light at the hyphal tip provoked a local rising in calcium levels (Hyde and Heath, 1997). The rise in cytoplasmic calcium concentrations induced by UV irradiation was followed by a precocious emergence of a branch (Grinberg and Heath, 1997). Authors of this study propose that calcium-sequestering systems that release calcium into the cytoplasm are targets for the UV irradiations. The local increase in cytoplasmic calcium levels activates a contractile system that transports factors responsible for branch
induction from a proximal region to the irradiated region. As possible candidates for these factors they suggest plasma membrane calcium channels or cell wall vesicles. This is opposite from the proposal in which calcium is assigned a branch inhibiting function.

1.2. Branching models. There are few developed models that describe branch initiation. These models can be roughly divided into two groups. One group of models suggests that the branch initiating factors originate from the growing hyphal tip. It is obvious that calcium ions and all the mentioned components contributing to the generation and maintenance of the cytoplasmic calcium gradient are candidates for this factor. In a model devised by Bartnicki-Garcia et al. (1989, 1990) the positioning of a vesicle supply center proposed to distribute vesicles containing cell wall and plasma membrane material to their final destination is thought to be crucial. One of the ways in which the authors explain the positioning of the vesicle supply center is by its physical attachment to the apical surface, probably by microfilaments. For this to be possible, the microfilaments must somehow be attached to the plasma membrane and cell wall.

Integrins were shown to co-localize with actin in the apical cortical cytoplasm of growing hyphae (Kaminskýj and Heath, 1995), and therefore qualify for this function. The same study showed that apical regions rich in integrin were more resistant to plasmolysis than surrounding regions, further supporting the function of integrin in connecting the cell wall and microfilaments. Another protein that may have a function in attaching microfilaments to the plasma membrane is spectrin. This actin-binding protein is localized at the extreme tip of growing hyphae in S. ferax (Degoussé et al., 1997). A study of plasmolysis in various Oomycetes and Ascomycetes revealed adhesion sites rich
in F-actin (Bachewich and Heath, 1997). The pattern of these adhesion sites along growing hyphae changed with increasing distance from the hyphal tip, and were most pronounced in the apical and transition zones. Treatment with hyphal growth inhibitors reduced the number of adhesions and F-actin at the extreme apex, indicating that the adhesions are involved in polarized hyphal growth, and supporting the possibility that there is a physical attachment of the apical cytoplasm and its components to the cell wall. It is also interesting that hyphae with abundant sub-apical adhesions produced sub-apical branches, indicating that the adhesions are also related to the process of branch formation.

The second group of models postulate that the factor, or factors, that induces branching originates from within the mycelial body of the fungus. Early studies of this kind suggest that the formation of a new branch is triggered when the elongating capacity of a hypha is exceeded (Katz et al., 1972). In these studies an accumulation of an unknown precursor in the cytoplasm was proposed as the triggering factor. If produced at a certain distance from the hyphal tip, candidates for this accumulating factor are hyphal components that are transported from the site of their production in the proximal mycelium to the hyphal apex. Vesicles containing plasma membrane and cell wall precursors qualify for this function. Up to 12 mm of protoplasm supplies the growing tip with necessary components for growth in Neurospora crassa (Zalokar, 1959). Collinge and Trinci (1974) pointed out the importance of the length of the peripheral growth zone, and the supply of vesicles that it provides, to the growth and extension rate of the hyphal tip. In a model developed by Trinci (1974) the transport and incorporation of vesicles has a central place in the interpretation of branch occurrence. According to this model a new
branch is initiated when the rate of vesicle incorporation exceeds a minimal threshold. The increase in vesicle incorporation is accompanied with an increase in the cytoplasmic volume, and branch induction occurs when the mean hyphal growth unit reaches a critical threshold. By definition, the hyphal growth unit presents the ratio between the total hyphal length and the total number of hyphal tips (Trinci, 1974). This model, in which branching is regulated by protoplasm distant from the hyphal tip, was further elaborated by Prosser and Trinci (1979). The authors propose that an increase in the concentration of vesicles and nuclei regulates hyphal growth and branch initiation. The model offers two options for branch induction. One is that a new hypha is developed when the rate by which vesicles accumulate at the hyphal tip exceeds the maximum rate by which the hypha can incorporate them. This results in the formation of a dichotomous branch, or a lateral branch close to the tip, in the apical compartment. The second option is effective when the septae become an obstacle for the passage of vesicles, and vesicles accumulate in the region prior to the obstruction site. The result, in this case, is a lateral branch.

Bartnicki-Garcia et al. (1989) also accept the vesicle basis for hyphal growth and branching but their model for generating cell shape is a distinct one. They postulate that vesicles arriving from the proximal regions of the mycelium are first gathered at a vesicle supply center close to the growing apex. From here they are distributed to their final destination at the hyphal tip. According to this model both unbranched and branched hyphal growth are a result of the displacement of the entire mechanism that causes movement of the vesicle supply center. One of the ways, in which the authors propose the displacement occurs, is by a mycelium-dependent pushing mechanism. The pushing force is suggested to act upon the mechanism that assembles the cytoskeleton throughout the
mycelium. The authors mention that a new vesicle supply center is created at each new branching site, but the question how this is done still remains. Based on the analysis of apical branching, Reynaga-Peña et al. (1997) propose that the Spitzenkörper acts as a vesicle supply center. The new vesicle supply centers are, according the authors, created de novo, by gradual condensation of vesicle clouds. Wiebe et al. (1992) found that hyphal extension and branch initiation are inhibited independently, and suggested these two processes are regulated independently. They agree with the previous authors that branch formation is induced as a response to an internal signal in the mycelium.

In a current model by Watters et al. (1999b, in press) the decision for branch initiation is proposed not to be controlled exclusively by the hyphal apex, and is, at least in part, attributed to a determining factor occurring at the previous branching site. This conclusion was based on the analysis of branch symmetry. There are still numerous unanswered questions in each of the models, and a substantial amount of information needs to be gathered before a satisfactory model integrating all aspects of branch initiation can be developed.

1.3. Cold sensitivity. Exposure of actin filaments to low temperature causes their depolymerization in controlled conditions in vitro (Hayashi. 1967). There are a number of in vivo studies in various organisms showing a connection between cell morphology and changes in the microfilament assembly at low temperatures. Winokur and Hartwig (1995) reported two processes initiated upon decreasing the temperature blood platelets were incubated at. One of them was uncapping of fast-growing actin filament ends, followed by an increase in the addition of new actin monomers. The second was severing of actin filaments resulting in an increased number of actin filament nucleation sites. The
severing was enhanced by an increase in internal calcium concentration that occurred during chilling. At low temperatures these two processes acted together in forming filopodia and lamellipodia. In some fungi the effect of chilling is opposite. In flagellates of Physarum polycephalum Schweinitz microfilaments were disrupted at 4°C, preventing the maintenance of an elongated cell shape (Uyeda and Furuya, 1986). In this plasmodial slime mould cooling increased the release of calcium from internal stores. The elevated intracellular calcium concentration then resulted in the disassembly of microfilaments. The action of calcium was proposed to be indirect because actin filaments are insensitive to high calcium concentrations in vitro (Kasai et al., 1962). In Neurospora crassa a temporary increase in branching follows the exposure of hyphae to low temperatures (Watters et al., 1999a, in press), indicating that a mechanism similar to the one in blood platelets may be involved.

Beside changes in the structure and function of actin filaments cold environmental conditions provoke a number of other reactions connected to changes in cell morphology. In blood platelets the use of the calcium sensitive probe Indo-1 showed that cooling induced a rise in the internal calcium concentration, as in P. polycephalum, and that it coincided with the formation of filopodia and lamellipodia (Oliver et al., 1999). Oliver et al. proposed four possible mechanisms that may cause the increase of calcium presence in the cytoplasm. The lipid phase transitions that happen in membranes during temperature shifts make it feasible that a leak in the membrane of an internal calcium store is responsible for the elevated calcium levels. Another possibility is that there is a imbalance between leaks and the function of calcium pumps at low temperatures. The third explanation is that proteins that sequester calcium decrease their affinity to bind to
calcium at reduced temperatures. Finally, membrane-dependent proteins were proposed to change their activity after chilling and affect signaling pathways that result in a shape change.

The exposure of blood platelets to low temperatures induced the disappearance of a marginal band of microtubules (White and Krivit, 1967). This disappearance was correlated with the change of cell shape from discoid to spherical with protrusions. A difference in the assembly of microtubules and microfilaments as a reaction to cold conditions can be seen in blood platelets. While microfilaments are synthesized, microtubules are disassembled. It is interesting that a category of microtubules adapted to cold exists in mammalian cells. To achieve cold stability, this category of microtubules utilizes different strategies (Wallin and Strömberg, 1995). These strategies include interactions with proteins that stabilize microtubules, formation of microtubule doublets or triplets, bundling, the association of microtubules with membranes, formation of tubulin isotypes and posttranslational modifications (Wallin and Strömberg, 1995). Soon after the temperature starts decreasing, phosphodiesterase activity in the blood platelet is inhibited (Zappia et al., 1976). Cooling also inhibits adenylate cyclase activity, but the onset of inhibition is at a later time and to a lesser extent. Zappia et al. (1976) propose that the timing of phosphodiesterase and adenylate cyclase inhibition results in a transient increment in cAMP, and that those temporarily elevated levels of cAMP influence the dephosphorylation of tubulin monomers. The change in the cell morphology as a reaction to chilling would be, according to these authors, a consequence of the disappearance of the marginal microtubule bundle. Low temperatures also enhanced the catabolism of ATP and decreased the total ATP and ADP content (Rao and Murphy, 1982).
accompanied with a malfunction in the secretion of ATP and ADP, which the authors attributed to a defect in maintaining ATP homeostasis.

Changes in the membranes during temperature reductions appear to be important for changes in cell morphology. Ando et al. (1974) found that the net negative charge of platelet surfaces is reduced as a response to cooling. They also reported reduced activity of thrombosthenin, a contractile protein that is a plasma membrane constituent, after chilling. In addition, phosphoinositides were proposed to be important in the interaction with proteins that cap actin filament ends during cooling (Winokur and Hartwig, 1995). In this case the aggregation of phosphoinositides would initiate the unveiling of the fast growing ends of F-actin. Polymerization of monomers would result in the increased production of microfilaments enabling modifications in the shape of the cell.

All these studies demonstrate that there are multiple and complex processes involved in the reaction of the cell to changes induced by low temperatures. In Neurospora crassa lowering the temperature increases the branching frequency of hyphae, implying that at least one of the changes induced by chilling is also involved in the regulation of branch initiation. Although it might be difficult to differentiate between primary and secondary effects of the shift to cold conditions, investigation of the changes induced by chilling in fungi can give valuable information with regards to the regulation of branching. Having in mind the potential it offers research in this area has not received the attention it deserves.

Mutants sensitive to low temperature were isolated in different species of fungi. In Aspergillus nidulans (Eidam) Winter selection for cold sensitivity provided single gene mutants with a range of defects coupled with cold-sensitivity (Waldron and Roberts,
The additional defects included auxotrophism, deficiencies in osmosis, sensitivity to deoxycholate, sensitivity or resistance to acetidione or abnormal ribosome sedimentation profiles at non-permissive conditions (Waldron and Roberts, 1974b). In other fungi sensitivity to cold conditions could only be obtained after several rounds of mutagenesis, and were a result of multiple mutations. One example is Aspergillus fumigatus Fres. in which a cold-sensitive strain was obtained as a result of five sequential mutations (Arseneau and Gregory, 1981). Besides being sensitive to cold temperatures, this strain was defective in either the assembly of ribosomal units or in ribosomal RNA processing. The first isolated Neurospora crassa cold sensitive strains were additionally osmotic-remedial, auxotrophic or were deficient in transport or incorporation of amino acids, and were mainly single gene mutations (Roberds and DeBusk, 1973). Another N. crassa cold-sensitive mutant strain crib-1 has a single gene mutation resulting in defective ribosome biosynthesis (Schlitt and Russell, 1974). This mutation was mapped to linkage group IV. Further analysis of crib-1 implied that this strain has a lesion affecting processing of ribosomal RNA (Russell et al., 1976). If actin is directly or indirectly involved in branch initiation, the sensitivity of F-actin polymerization to chilling gives the possibility that cold sensitivity is, at least in some cases, a result of defects related to branching processes.

The goal of this study is to determine which genes are involved in the branching process in Neurospora crassa. The study is primarily designed to detect the involvement of actin and actin-related genes in the initiation and formation of branches, but also gives space for revealing the involvement of genes not directly related to actin. Both low temperatures and cytochalasin A affect microfilament assembly. Therefore, mutations
causing cytochalasin A resistance and cold sensitivity were selected for. The strains carrying these mutations were expected to differ from the wild type in their branching pattern and/or frequency in corresponding conditions, indicating in this way that actin or actin-related proteins are involved in branching. The obtained mutant strains were morphologically characterized and the mutated loci were mapped. In this way two useful categories of mutants are added to the already existing pool of mutant strains with the potential to elucidate questions related to branching events in fungi.
CHAPTER II
MUTANT STRAINS RESISTANT TO CYTOCHALASIN A

2.1. Introduction. A microfilament network is involved in hyphal tip growth (Heath, 1990). If this network is directly or indirectly involved in the branching process, then by changing the actin structure and assembly a change in the branching pattern is expected to occur in a defined environment. When this connection is established, the involvement of actin, or actin-related proteins, in branching will become clearer. To obtain the mentioned changes mutations in the genes involved in building the apical microfilament network were induced. Selection of such mutant strains was made possible by designing a screen for cytochalasin resistance.

Cytochalasins are low molecular weight compounds produced by fungi (Aldridge et al., 1967; Yahara et al., 1982). They reversibly bind to fast-growing barbed ends of F-actin filaments, cap them, and prevent further addition of G-actin monomers (Brown and Spudich, 1981; Cooper, 1987). Beside the binding site at the filament end, some reports indicate that there could be more than one cytochalasin-binding site on an actin filament (Urbanik and Ware, 1989), but this was not confirmed. If cytochalasins bind to more than one site on the F-actin filament, there could be several ways in which they can inhibit microfilament assembly. Additional effects of cytochalasins on cells of some organisms include inhibition of cellulase synthesis (Thomas et al., 1974) and respiratory inhibition (Manavathu and Thomas, 1980). The conclusions based on effects of cytochalasin must therefore be considered cautiously. Although a number of cytochalasins are characterized, cytochalasin A (fig. 2.1.) was chosen for this study, as it was proven to be most effective in Neurospora crassa (Allen et al., 1980).
Mutations in the actin gene could lead to such conformational changes in the produced actin protein that would prevent, or modify, the capping of F-actin filaments. Possible modifications in actin-related proteins could alter the cytochalasin binding sites, with the same outcome. In both cases this would result in a cytochalasin resistant phenotype and/or an altered branching pattern. In wild type strains of Neurospora crassa, the initial response to the addition of cytochalasin A was an increase in branching (Allen et al., 1980). This response started to be manifested at the concentration of 1µg/mL cytochalasin A. An increase in cytochalasin A concentration resulted in a further increase in branching, appearance of swollen hyphae and an increase in growth inhibition. Cell wall depositions were also observed (Allen et al., 1980). Cytochalasin resistant strains were not expected to show the response characteristic for the wild type at corresponding cytochalasin A concentrations. The response, if any, was expected to occur at higher concentrations.

2.2. Existing strains resistant to cytochalasin A.

2.2.1. Introduction. In a previous project to determine if it was possible to obtain cytochalasin A resistant mutants, several cytochalasin A resistant mutant strains were isolated. A conidiospore suspension of the wild type 74-OR23-1A strain was exposed to an amount of UV radiation that caused a 50% spore kill. The conidiospores were then diluted and plated on solid Vogel’s minimal medium containing 5µg/mL cytochalasin A. Colonies that grew faster than the wild type from which they derived were isolated, and purified (O. Van Marle, and A. Finch, unpublished results). Most of the strains were not characterized morphologically, and none of them were mapped.
Cytochalasin A resistant strains are a new group of mutant strains with different morphologies. Originally they were named car (cytochalasin A resistant), but they were subsequently renamed because a mutant strain with a deficiency in low-affinity glucose transport already bears that name (Perkins et al., 1982). The current name is ccr (cytochalasin A resistant). They are useful for determining the involvement of microfilaments in various processes in the cell (see section 2.3.).

The existing cytochalasin A resistant strains were characterized to select strains for further study. To determine if the mutations occurred in one or more genes all the existing cytochalasin A resistant strains were backcrossed with the wild type strain. To purify the strains from mutations in loci unrelated to branching, and to separate the ones involved in branching, crosses with the wild type strain were repeated. The most resistant strain and the wild type strain were tested under varying media to determine under which conditions was the difference between them maximized. These conditions were applied to the screens for cytochalasin A resistance to facilitate the isolation of cytochalasin A resistant strains.

2.2.2. Materials and methods. The strains used in the following experiments were the wild type strains FGSC #987 (wild type 74-OR23-1A) and FGSC #988 (wild type 74-OR8-1a), as well as FGSC #987 carrying the mutated alleles ccr-4 A, ccr-5.2 A, ccr-5.4 A, ccr-9 A, ccr-27 A, ccr-29 A, ccr-35.1 A, ccr-35.2 A, ccr-L3516 A, ccr-50 A. In the remainder of this thesis the strains carrying the ccr mutated alleles will be referred to as ccr strains or mutants. The ccr-5.2 and ccr-5.4 strains were obtained from purification of the original ccr-5 strain. Similarly, the strains ccr-35.1 and ccr-35.2 were obtained by purification of the original ccr-35 strain. Some strains showed variable colony
colony morphologies at 25°C, so all the strains were purified once more. All the strains were tested for growth on solid Vogel’s minimal medium at 6°C, 8°C, 25°C, and 37°C, as well as on solid Vogel’s minimal medium containing 5μg/mL and 7μg/mL cytochalasin A at 25°C. All media were prepared as described by Davis and De Serres (1970). Alterations in media composition are noted.

Cytochalasin A was dissolved in dimethyl sulfoxide to a concentration of 2mg/mL. This stock solution was used to make appropriate dilutions. The cytochalasin A solution was added after autoclaving and cooling the medium to about 54°C. This prevented degradation of the compound. Different batches of cytochalasin A varied significantly in strength. The solute of the stock solution, dimethyl sulfoxide, was reported to increase branching and reduce growth rates in Neurospora crassa (Aiuto and Sussman, 1976). After dilution in the medium, the concentrations of dimethyl sulfoxide were in all cases at levels that do not have any effect on the morphology.

The wild type strain and the ccr-50 strain were further tested on:

- Solid complete medium,
- Solid complete medium containing 7μg/mL cytochalasin A,
- Solid complete medium containing 14μg/mL cytochalasin A,
- Solid crossing medium,
- Solid crossing medium containing 7μg/mL cytochalasin A,
- Solid crossing medium containing 14μg/mL cytochalasin A,
- Solid plating medium with a 50% reduction in the sorbose content,
- Solid plating medium with 85% reduction in the sorbose content, and
- Solid plating medium without added sorbose.
- Solid Vogel’s minimal medium containing 5mM, CaCl$_2$·2H$_2$O,
- Solid Vogel’s minimal medium containing 50mM, CaCl$_2$·2H$_2$O,
- Solid Vogel’s minimal medium containing 100mM CaCl$_2$·2H$_2$O, and
- Solid Vogel’s minimal medium containing 300mM CaCl$_2$·2H$_2$O.

Growth on various media was measured on 85mm Petri dishes.

UV irradiation, crosses and purification were carried out as described by Davis and De Serres (1970). From each cross 10 random ascospores and 12 sets of octads were isolated on solid Vogel’s minimal medium. The isolation of octads was based on the method described by Perkins (1966).

2.2.3. Results and discussion. There was no difference between most of the cytochalasin A resistant and wild type strains at 25°C (figs. 2.2.A, 2.2.B) and 37°C (figs. 2.2.C, 2.2.D). On this and all further graphs the colony diameter maximum that the strains reached at 85 mm was due to the size of the Petri dish containing the medium. The only mutant strain that showed a marked reduced colony diameter and growth rate at both of these temperatures was $ccr$-$L3516$. Three cytochalasin A resistant strains, $ccr$-$29$, $ccr$-$35.2$, and $ccr$-$L3516$ were cold sensitive at 8°C, and did not grow in the 22 days they were observed. The rest of the strains overgrew the medium in 15 to 22 days (figs. 2.3.A, and 2.3.B). There was no growth in any of the strains after four days at 6°C. On medium containing cytochalasin A all mutant strains had higher colony growth rates than wild type strains (figs. 2.3.C, and 2.3.D).

Crosses of the wild type strain with mutants $ccr$-$4$, $ccr$-$9$, $ccr$-$27$, $ccr$-$29$, and $ccr$-$50$ resulted in intermediate phenotypes in the isolated octad progeny (tab. 2.1.). There were four non-viable ascospores in each octad from the cross with mutant $ccr$-$L3516$.
Mutants ccr-5.2, ccr-35.1, and ccr-35.2 showed a 1:1 segregation of parental phenotypes in the progeny. Mutant ccr-50 seemed to show a 1:1 ratio in the progeny after the first backcross, but in some octads one of the resistant progeny had a slightly less expressed resistance than the other three. After a second backcross, the segregation of 1:1 was observed. The distribution of phenotypes in the randomly picked progeny generally supported the results obtained from octad analysis (tab. 2.1.). Lack of correlation between the results of octad analysis and analysis of randomly picked progeny was due to either very weak resistance that made it difficult to differentiate from the wild type, or possible contamination during the addition of the male conidiospores. Progeny with intermediate phenotypes indicated that mutations in more than one locus took place. When the mutation occurred at only one locus, the cytochalasin A resistant and wild type phenotypes segregated in a 1:1 ratio in the progeny.

Mutants ccr-50 and ccr-35.2 were initially chosen for further study. Mutant ccr-50 had a phenotype that closely resembled the wild type phenotype except for its cytochalasin A resistance. Mutant ccr-35.2 had a slower growth rate and more aerial hyphae than the wild type in all conditions, giving it a fluffy appearance. This strain was also cold sensitive. Mutant ccr-50 showed an inconsistency in its resistance to cytochalasin A indicating that there are modifying genes present that influence the resistance. The same inconsistency was noticed in mutant ccr-35.2. Therefore, both of these strains were abandoned.

A potential problem in measurements of growth was the fact that aerial hyphae tend to bend over and continue growing beyond the colony margin. To avoid this, two techniques were tested. A dialysis membrane was spread over solid Vogel's minimal
medium and overlaid with a thin layer of 1% agar before inoculation. The cytochalasin A resistant mutant *ccr-50* and the wild type strain were inoculated and covered with another layer of dialysis membrane. As a result, hyphae did not extend into the medium or above it, and their growth was not denser than without the use of dialysis membrane. However, the layer of 1% agar was not thin enough to prevent three-dimensional growth of the hyphae, which would hinder precise measurements of growth. Due to its optical qualities, the double layer of dialysis membrane also decreases the quality by which the hyphae can be observed under the microscope. If the layer of 1% agar was omitted, growth of both wild type and the cytochalasin A resistant strains were denser than without dialysis membranes, and slightly reduced in their growth rate. This increase in density was also present if cellophane was used instead of dialysis membranes. Another option was to grow the strains in inverted Petri dishes. Gravity prevented aerial hyphae from overtaking the colony front, and the density and the growth rates of hyphae on the colony surface did not change. In addition, observation under the microscope was clearer. Therefore the latter method was used in all the experiments concerning hyphal growth.

The strain most resistant to cytochalasin A, *ccr-50*, and the wild type strain were tested for growth on different media. This was done to select for a medium that would facilitate differentiation of wild type and cytochalasin A resistant strains. On complete medium (fig. 2.4.A), complete medium containing 7μg/mL (fig. 2.4.B) or 14μg/mL (fig. 2.4.C) cytochalasin A, crossing medium (fig. 2.4.D), and crossing medium containing 14μg/mL cytochalasin A (fig. 2.4.F) there was no obvious difference in the colony growth rates of these strains. On crossing medium containing 7μg/mL cytochalasin A (fig. 2.4.E) mutant *ccr-50* and the wild type strain did not grow, probably because of
inoculation with non-viable conidia. The wild type strain and the cytochalasin A resistant strain could also not be differentiated on plating medium with a 50% reduction in the sorbose content (fig. 2.5.A), plating medium with an 85% reduction in the sorbose content (fig. 2.5.B) and plating medium without added sorbose (fig. 2.5.C), or solid Vogel’s minimal medium (fig. 2.7.A). Growing the strains on solid Vogel’s minimal medium containing different concentrations of CaCl₂·2H₂O did also not significantly increase the difference (figs. 2.6.A, and 2.6.B). On solid Vogel’s minimal medium containing 100mM and 300mM CaCl₂·2H₂O the cytochalasin A resistant ccr-50 mutant grew just slightly slower, but the difference was not distinctive enough (figs. 2.6.C, and 2.6.D). As a result I chose solid Vogel’s minimal medium containing up to 7µg/mL cytochalasin A and solid plating medium containing up to 7µg/mL cytochalasin A for the screens selecting for cytochalasin A resistance.

High concentrations of cytochalasins were reported to inhibit germination of spores and growth in various fungi (Allen et al., 1980; El Mougith et al., 1984; Sabanero, 1994). Therefore growth on various concentrations of cytochalasin A were tested to determine which of these conditions would best facilitate differentiation of cytochalasin A resistance. On solid Vogel’s minimal medium containing 8µg/mL (fig. 2.7.B), 10µg/mL (fig. 2.7.C), 12µg/mL (fig. 2.7.D), 14µg/mL (fig. 2.7.E), and 20µg/mL (fig. 2.7.F) cytochalasin A, the cytochalasin A resistant ccr-50 strain tended to have higher colony growth rates. Cytochalasin A did not inhibit germination. The difference in growth between the mutant and wild type strains at 20µg/mL was very similar to the difference at 10µg/mL cytochalasin A. The cytochalasin A used for this experiment had reduced strength. Some exceptions were observed due to uneven distribution of
cytochalasin A in the medium. In these cases after distribution of the medium into Petri dishes any specific strain showed significantly different growth rates on different Petri dishes that were supposed to contain the same cytochalasin A concentration. This indicated that different Petri dishes contained different concentrations of cytochalasin A. To ensure the concentration of cytochalasin A in the medium is homogenous in the following experiments the medium was stirred after the addition of cytochalasin A and before the distribution into Petri dishes.

2.3. New cytochalasin A resistant strains.

2.3.1. Introduction. There are a number of mutant strains of *Neurospora crassa* with an alteration in their branching pattern, and modified growth rates (Perkins *et al.*, 1982). Most of the mutations causing them have not been characterized. They represent a valuable source of mutations potentially involved in the branching process. Adding a pool of cytochalasin A-resistant strains to this group has advantages. The cytochalasin A resistant strains contribute to the diversity of mutations at loci involved in tip growth and branching. Cytochalasin A resistance at the same time narrows the scope of possible mutations to ones involved in microfilament assembly. Mutations at individual loci are particularly useful because of the possibility to determine the effect of a single gene on the branching process. Intercrossing different mutant strains can reveal allelism and interaction of such genes, and facilitate their functional grouping.

Preliminary screens for cytochalasin A resistance were carried out to determine which concentration of conidia, and which concentration of cytochalasin A in the medium would give the highest yield of the cytochalasin A resistant mutants. Vogel's minimal medium was chosen because it selects against auxotrophic mutant strains.
Plating medium was additionally tested because it enables screening of a larger number of conidia than Vogel’s minimal medium, due to its property to restrict colony growth. The strains obtained from the main screen for cytochalasin A resistance were characterized. The possible connection between cytochalasin A resistance and cold sensitivity was also tested.

2.3.2. Material and methods. The strains used were FGSC #987 (wild type 74-OR23-1A) and FGSC #988 (wild type 74-OR8-1a), FGSC #278 (pk;a), FGSC #793 (gra; a), FGSC #1177 (col-4;A), FGSC #1373 (col-17;A) and FGSC #3884 (ipa;a). In the preliminary screen for mutant strains, a suspension of 1X10⁷ wild type (74-OR23-1A) conidia was irradiated with UV light. The length of irradiation was 1 minute. As shown in previous studies, this length of exposure to UV light proved to be lethal for 50% of the conidia, and induced mainly single point mutations (O. Van Marle, A. Finch, unpublished results). The conidia were overlay plated on a variety of media in three different concentrations (1X10⁶, 1X10⁵ and 1X10⁴) and incubated at 25°C. The media used for plating were solid plating medium and solid Vogel’s minimal medium, as well as these media with the additions of 0.3µg/mL, 3µg/mL, 5µg/mL and 7µg/mL of cytochalasin A. As a control, non-irradiated conidia were plated under the same conditions.

The main screen was carried out on solid Vogel’s minimal medium containing 7µg/mL cytochalasin A. A suspension of 1X10⁷ UV irradiated conidia was diluted to a concentration of 1X10⁴ and overlay plated on the media. Strains with better growth than wild type were isolated and purified. The cytochalasin A resistant strains ccr-100, ccr-101, ccr-119, ccr-120, ccr-128 and the wild type strain were all tested on solid Vogel’s
minimal medium, solid Vogel's minimal medium containing 7μg/mL cytochalasin A and complete medium.

To test wet and dry weight mutant ccr-100 and the wild type (74-OR23-1A) strain were grown on liquid Vogel's minimal medium without cytochalasin A and liquid Vogel's minimal medium containing 3μg/mL and 7μg/mL cytochalasin A. Each concentration was tested in three repeats. Each culture was grown in 50 mL of adequate liquid medium and shaken at 100 rpm throughout their incubation at 25°C. The mycelia were vacuum drained and wet weight was measured. To measure dry weight, the mycelia were dried in an oven at 70°C overnight and then weighed. For measurements of the distance between branching sites, mutant ccr-100 and the wild type strain were grown on solid Vogel's minimal medium, solid Vogel's medium containing 3μg/mL cytochalasin A, and Vogel's medium containing 7μg/mL cytochalasin A. The same media were used for measurements of dichotomous branches versus lateral branches. Mutant ccr-100 was grown on solid Vogel's minimal medium containing 30mM adenosine 3', 5'-cyclic monophosphate (cAMP) to test suppression with cAMP. Suppression with calcium was tested by growing the same strain on Vogel's minimal medium containing 5mM, 50mM, 100mM and 300mM CaCl2·2H2O. In tests for determination of cold sensitivity, colonies were grown on solid Vogel's minimal medium for 24 hours at 25°C, and then transferred to a cooler temperature for the next two weeks. The low temperatures tested were 17°C, 14°C, 10°C and 7°C. In all experiments the wild type strain was tested in parallel as a control.

Measurements of growth of individual hyphae were carried out on solid Vogel's minimal medium at 25°C. For analysis of hyphal morphology and dichotomous
branching cultures were observed under a Zeiss Axioskop microscope and photographs were taken with a 35mm camera on TMX400 film. A Leitz dissecting microscope was used to measure distances between branching sites.

Except for the crosses summarized in table 2.2., 100 or more random ascospores were picked from individual crosses. The rest of the methods were the same as in section 2.2.

2.3.3. Results and discussion. Several potential mutant strains were isolated from the preliminary screen. Mutant *ccr-100* was isolated from plating medium containing 7μg/mL cytochalasin A. Medium containing 0.3μg/mL cytochalasin A yielded mutants *ccr-101* to *ccr-104*. On plating medium containing 3μg/mL cytochalasin A mutant *ccr-105* showed better growth than wild type, and was isolated. Finally, mutant *ccr-106* was isolated from solid Vogel’s minimal medium containing 7μg/mL cytochalasin A. All of these strains showed cytochalasin A resistance expressed through faster growth than wild type strains on medium containing cytochalasin A.

Three presumptive mutant strains, *ccr-107*, *ccr-108* and *ccr-109* that did not have reduced branching were isolated from the main screen. Several other potential mutant strains, *ccr-110* to *ccr-128*, all with reduced branching were also isolated. The purified strains were tested for growth on solid Vogel’s minimal medium, and solid Vogel’s minimal medium containing 7μg/mL cytochalasin A. Three strains that were most resistant to cytochalasin A (*ccr-100*, *ccr-120* and *ccr-128*) and two strains that showed less branching (*ccr-101* and *ccr-119*) were purified and further tested. The difference in colony growth rates on medium containing cytochalasin A was the largest between *ccr-100* and the wild type strains.
A major concern was that the faster growth and the lack of a response typical for the wild type could be a result of a mutation preventing cytochalasin uptake. To clarify this, growth of the ccr strains on Vogel's minimal medium (fig. 2.8.A) and the same medium containing 7μg/mL cytochalasin A (fig. 2.8.B) were compared. The newly obtained strains did not show a reaction as severe as in the wild type on all media containing various concentrations of cytochalasin A. However, a reduction in the growth rate occurred on medium containing cytochalasin A compared to growth on solid Vogel's minimal medium. Because the only difference between the two media was the presence of cytochalasin A, this compound had to cause the reduction in the growth rate, demonstrating that uptake of cytochalasin A was not prevented.

Two other possibilities are that the uptake of cytochalasin A is slowed down or reduced. If the uptake was slowed down, the severity of the reaction to cytochalasin A would increase with longer exposure to medium containing cytochalasin A. This did not happen and therefore this possibility was ruled out. Wild type strains react to the presence of cytochalasin A with a reduced growth rate and altered branching pattern and frequency. The severity of the reaction increases with increasing concentrations of cytochalasin A. A simple reduction in cytochalasin A uptake would result in the expression of a reaction typical for the wild type strains at low cytochalasin A concentrations when high concentrations of cytochalasin A are present in the medium. Although all the ccr strains had a reduced growth rate in the presence of 7μg/mL cytochalasin A, their branching pattern and frequency were different than that of the wild type at low concentrations of cytochalasin A. The different branching pattern and
frequency can be a result of a mutation at a locus involved in branching regardless of whether it is reducing cytochalasin A uptake.

To test whether the mutation occurred at one locus, these original strains were backcrossed with the wild type strain (tab. 2.2.). The distribution of the cytochalasin A resistant and sensitive progeny in octads showed a 1:1 ratio. This was supported by the segregation in the randomly picked progeny, except in the case of the cross between the wild type strain and mutant ccr-128, in which the proportion of the resistant was higher than the sensitive progeny. This disproportion is probably a result of chance, because the segregation in the octad progeny was consistently 1:1.

The new strains were grown on complete medium (fig. 2.8.C), but did not show a difference in colony morphology and had similar growth rates compared to growth on solid Vogel’s minimal medium. This demonstrated they were not auxotrophs. The mutants that additionally showed low branching regained the standard branching pattern very early (before the colony diameter reached 1cm), rendering them difficult to work with regards to this property. Out of the three remaining mutant strains, the cytochalasin A resistance of strains ccr-120 and ccr-128 was significantly lower than strain ccr-100. For this reason only mutant ccr-100 remained for further study.

The characteristics of the ccr-100 strain were investigated in detail. On solid Vogel’s minimal medium at 25°C mutant ccr-100 had a slower colony growth rate than the wild type strain (fig. 2.9.A). On the same medium with the addition of 3µg/mL (fig. 2.9.B) and 7µg/mL (fig. 2.9.C) cytochalasin A, the growth rate of the wild type strain was slower than in mutant ccr-100, and the reduction was proportional to the amount of cytochalasin A added to the medium. Wet and dry weights were measured on
liquid media to determine if the biomass production followed the trends shown by growth on solid media. The biomass expressed through wet and dry weight of both mutant *ccr-100* and the wild type strain supported the results obtained for colony growth rates on solid media, except that they were generally more severe (fig. 2.10.). This was most likely the result of a greater surface exposure and resultant penetration of cytochalasin A in liquid medium. On solid Vogel’s minimal medium at 8°C both mutant *ccr-100* and the wild type strain showed reduced growth rates (fig. 2.11.A). Although this was expected, mutant *ccr-100* stopped growing after reaching a colony diameter of 10mm, while the wild type strains continued growing with a reduced growth rate compared to growth at 26°C (fig. 2.11.B). Growth of the mutant and wild type strains on complete medium at 25°C was similar to growth on Vogel’s minimal medium confirming that mutant *ccr-100* is not an auxotroph (fig. 2.11.C).

When observed with the naked eye or under the microscope it was possible to see that the colony morphology of mutant *ccr-100* changed depending on the age of the colony. There were four successive stages of growth on solid Vogel’s minimal medium at 25°C. The division into these stages was for description purposes, and was arbitrary.

The first stage was named the initial stage. It lasted up to 28 hours from the time of inoculation. In this period the hyphae started growing similar to the wild type from which this mutant strain was derived. The distance between branching points corresponded with the same distances in the wild type. The major difference was in the branching pattern, which was mainly dichotomous in mutant *ccr-100*, and mainly lateral in the wild type strain (fig. 2.12.A). The branching pattern remained predominantly
dichotomous throughout all the stages of growth. Very few aerial hyphae were developed in this stage; and the colony appeared flat on the medium surface (fig. 2.13.A).

In the second, intermediate stage, the intervals between adjacent branching sites decreased in length (fig. 2.12.B). The development of aerial hyphae intensified towards the end of this period that lasted about 12 to 24 hours. The colony still appeared flat, but thicker (fig. 2.13.B).

The third stage was characterized by the development of aerial hyphae. This was accompanied with the abundant production of conidia. Colony parts above the surface resembled the shape of a crater at this stage (fig. 2.13.C). The distance between new branching sites continued to decrease (fig. 2.12.C). Because of copious aerial hyphae and conidia production this stage was named the conidiating stage.

About 24 hours after entering the conidiating stage, the constant phase took over. This stage lasted until the colony reached the limits of the medium (around 85 mm, in a Petri dish). The new branches formed at a smaller distance compared to the previous three stages (fig. 2.12.D) and continued growth did not result in a further decrease in the distance between branches. For this reason this stage was named the constant or the continual stage. Aerial hyphae and conidia continued to develop, although they were slightly less abundant than in the previous stage (fig. 2.13.D). Throughout the observation period the wild type strain branched alternately, without a change in the mean distance between branching loci (fig. 2.12.E). This mean distance in wild type corresponded to the one observed in mutant ccr-100 in the initial phase.

It is interesting to note that these four successive stages could be seen if a conidium, an ascospore or a small amount of conidia or ascospores were used as an
inoculum. If a small fragment from any part of a mycelium that has reached the constant phase was used as the inoculum, hyphae continued growing with the same branching frequency and pattern as in the constant phase (fig. 2.14.). This finding was very intriguing since it suggested that there is a difference in the regulation of branch initiation in the initial phase and the constant phase (see Chapter V for discussion).

A cross between two mating types of mutant ccr-100 resulted in the production of protoperithecia and the darkening of perithecia, but no ascospores were formed. This suggested that the mutated locus influenced not only the branching pattern, but also some process in the sexual cycle.

The lengths between branching sites in mutant ccr-100 in the initial, intermediate and constant phases on solid Vogel's minimal medium and solid Vogel's minimal medium containing 10μg/mL and 20μg/mL cytochalasin A were measured. Cytochalasin A used in these experiments was from a batch that had reduced strength. Based on the morphology of the wild type control at the given concentrations of cytochalasin A the cytochalasin A concentration was estimated to correspond to the concentration of 3μg/mL and 6μg/mL of normal strength cytochalasin A. On all media the median lengths of distances between branching sites were the longest in the initial phase and the shortest in the constant phase (fig. 2.15.). The median length between branching sites was not reduced on Vogel's minimal medium containing cytochalasin A compared to Vogel's minimal medium without additions, as was the case with the wild type control. This indicated that the action of the factor(s) determining when and/or where a new branching point will occur is insensitive to the action of cytochalasin A itself. At these same concentrations cytochalasin A increased branching in wild type. It is therefore possible
that the mutated locus is involved in determining the site of branch initiation, and is at the same time related to actin (for detailed discussion see Chapter V).

The overall and stage-specific proportion of dichotomous branches and lateral branches was measured on solid Vogel’s minimal medium and Vogel’s minimal medium containing 10μg/mL and 20μg/mL cytochalasin A (fig. 2.16.). In all stages and on all media the overall mean was similar, having the average ratio of 85 dichotomous:15 lateral. This ratio was 1 dichotomous:99 lateral in the wild type control. The presence of such a constantly different branching pattern in mutant ccr-100 suggests that the mechanism involved in the production of dichotomous branches is different than the one involved in the formation of lateral branches. In this case the mutation at the ccr locus would be responsible for inducing a switch from mainly lateral to mainly dichotomous branching. Another possibility is that the same branching mechanism is active in both apical and lateral branching and the mutation in the strain ccr-100 is responsible for the modification from lateral to apical branching (for detailed discussion see Chapter V).

Individual growth rates of hyphae were also recorded. The increase in length through time was linear, and lower in ccr-100 (fig. 2.17.A) than in the wild type control (fig. 2.17.B). The average growth rate of individual ccr-100 hyphae was 2.46μm/min, which was considerably less than the 8.71μm/min recorded in wild type. The lower growth rates of mutant ccr-100 individual hyphae compared to individual hyphae of the wild type strain corresponded with results from colony growth rate measurements.

Apical growth rates were also measured in the ccr-100 strain for pairs of dichotomous apical branches (fig.2.17.A). There was no reduction in the growth rate of a main hypha before or during the branching occurrence. From the earliest stages the newly
formed branch grew at a growth rate equal to the growth rate of the hypha it came from. The constant growth rate suggests that there is no need for de novo production of precursors necessary for apical growth. This is consistent with reports that the growing hyphal tip is supplied with precursors by up to 12mm of supporting protoplasm (Zalokar, 1959; Trinci, 1974).

The course of a dichotomous branching event was recorded by time lapse photography. The first sign that indicated the branching process begun was a swelling of the extreme apex (figs. 2.18.A and B). The apical region became less tapered, and almost cylindrical prior to the emergence of the two new branches. Out of the two new branches, one of them appeared to fall behind in length compared to the other branch (figs. 2.18.C, and 2.18.D). The difference disappeared about 10 minutes after its emergence (figs. 2.18.E, and 2.18.F). As in the ccr-100 mutant of Neurospora crassa, in the ramosa-1 mutant of Aspergillus niger v. Tieghem one of the two apical branches appear shorter at the initial phase of growth when observed by light microscopy (Reynaga-Peña and Bartnicki-Garcia, 1997). The magnification did not allow the precise observation of intracellular events underlying the changes in hyphal morphology during apical branching in the present study.

The hyphal morphology of mutant ccr-100 was different than the wild type in that the hyphae meandered more profoundly during their growth (fig. 2.19.A). During the transition from the initial to the intermediate stage, the diameter of the hyphae at the colony margin increased as shown in figure 2.19.B. However, this was not reproducible at every transition, so the changed morphology of the apical region was probably due to changes in the environment that coincided with the transition period.
The cytochalasin A resistant strain \textit{ccr-100} shows some similarities with other \textit{Neurospora crassa} strains. Dichotomous branching, although not so abundant as in the strain \textit{ccr-100}, is also present in the strain \textit{pk} (Perkins, 1959). To determine if the mutated loci were allelic, the two strains were crossed. Viability of the shot ascospores was 26\%, which was very low. The cross between strains \textit{pk} and \textit{ccr-100} using strain \textit{pk} as a maternal parent showed the presence of recombinant phenotypes, and demonstrated that the loci were not allelic (tab. 2.3.). The ratio of parental to recombinant phenotypes, however, indicated that the two mutated loci were linked. The estimated distance, based on the recombinant frequency, was 8 map units. Subsequent crosses of the same type, made to confirm these results, failed to produce ascospores. This was probably a result of slightly different conditions in which the crosses matured. When mutant \textit{ccr-100} was used as a maternal parent the cross did not produce ascospores. In crosses with some other strains (see the remainder of this section) mutant \textit{ccr-100} produced ascospores when it was used as a maternal parent, but the number of viable ascospores was severely reduced.

The manner in which hyphae meandered in strain \textit{ccr-100} made them resemble aborted branches or protrusions (fig. 2.19.A). These protrusions of the hyphal surface were similar to the protrusions in the \textit{gran} mutant (Garnjobst and Tatum, 1967). To reveal possible allelism, I crossed the two mutants. As in the cross with the mutant \textit{pk}, the mutated loci were not allelic (tab. 2.4.). They were linked, and the distance was 6 map units. Both \textit{pk} and \textit{gran} loci are members of linkage group V. This strongly suggests that the \textit{ccr-100} locus also belongs to this linkage group.
Mutant strains col-4, col-17 and ipa were randomly chosen for crosses with strain ccr-100 because they were in use in the laboratory. In addition, crosses with the ipa strain are interesting because this strain has a reduced frequency of branching in early stages of growth. The ipa gene is a member of linkage group I, col-4 of linkage group IV, and col-17 belongs to linkage group VII (Perkins et al., 1982). Random ascospores were picked in all crosses, and parental phenotypes and recombinant phenotypes were scored. The cross of mutant col-4 with mutant ccr-100 showed independent segregation in the progeny (tab. 2.5.). When mutant ccr-100 was used as a maternal parent very few ascospores formed, and only 10% of them were viable (tab. 2.6.). The low viability is likely to be the result of the influence of the ccr-100 locus on the sexual cycle. The cross of strain col-17 with strain ccr-100 produced very few ascospores. The number of viable recombinants with characteristics from both parents was reduced compared to the remaining three categories of phenotypes (tab. 2.7.). This could be a result of a reduced viability due to the presence of both loci. Repeated crosses with col-17 did not result in the production of viable ascospores regardless of which strain was used as the maternal parent. In the progeny from the cross of strain ccr-100 with strain ipa both parental phenotypes were present, but only the recombinant category with the wild type phenotype was encountered (tab. 2.8). Although there was no progeny expressing phenotypes of both parents there was twice as many progeny expressing the ccr-100 phenotype than expected. This could be interpreted to mean that the ccr-100 allele is epistatic to the ipa allele. The occurrence of the wild type recombinant phenotype in the progeny indicated that the gene determining the ccr-100 phenotype was not allelic with genes at the col-4, col-17, or ipa loci.
Cyclic AMP has a range of functions in fungi, from enhancing the catabolism of carbon reserves, to inducing a transition from mycelial to yeast-like growth (Pall, 1981). In development of *Neurospora crassa*, cAMP stimulates the production of aerial hyphae and prevents premature conidiation (Terenzi et al., 1976). A *Neurospora crassa* morphological mutant deficient in adenylate cyclase activity, *crisp-1*, shows lowered levels of intracellular cyclic adenosine 3’, 5’-monophosphate (Perkins et al., 1982). A single gene encoding adenylate cyclase in *Neurospora crassa* was characterized and named *nac* (Kore-Eda et al., 1991). At an early stage of colony formation mutant *ccr-100* produces a large amount of aerial hyphae. In this aspect it is similar to mutant *crisp-1*. To test whether there is a similar defect in the *ccr-100* mutant, I followed the growth on medium containing 30 mM cyclic adenosine 3’, 5’-monophosphate. This concentration was reported to revert the mycelial elongation rate in *crisp-1* by 38% (Rosenberg and Pall, 1979). The *ccr-100* phenotype did not revert to wild type to any extent. However, it did reduce conidiation of both the wild type strain and mutant *ccr-100*. This was expected since cyclic adenosine 3’, 5’-monophosphate inhibits conidiation. What was not expected was that cyclic adenosine 3’, 5’-monophosphate changed the hyphal morphology of wild type, rendering it similar to mutant *ccr-100*. In these conditions wild type hyphae (fig. 2.20.B) formed protrusions in a similar manner as mutant *ccr-100* (fig. 2.20.A). The branching pattern and the length between branching sites remained the same both in mutant *ccr-100* and the wild type strain. This result points to possible raised levels of cyclic adenosine 3’, 5’-monophosphate in the mutant strain. The good conidiation of mutant *ccr-100* suggests that even if a raised level of cyclic adenosine 3’, 5’-monophosphate existed, the raise can not be extreme, and is probably a secondary effect.
Tip high gradients of calcium were suggested to have an inhibitory effect on branching at the apex (see Chapter I). To determine if the lack of intracellular calcium at the hyphal tip is causing the ccr-100 phenotype, the mutant strain was grown on solid Vogel’s minimal medium with added 5mM, 50mM, 100mM or 300mM CaCl$_2$·2H$_2$O. On medium containing 5mM (fig. 2.21.B), 50mM (fig. 2.21.C), and 100mM CaCl$_2$·2H$_2$O (fig. 2.21.D), the growth of both the mutant ccr-100 and the wild type strain did not show any difference compared to growth on Vogel’s minimal medium (fig. 2.21.A). Medium containing 300mM CaCl$_2$·2H$_2$O (fig. 2.21.E) slightly slowed down the growth of wild type strain, but did not affect mutant ccr-100. There were no noticeable changes in the morphology in all cases.

A cold environment affects polymerization of F-actin filaments (see Chapter I). If the ccr-100 mutant is defective in microfilament assembly, the defect may also reflect on cold sensitivity. After shifting the temperature from 25°C to 6°C growth of mutant ccr-100 completely stopped. The wild type control strain continued growing, although at a reduced rate, even after a shift from 25°C to 6°C. The cold sensitivity of mutant ccr-100 was reversible, and growth resumed once the temperature was restored to 25°C. After changing the temperature from 25°C to 17°C, 14°C or 10°C the growth rates decreased. As expected, the decrease in the growth rate was bigger the lower the final temperature was. In all cases the dichotomous branching pattern of the mutant did not change. To test at what temperature the cold sensitivity starts to be expressed mutant ccr-100 was transferred from 25°C to 7°C. After the temperature decreased to 7°C most of the hyphae changed their direction randomly and continued growing in the new direction at very slow growth rates (figs. 2.22.A and B). The rest of the hyphae retained their direction, but
decreased their growth rate, and eventually stopped growing. After 7 to 10 days growth of all hyphae ceased completely. The branching pattern remained dichotomous in all cases. The change in hyphal direction showed that the abrupt shift to 7°C temporarily disoriented the assembly at the hyphal tip without altering the branching pattern. Unless the change in growth direction is a secondary effect of the gene, this gives the possibility that one gene is involved in the control of both processes. The reversible nature of the cold sensitivity indicates that the change provoked at low temperatures does not result in irreparable damage.

2.4. Transformation of strain ccr-100.

2.4.1. Introduction. The cytochalasin A resistant mutant has a phenotype that is different from the wild type. The modified phenotype is a result of a change in a cell function. To establish what is causing this change it is necessary to identify and characterize the mutated gene locus. A gene encoding actin in *Neurospora crassa* was recently characterized (Tinsley *et al.*, 1998). The cosmid containing the actin gene was isolated from the Orbach/Sachs cosmid library of *Neurospora crassa* (Orbach, 1994), and the gene was mapped by RFLP analysis. It is a member of linkage group V and is situated in the close vicinity of *inl*. The *ccr-100* locus is a member of the same linkage group, and is 8 map units apart from *inl* (see chapter IV). The closeness of both the mutated *ccr-100* site and the actin gene to *inl* raises the possibility that the *ccr-100* mutation is an actin mutation, or a mutation at a locus close to actin. To test these two possibilities, the cosmid containing the actin gene was used to transform the *ccr-100* strain.

2.4.2. Materials and methods. The cosmid clone G17B12 from the Orbach/Sachs cosmid library of *N. crassa* was isolated from *E. coli* cells with a QIAp...
Miniprep Kit (Qiagen). The DNA concentration was measured. The presence of contaminating *E.coli* genomic DNA was checked by electrophoresis. The plasmid solution was stored at -20°C.

Generation of spheroplasts and their transformation were based on methods described by Vollmer and Yanofsky (1986). Mycelium of mutant *ccr-100* was grown on Vogel’s minimal medium for 7 days. Conidiospores were harvested with a cotton swab, and suspended in sterile distilled water. The suspension was filtered through glass wool to eliminate mycelial fragments, and their concentration was counted on a haemocytometer to determine their concentration. This was added to liquid Vogel’s minimal medium and incubated at 30°C until germination tubes, not longer than twice the diameter of the conidia, emerged. The suspension was then washed with 1M sorbose, and gently mixed with lysing enzymes. Novozyme 234 (Sigma) was dissolved in 1M sorbitol to a concentration of 5mg/mL and filter sterilized before use. The mixture was incubated at 30°C in a shaker at 100rpm. Cell wall degradation was followed microscopically, and the reaction was stopped when most of the germinating conidiospores displayed a spherical shape. After washing with 1M sorbitol conidiospores were re-suspended in STC to a concentration of 1X 10^8 conidiospores/mL, and placed on ice. To each 0.4mL of the obtained suspension 5µL of dimethyl sulfoxide and 0.1mL of PTC were added. For storage, this solution was divided in 50µL aliquots each containing 5 X 10^7 conidiospores/mL, frozen in liquid nitrogen, and preserved at -70°C.

Fresh or frozen spheroplasts were used for transformation of mutant *ccr-100* cells with the cosmid DNA solution. If frozen cells were used, they were defrosted on ice before treatment. A mixture of 1µL heparine solution and 0.4µL spermidine solution was
added to the plasmid solution containing 500ng of DNA, and left on ice. After several minutes 10μL of the spheroplast solution containing 1X 10⁷ conidiospores/mL was added and incubated on ice for 50 minutes. This was followed by the addition of 110μL of PTC, storage at room temperature for 20 minutes, and the addition of 500μL STC. Finally, 200μL of the resulting solution was mixed with cooled top medium, and overlayed on bottom medium.

Both a positive and a negative control were present. In the positive control a mutant strain was transformed with DNA known to rescue the mutant phenotype. In the negative control no DNA was added.

2.4.3. Results and discussion.

In all the experiments no transformant colonies with a wild type phenotype were isolated. This implies that the actin gene is not mutated in strain ccr-100. However, the same result could be due to the poor transformation potential of defrosted spheroplasts, or the resistance of conidia to lysing enzyme during sphaeroplast production. It is possible that the nature of the ccr-100 mutant is causing abnormal cell wall depositions in conidia, which would result in such a difficulty. For this reason transformation with the actin gene deserves another attempt. If the actin gene were intact, according to the obtained mapping results (see Chapter IV) the mutation would have to be at a locus close to the actin gene. In future studies the position of this gene in the genome can be determined by chromosome walking. Once the gene is mapped, the characteristics of this gene and its product can be determined, and the results incorporated in relevant cytological processes.
Figure 2.1. Structure of cytochalasin A.
Figure 2.2. Growth of cytochalasin A resistant and wild type strains on solid Vogel’s minimal medium at 25°C (A and B). Growth of cytochalasin A resistant and wild type strains on solid Vogel’s minimal medium at 37°C (C and D).
Figure 2.3. Growth of cytochalasin A resistant and wild type strains on solid Vogel's minimal medium at 8°C (A and B). Growth of cytochalasin A resistant and wild type strains on solid Vogel's minimal medium containing 5μg/mL cytochalasin A (C and D).
Figure 2.4. Growth of mutant ccr-50 and the wild type strain on complete medium (A), complete medium with the addition of 7μg/mL cytochalasin A (B), complete medium with the addition of 14μg/mL cytochalasin A (C), crossing medium (D), crossing medium with the addition of 7μg/mL cytochalasin A (E), and crossing medium with the addition of 14μg/mL cytochalasin A (F).
Figure 2.5. Growth of mutant \textit{ccr-50} and the wild type strain on plating medium with a 50\% reduction in sorbose contents (A), with an 85\% reduction in sorbose contents (B), and without sorbose (C).
Figure 2.6. Growth of mutant ccr-50 and the wild type strain on solid Vogel’s minimal medium containing 5mM CaCl$_2$·2H$_2$O (A), 50mM CaCl$_2$·2H$_2$O (B), 100mM CaCl$_2$·2H$_2$O (C), and 300mM CaCl$_2$·2H$_2$O (D).
Figure 2.7. Growth of mutant \textit{ccr-50} and the wild type strain on solid Vogel’s minimal medium (A), solid Vogel’s minimal medium containing 8\,\mu\text{g/mL} cytochalasin A (B), 10\,\mu\text{g/mL} cytochalasin A (C), 12\,\mu\text{g/mL} cytochalasin A (D), 14\,\mu\text{g/mL} cytochalasin A (E), and 20\,\mu\text{g/mL} cytochalasin A (F).
Figure 2.8. Growth of cytochalasin A resistant and wild type strains at 25°C on solid Vogel's minimal medium (A), solid Vogel's minimal medium containing 7μg/mL cytochalasin A (B), and solid complete medium (C).
Figure 2.9. Colony diameters of mutant ccr-100 and the wild type strain on solid Vogel’s minimal medium at 25°C without any additions (A), with the addition of 3μg/mL cytochalasin A (B), and with the addition of 7μg/mL cytochalasin A (C).
Figure 2.10. Growth of mutant ccr-100 and the wild type strain on liquid Vogel's minimal medium at 25°C. A. Wet weight. B. Wet weight on Vogel's minimal medium containing 3 μg/mL cytochalasin A. C. Wet weight on Vogel's minimal medium containing 7 μg/mL cytochalasin A. D. Dry weight. E. Dry weight on Vogel's minimal medium containing 3 μg/mL cytochalasin A. F. Dry weight on Vogel's minimal medium containing 7 μg/mL cytochalasin A.
Figure 2.11. Growth of mutant ccr-100 and the wild type strain on solid Vogel’s minimal medium at 8°C (A), solid Vogel’s minimal medium at 26°C (B), solid complete medium at 25°C (C).
Figure 2.12. Colony morphology of strain ccr-100 on solid Vogel's minimal medium at 25°C. A. After 24 hours, initial stage. B. After 48 hours, intermediate stage. C. After 36 hours, conidiating stage. D. After 48 hours, constant stage. E. After 7 days, constant stage.
Figure 2.13. Distance between branching sites in mutant *ccr-100* and the wild type strain on solid Vogel’s minimal medium at 25°C. 

A. One-day-old colony of strain *ccr-100*, initial stage. 
B. Two-day-old colony of strain *ccr-100*, intermediate stage. 
C. Three-day-old colony of strain *ccr-100*, conidiating stage. 
D. Four-day-old colony of strain *ccr-100*, constant stage. 
E. Wild type strain after 1 day. Bar=37μm.
Figure 2.14. Growth of an 8-hour-old strain *ccr-100* after inoculation of a mycelial fragment on solid Vogel's minimal medium at 25°C. Bar=84μm.
Figure 2.15. The median of lengths between branching sites of strain ccr-100 on solid Vogel's minimal medium containing 10 μg/ml cytochalasin A (VM+10), 20 μg/ml cytochalasin A (VM+20), and without cytochalasin A (VM) at 25°C.
Figure 2.16. Percent of lateral and dichotomous branches in strain ccr-100 on solid Vogel's minimal medium containing 10 μg/ml cytochalasin A (VM+10), 20μg/ml cytochalasin A (VM+20) and without cytochalasin A (VM) at 25°C.
Figure 2.17. A. Growth of a one-day-old mutant ccr-100 hypha and a newly formed branch during apical branching on solid Vogel’s minimal medium at 25°C. The black squares indicate the beginning of an apical branching event. B. Growth of a 12-hour-old wild type strain hypha on solid Vogel’s minimal medium at 25°C.
Figure 2.18. Branching of a hypha of strain ccr-100 in the initial phase on solid Vogel’s minimal medium at 25°C. A. At 0min. B. After 5.5min. C. After 7min. D. After 8.5min. E. After 10min. F. After 12min. Bar=37μm.
Figure 2.19. A. Morphology of one-day-old hyphae of strain ccr-100 with cell wall protrusions on solid Vogel’s minimal medium at 25°C. Bar=37 μm. B. Morphology of one-day-old ccr-100 hyphae showing diameter increase at the beginning of the intermediate phase on solid Vogel’s medium at 25°C. Bar=37 μm.
Figure 2.20. Morphology of 2-day-old hyphae on solid Vogel’s minimal medium containing 30mM cAMP at 25°C. Bar=37μm. A. Strain ccr-100. B. Wild type strain.
Figure 2.21. Growth of mutant ccr-100 and the wild type strain on solid Vogel’s minimal medium at 25°C without any additions (A), with the addition of 5mM CaCl$_2$·2H$_2$O (B), with the addition of 50mM CaCl$_2$·2H$_2$O (C), with the addition of 100mM CaCl$_2$·2H$_2$O (D), and with the addition of 300mM CaCl$_2$·2H$_2$O (E).
Figure 2.22. A. and B. Hyphae of strain ccr-100 after growth on solid Vogel’s minimal medium at 25°C for one day, then at 7°C for 3 days (explanation in Chapter II). Bar=37 μm.
3.1. Introduction. Low temperatures affect polymerization of F-actin filaments (see Chapter I). As a consequence, cellular processes in which microfilaments are involved are also affected by cold conditions. Wild type strains of *Neurospora crassa* show a cold-shock reaction when they are transferred from 25°C to 4°C (Watters et al., 1999a, in press). The cold-shock consists of three arbitrary stages. In the initial stage hyphae go through a lag period in which they elongate without the formation of new branches. This is followed by a period of intensive branching in which the hyphae reduce the distance between branching sites to the extent that they appear to form multiple apical branches. In the third stage segments separating adjacent branching sites increase in length, and approach values similar to those at 25°C. In all the three stages growth rates are reduced compared to the growth rates at 25°C. Actin in the form of microfilaments is involved in branching (Heath, 1990), and this involvement can also be applied to branching during the cold-shock response.

Behaviour of hyphae that differs from wild type at low temperatures, e.g., a lack of the cold-shock response or a change in the branching pattern, can point to modifications of actin, or actin related proteins, in mutant strains. Among these non-standard behaviours is cold sensitivity. Sensitivity to cooling can also be a result of a wide range of molecular and cellular events (Roberds and DeBusk, 1973; Waldron and Roberts, 1974a) not related to actin and microfilaments. Selecting for mutations that cause cold sensitivity as a result of microfilament modifications is possible by choosing
strains that are additionally resistant to cytochalasin A, or show a different branching behaviour.

In addition to the screen for resistance to cytochalasin A, I performed another screen for cold sensitivity. The advantage of obtaining cold sensitive mutants is that they increase the variety of mutations related to branching. At the same time a category of mutations related to actin is selected for within a larger pool of mutations. For this reason I constructed a screen to select for cold sensitive mutants by filter concentration. The obtained cold sensitive strains were purified and their morphology characterised.

3.2. Material and methods. The wild type used was strain FGSC #987 (wild type 74-OR23-1A). UV irradiation was carried out as described in Chapter II. The suspension for irradiation contained $1 \times 10^7$ conidia/mL. Mutant enrichment by filter concentration was based on the method described by Yoder (1979). The mutant enrichment lasted 36 days. The irradiated conidia were divided in two batches. Each batch contained $1 \times 10^7$ conidia suspended in 250mL of Vogel’s minimal medium and was incubated while shaking at 7°C. Every 2-3 days the medium was filtered through 4 layers of cheesecloth. In the course of the experiment, each batch of medium with irradiated conidia was filtrated 15 times. When no growth was observed for four days, the liquid medium with conidia was mixed with warm complete medium containing 15% agar at a ratio of 1:2, left to solidify, and incubated at 25°C. This gave approximately 10 to 15 colonies per Petri dish. After the viable conidia germinated, they were transferred to solid plating medium and incubated for one day at 25°C. A total of 619 conidia were viable. The colony margins were marked, and the plates with the colonies were then shifted back to 8°C. Strains that contained alleles that conferred cold sensitivity stopped growing after
the temperature shift and were selected. These strains will be referred to as cold sensitive strains in the remainder of this thesis. All the cold sensitive strains were also tested for growth on complete medium to eliminate auxotrophs.

The cold sensitive strains were purified and backcrossed as explained in Chapter II. The materials and methods for measurements of growth are also described in Chapter II. Growth of the cold sensitive strains was tested for growth on plating medium containing 3μg/mL cytochalasin A at 25°C. The wild type strain was used as a control.

3.3. Results and discussion. The main screen selecting for cold sensitivity gave 19 new potentially cs (cold sensitive) strains. Some of the strains continued growing at rates approximately the same as the wild type strain at 7°C and were eliminated from further studies. This left 11 cs strains that were cold sensitive at 7°C. These strains did not grow, or grew very little compared to the wild type strain at 7°C. The strains cs-24, cs-43, cs-245, cs-259, cs-304, cs-320, cs-441, cs-460, cs-466, cs-566, and cs-611 were purified and backcrossed twice with the wild type strain. The strain cs-304 grew particularly slowly and produced conidia in small amounts. Crosses of wild type and cs-304 strains were slow for this reason, and the ccr-304 strain was eliminated from further studies. The segregation in the progeny was 1:1 in the crosses of the wild type strain with strains cs-24, cs-43, cs-245, cs-259, cs-460, cs-466, cs-566, and cs-611. This indicated that single loci were mutated in the cold sensitive strains. In the crosses of the wild type strain with strains cs-320 and cs-441 intermediate phenotypes were encountered in the progeny. Intermediate phenotypes in these crosses suggested that more than one locus was mutated. To separate the mutated loci strains cs-320 and cs-441 were backcrossed once more. The progeny from both crosses indicated that two loci were mutated.
On solid plating medium containing 3µg/mL cytochalasin A strains cs-304 and cs-320 showed visibly larger colony diameters than the wild type strains, demonstrating that they are resistant to cytochalasin A. Only slightly increased colony diameter compared to the wild type strain was observed in the strain cs-611. This means that the cytochalasin A resistance and cold sensitivity are in at least some cases related. At the same time it suggests that the mutated loci in these strains are related to microfilament assembly.

All the strains with a mutation at only one locus, i.e. cs-24, cs-43, cs-245, cs-259, cs-460, cs-466, cs-566, and cs-611, were tested for growth on solid Vogel’s minimal medium (fig. 3.1.A) and solid complete medium (fig. 3.1.B). Growth was not inhibited on solid Vogel’s minimal medium showing that the cold sensitive strains were not auxotrophs. All the strains produced conidia and ascospores, showing that the asexual and sexual cycles could be completed. The branching pattern in all the strains was lateral.

Colony morphology and growth rates of strain cs-24 on solid Vogel’s minimal medium are similar to the wild type strain at 25°C. Morphology of individual hyphae (fig. 3.2.) do not differ from the wild type strain (fig. 3.3.) at 25°C. The same similarity could be seen in growth rates (figs. 3.4.A and 3.4.B) and distances between branching sites (fig. 3.5.). It is interesting that the main hyphae had a higher growth rate than the lateral branches. This is different than in the strain ccr-100, where the two branches that resulted from an apical branching event continued growing at the approximately same growth rate as the main hypha. This cold sensitive strain was grown on solid Vogel’s minimal medium or solid plating medium for 1 day and then shifted to 8°C for at least a week. Growth stopped completely several hours after the temperature shift. This was
followed with the swelling and bursting of most of the tips of leading hyphae. The compartments behind the apical ones successively underwent the same bursting process in the following days. This behaviour of hyphal compartments is a result of a low temperature-induced defect in cell function. The defective protein encoded by the mutated locus could be a component of the plasma membrane, cytoskeleton or apical microfilament network. It could also be a protein involved in the regulation of the production or assembly of the mentioned cell constituents.

Colonies of strain cs-43 were more compact than wild type on solid Vogel’s minimal medium at 25°C. Aerial hyphae formed early, and were abundant. The growth rate of the colony was low, but conidiation occurred at approximately the same time as in the wild type. For this reason the colony conidiated at a smaller diameter. The diameters of hyphae were reduced and branching was increased compared to the wild type (fig. 3.6.). This reflected on the average and median of lengths between branching sites. (fig. 3.5.). The compact growth of strain cs-43 was a result of the more common occurrence of secondary tertiary and quaternary branches. When strain cs-43 was transferred to 8°C it completely stopped growing. The mutated locus causes changes at 25°C as well as at 8°C. It is likely that the mutation is related to processes involved in branching because there is an increase in the occurrence of branches under all tested conditions.

Colonies of strain cs-245 appeared less dense than the wild type strain on solid Vogel’s minimal medium at 25°C. They had a low growth rate compared to the wild type strain. At early stages of growth many hyphae were submerged in the medium. Aerial hyphae and conidia were produced later than in the wild type strain. Hyphae had a smaller diameter and the distance between branches was larger (figs. 3.7. and 3.5.).
Secondary and tertiary branches were sparser than in the wild type strain, particularly at early stages of growth. After a shift to 8°C, growth completely stopped both on solid Vogel’s minimal medium and on solid plating medium.

Growth of strain cs-259 was at a reduced rate compared to the wild type strain. As a consequence, this strain formed aerial hyphae and conidiated later than the wild type strain. The morphology of individual hyphae (fig. 3.8.) and the distances between branching sites (fig. 3.5.) were similar to the wild type. Growth ceases entirely when the temperature is reduced from 25°C to 8°C.

The strain cs-460 did not grow on solid Vogel’s minimal medium at 8°C after growth on 25°C. This cold sensitive strain has a very irregular colony margin at the early stage of growth. The colony growth rates, colony morphology, hyphal morphology (fig. 3.9.), and the distances between branching sites (fig. 3.5.) were all similar to the wild type strain at non-restrictive temperatures.

The main characteristic of strain cs-466 is that the lengths between adjacent branching sites are longer than in the wild type strain (fig. 3.5.). The difference is most obvious in the length between the tip of a leading hypha and the first branch behind the hyphal tip (fig. 3.10.). The angles between the leading hyphae and the branches were less acute than in the wild type. In some cases the branches formed right angles. These two characteristics (reduced branching and less obtuse branches) render the ccr-466 strain phenotype as very unusual. Both these characteristics are very rare in Neurospora crassa. Aerial hyphae and conidia were produced at a later stage than in the wild type strain. A lower hyphal diameter was also typical for strain cs-466. This was accompanied by reduced growth rates of individual hyphae (figs. 3.4.C and 3.4.D). After reaching a
diameter of 40-50mm on solid Vogel's minimal medium, the hyphae increase their branching, and reduce the distance between branching sites to the wild type values in some regions of the growing mycelium. In other regions the growth remains the same. When one-day-old colonies grown on 25°C are shifted to 8°C, growth stops as soon as the lower temperature is reached.

The formation of branches at a greater distance from the hyphal tip could be a result of inhibition of branch initiation. This would support the hypothesis that apical branching is inhibited by higher concentrations of intracellular calcium, or some other inhibiting factor, at the hyphal tip (see Chapter I). The increased distance between branching sites could be a result of a reduced production of a factor that initiates branching. It could also reflect a deficiency in the transport or localisation of such a factor to its target site.

The colonies of strain cs-566 had reduced growth and a lower growth rate than wild type (figs.3.4.E and 3.4.F). Consequently, the production of aerial hyphae and conidiation was delayed in this strain compared to the wild type strain. The hyphae formed more secondary and tertiary branches giving the colony a dense look. Unlike strain cs-43, the increased density was not accompanied with a decrease in the distance between branching sites in the cs-566 strain (fig. 3.5.). A smaller hyphal diameter than in the wild type strain was also a characteristic of strain cs-566 (fig. 3.11.). This strain was only partially sensitive to low temperatures, and continued growing after lowering the temperature from 25°C to 8°C. However, the growth rate at 8°C was much lower than in the wild type strain.
At early stages of colony growth a large number of hyphae are submerged in the medium in strain *cs-611*. Branches form less acute angles with leading hyphae than the wild type (fig. 3.12.). The distance between branching sites is increased the first two to three days after germination, but it regains the wild type values after that (fig. 3.5.). Occasionally hyphae branch at the tip, which is more often on solid complete medium than on solid Vogel’s minimal medium. Growth completely stops when colonies are transferred from 25°C to 8°C.

The strains that are most resistant to cytochalasin A and therefore very interesting for the present study, 320 and *cs-441*, remain to be characterised. These strains show intermediate phenotypes when they are crossed to the wild type, and probably have more than one mutated locus. To know the contribution of each of the mutations to the cytochalasin resistance, it is necessary to separate the mutations. For that reason further crosses and tests will be performed with these strains.
Figure 3.1. Colony diameters of cold sensitive strains and the wild type strain at 25°C on solid Vogel’s minimal medium (A), and on solid complete medium (B).
Figure 3.2. Morphology and branching of a mutant cs-24 leading hypha after 1 day of growth on solid Vogel's minimal medium at 25°C. Bar=268μm.

Figure 3.3. Morphology and branching of a wild type (74-OR23-1A) leading hypha after 1 day of growth on solid Vogel's minimal medium at 25°C. Bar=268μm.
Figure 3.4. Growth (A) and growth rate (B) of a mutant cs-24 one-day-old hypha and its branches on solid Vogel’s minimal medium at 25°C. Growth (C) and growth rate (D) of a mutant cs-466 two-day-old hypha on solid Vogel’s minimal medium at 25°C. Growth (E) and growth rate (F) of a mutant cs-566 two-day-old hypha on solid Vogel’s minimal medium at 25°C.
Figure 3.5. The averages and medians of lengths between branching sites in 1-day-old cold sensitive and wild type strains on solid Vogel's minimal medium at 25°C.
Figure 3.6. Morphology and branching of a mutant cs-43 leading hypha after 1 day of growth on solid Vogel's minimal medium at 25°C. Bar=268μm.

Figure 3.7. Morphology and branching of a mutant cs-245 leading hypha after 1 day of growth on solid Vogel's minimal medium at 25°C. Bar=268μm.
Figure 3.8. Morphology and branching of a mutant cs-259 leading hypha after 1 day of growth on solid Vogel’s minimal medium at 25°C. Bar=268μm.

Figure 3.9. Morphology and branching of a mutant cs-460 leading hypha after 1 day of growth on solid Vogel’s minimal medium at 25°C. Bar=268μm.
Figure 3.10. Morphology and branching of a mutant cs-466 leading hypha after 1 day of growth on solid Vogel’s minimal medium at 25°C. Bar=268μm.

Figure 3.11. Morphology and branching of a mutant cs-566 leading hypha after 1 day of growth on solid Vogel’s minimal medium at 25°C. Bar=268μm.
Figure 3.12. Morphology and branching of a mutant cs-611 leading hypha after 1 day of growth on solid Vogel’s minimal medium at 25°C. Bar=268μm.
CHAPTER IV

MAPPING

4.1. Introduction. The obtained cytochalasin A resistant and cold sensitive strains are very interesting regarding their involvement in branching. The final goal of the project of which this study is a part is to determine what genes are implicated in the branch initiation and development processes, and in what way. Precise determination of the location of a new gene is necessary for gene characterization and manipulation. This was why the obtained mutants were mapped. The linkage tester strain alcoy was used for this purpose (Perkins et al., 1969).

The strain alcoy contains three independent reciprocal translocations. Markers are present at, or close to, each breaking point to facilitate scoring. The segregation of a marker with the mutant phenotype indicates the linkage of the mutation to one of two linkage groups. In just one subsequent cross the exact linkage group can be determined. In the case of linkage to group I or II, a cross with a strain containing markers aur and arg-5 is necessary. A cross with a strain containing markers cot-1 and inl is sufficient if there is linkage to group IV or V. Determining linkage to group III or VI is possible by crossing the mutant with a strain containing markers tryp-1 and ylo-1. Perkins et al. (1982) described the phenotypes of these markers. Failure of the mutant phenotype to segregate with any of the markers shows it is either a member of linkage group VII or it is located far away from the breakage point and the marker. In these cases additional crosses are necessary to confirm the location of the mutation.

It is possible that the cytochalasin A resistant mutant ccr-100 and at least some of the strains sensitive to cold have mutations at the same locus, and are allelic. To reveal
possible allelism the strains were intercrossed. Interactions between the ccr-100 mutant and the cold sensitive strains were determined in this way.

4.2. Material and methods. The strains that were crossed with the linkage tester were ccr-100, cs-24, cs-43, cs-245, cs-259, cs-460, cs-466, cs-566, and cs-611. Perkins (1964) described the crossing method. The alcoy strain was grown on slants of solid crossing medium for 5 days to a week until protoperithecia formed. A conidiospore suspension was then added and the cross was incubated at 25°C for 10-14 days, until the ascospores started shooting. One week after shooting dark ascospores were picked and placed separately on solid Vogel’s minimal medium slants. They were left to mature for another week and then heat-shocked for 40 minutes at 60°C. Activated ascospores were incubated at 37°C for 3-4 days to score progeny with the cot-1 phenotype. This was followed by a temperature shift to 25°C which allowed the growth and scoring of the rest of the progeny. The segregation of the mutant phenotype with each of the markers was followed separately, and the frequency of recombinant progeny was recorded. This assigns the mutated locus to one of two possible linkage groups. A total of 100 ascospores were isolated from the cross with strain ccr-100, and 300 from each of the crosses with the cold sensitive strains. The increased number of isolated ascospores derived from crosses with the cold sensitive strains was due to very low viability in the first 100 isolated progeny.

For the mutant ccr-100 in the following step a cross was made to assign the linkage to one of two possible groups. To differentiate between linkage group IV and V, the strain VI (aur, cot-1, inl) A was used for additional crosses.
The ccr-100 and cs strains were intercrossed by the same method as described earlier in this section. A total of 6 octads were isolated from each cross.

4.3. Results and discussion. The progeny of mutant ccr-100 from the cross with the alcoy strain segregated with the cot-1 marker, indicating it was a member of linkage group IV or V (tab. 4.1.). In the next step mutant ccr-100 was crossed with VI (aur, cot-1, inl), a strain containing both cot-1 and inl. The marker cot-1 is in linkage group IV, and inl is located in linkage group V. The ccr phenotype segregated with inl, showing it belongs to linkage group V (tab. 4.2). This is consistent with the results from crosses of mutant ccr-100 with strains pk and gran. In both cases linkage was shown, confirming that the ccr-100 mutation is in linkage group V.

The linkage tester strain alcoy was also crossed with the cold sensitive strains. The cross with strain cs-259 produced a very small number of ascospores that were not viable, so the assessment of the position of this mutation was not possible. Progeny from crosses with strains cs-43, cs-245, cs-460, and cs-566 did not segregate with any of the markers. This indicates that the mutated loci are either associated with linkage group VII, or are situated at loci distant from the markers. A connection of the mutated locus to linkage group III, or VI, was displayed in the cross with mutant cs-24. The remaining two strains, cs-466 and cs-611, were linked to cot-1. This indicated an alliance of the mutated sites with linkage group IV, or V. These two strains, one with low branching and the other with weak cytochalasin A resistance, show the same segregation with the marker cot-1 as mutant ccr-100. The summary of the crossing results is presented in table 4.3.

The cytochalasin A resistant ccr-100 strain was crossed with the cold-sensitive strains to determine allelism. All the octads from the crosses with strains cs-460 and cs-
611 were incomplete. The inability of mutant cs-611 to produce complete octads is consistent with the small number of viable conidia in all the crosses engaging this strain, and implicates the mutated locus in some process of the sexual cycle. Some of the progeny from the cross between strains ccr-100 and cs-460 had a wild type phenotype, showing that the mutated loci were not allelic. Recombinant progeny were also present in the crosses between mutant ccr-100 and the cold sensitive strains cs-43, cs-245, and cs-566, respectively. At least one tetratype octad was registered in all of these crosses. Recombinant progeny were not present in the crosses with strains cs-24 and cs-259. Here the ccr and cs phenotypes were present in a 1:1 ratio. The presence of exclusively parental ditypes indicates that the mutations occurred at the same locus, and that they are allelic, or that they occurred at different loci very close to each other. However, mapping with the alcoy strain showed that the cs-24 mutation is situated in linkage group III or VI and that the ccr-100 mutation is in linkage group V. The presence of a parental ditype in all octads from the cross between strains cs-24 and ccr-100 is therefore a result of the same pattern of allele distributions during meiosis.
5.1. Summary and discussion of the mutant strain $ccr-100$ characteristics.

The $ccr-100$ mutant differs from wild type in its branching pattern and frequency, cytochalasin A resistance, and cold sensitivity. Although only one locus is mutated according to the presented mapping results, mutant $ccr-100$ differs from the wild type strain in several characteristics. This could mean that the mutated locus is pleiotropic, or that some of the phenotypic expressions are secondary. To distinguish between these two possibilities it will be necessary to engage molecular analysis of the mutated locus. Until then, it is still possible to sort out the possible changes that might have occurred based on the data that is accessible.

One consistent property of strain $ccr-100$ is its resistance to cytochalasin A. Although cytochalasin A can affect processes other than microfilament assembly in some fungi, such effects have not been shown in *Neurospora crassa*. This does not exclude the possibility that other effects occur, but it is reasonable to believe that the primary effect of cytochalasin A in *Neurospora crassa* is the capping of F-actin filaments at the fast growing ends (Cooper, 1987). This capping could increase the rigidity of the apical microfilament network and reduce the extension capacity of the hyphal tip. Any factor that reduces the elongation rate of the hypha but does not impair the rate of vesicle production and transport to the tip is expected to intensify the frequency of branch initiation (Trinci, 1974). In wild type the reduction of hyphal tip extension after cytochalasin A treatment could therefore be accompanied by an increase in the
incorporation of vesicles containing plasma membrane and cell wall material per unit of apical surface, providing material for the new branches.

The most obvious explanation for cytochalasin A resistance is that the mutation occurred in the actin gene at a region coding for cytochalasin A binding sites. The involvement of actin in multiple essential processes in the cell would predict that even small changes in the actin gene would result in severe effects at the cellular level. Still, mutational analysis of the actin gene in yeast yielded some strains with mutations in the actin gene that did not phenotypically differ from the wild type (Wertman et al., 1992). This makes it difficult to exclude the possibility of an actin mutation in strain ccr-100.

The interaction of F-actin with numerous actin-binding proteins gives the possibility that the mutation is occurring in an actin binding protein that alters the sensitivity and binding of F-actin to cytochalasin A. Actin binding proteins can be actin related, with an amino acid sequence similar to actin (Tinsley et al., 1998). One of the ways in which this could happen is by the attachment of an altered actin binding protein to, or close to, the site on the actin filament to which cytochalasin A usually binds. This could physically prevent the attachment of cytochalasin A molecules, or it could modify the cytochalasin A binding site on actin and reduce its specificity to cytochalasin. Another option is that a modified protein unrelated to actin and the microfilament assembly could be binding to cytochalasin A and preventing its interaction with F-actin. In this case, even if the protein were not binding to actin it would still be useful to genetically dissect the branching process in Neurospora crassa.

Dichotomous branching is another feature that is expressed in mutant ccr-100 in all the tested conditions. Wild type strains of Neurospora crassa form predominantly
lateral branches (Zalokar, 1959). This contrasts with the ccr-100 strain in which approximately 85% of branching events are situated at the apex. The regulation of the branching pattern is different in these strains, and it is not clear whether these two branching patterns are modifications of a single regulating mechanism, or the result of the existence of two alternative mechanisms. In the latter case the ccr-100 mutation would cause the loss of function of a component crucial for lateral branch regulation, allowing the default apical branching mechanism to be expressed. The ccr-100 strain could provide information on the nature of the switch between the two mechanisms, and on the characteristics of the usually masked inherent regulating mechanism resulting in dichotomous branching. If the same mechanism is regulating both types of branching patterns, and an “initiating factor” that marks the time and site where a branch will emerge exists, the result of the switch could be an altered deposition of this “initiating factor”. One of the upstream components of the regulatory system that could be regulating the deposition could be a member of the cytoskeleton.

Apical branching always results in two branches in the ccr-100 strain of Neurospora crassa. Two questions immediately arise from the observations of apical branching. First, why and how does a dichotomous branch always result in two, instead of multiple branches? Second, why and how do the branches always emerge at an approximately same angle from the axis of the hypha they derived from, and at opposite sides of the hypha? Because it is associated with growing hyphae in most fungi (Grove and Bracker, 1970), a candidate for the regulation of both these characteristics of apical branching is the Spitzenkörper. During apical branching in a mutant strain of Aspergillus niger the two new Spitzenkörper are produced de novo by accumulation of vesicle
clouds (Reynaga-Peña and Bartnicki-Garcia, 1997). Detailed analysis of the
Spitzenkörper in this strain of *A. niger* showed bilateral, rather than radial symmetry
(López-Franco and Bracker, 1996). If the processes occurring in *A. niger* are generally
present in fungi, both the apical branching characteristics under consideration could be
influenced by the bilateral symmetry of the Spitzenkörper. In this case the accumulation
of vesicle clouds would be nucleated at the bilateral ends of the Spitzenkörper. Because
the Spitzenkörper disperses prior to the formation of new branches, this would mean that
a cue (or cues) would have to be left behind to mark the new sites of nucleation. This
would also imply that the same cue that is nucleating the Spitzenkörper is controlling, at
least in part, branch initiation. The nature of this cue is difficult to predict having in mind
that the Spitzenkörper itself has not been completely defined. However, microfilaments
that are proposed to hold the components of the Spitzenkörper together (Howard, 1981),
and that may not be visible after the Spitzenkörper disperses, could be a part of the signal.

Another question is what is causing the Spitzenkörper to disperse? In *ramosa-l*, a
temperature-sensitive mutant of *Aspergillus niger*, the first manifestation of branching
that precedes the disappearance of the Spitzenkörper is a short transient cytoplasmic
contraction (Reynaga-Peña and Bartnicki-Garcia, 1997). The magnification of the light
microscope in the present study was a limiting factor that prevented the observation of
the Spitzenkörper and the detailed changes in the cytoplasm preceding the emergence of
new branches. It is possible that the same kind of contraction also occurs in the *ccr-100*
strain of *Neurospora crassa*. Whatever is causing the cytoplasmic contraction may itself
be the cause of the Spitzenkörper dispersal, or the cytoplasmic contraction may
contribute to an increased rate of vesicle migration to the hyphal tip. The way in which
the increased number of vesicles that arrived to the tip may be disrupting the organization of the Spitzenkörper is purely speculative because of the elusive nature of this apical body. It is possible that the copious vesicles arriving to the hyphal tip somehow destabilize the integrity of the Spitzenkörper, maybe by lowering the affinity of vesicles to a core-organizing component. Another possibility would be that the factor, or factors, that localize the site of branch emergence (Katz et al., 1972) and that are normally present at the tip at very low levels are transported to the tip by the contraction.

Going back another step would raise the question of what is causing the cytoplasmic contraction. One of the possibilities is that there is a mutated gene coding for a tension-bearing component of the cytoskeleton that changes the structure of the cytoskeleton in a way that reduces the tension-bearing function. As a result the protein would not be able to withstand as much tension as the wild type protein, leading to the breakage of bonds it makes with other cytoskeletal constituents at a critical tension level. The breakage would induce a forward movement of the rest of the cytoskeleton together with the associated cytoplasm and cytoplasmic components (fig. 5.1.). A change in a protein responsible for the attachment of actin filaments and the cytoskeleton to the plasma membrane could have the same effect. Candidates for these modified actin-binding proteins would include proteins such as filamin (Alberts et al., 1989) or ankyrin (Bennet and Gilligan, 1993). The change in the structure leading to the loss of the tension bearing function of the cytoskeleton could also be a result of an accumulation of a critical level of a cytoplasmic component that regulates the conformation and/or assembly of the actin-binding protein. Secondary messengers like calcium and cAMP involved in cell signaling processes could be responsible for changes of this kind (Alberts et al., 1989).
The function of actin and actin binding proteins is regulated by calcium ions (Yin et al., 1981; Constantin et al., 1998). Besides actin, the plasma membrane associated network consists of various actin binding and plasma membrane binding proteins. Members of the spectrin superfamily, alpha-actinin, dystrophin, fimbrin, and other actin-binding proteins are components of this network that binds to both actin and proteins of the plasma membrane (Bennet, 1990; Bennett and Gilligan, 1993).

The growth of two new hyphae would involve twice as much material than would be required for only one hypha. If the material for the extra hypha were produced de novo, then a reduction in the growth rate would be expected prior to branching. Growth continues at the same pace during apical branching in Neurospora crassa, so components that are incorporated in the new branches must be present in excess in the cytoplasm. For this reason the production of vesicles is not likely to be the main component of the regulation mechanism for branch initiation, although vesicle production is obviously necessary for tip growth and branching. The absence of a reduction in the growth rate could also be a result of the limitations in the resolution of the microscope and the absence of continual tracking of events at the tip that would reveal dynamic changes through short periods of time. The equality in growth of apical branches is a new observation that has not been reported in Neurospora crassa until the present study.

If the proposed mode of dichotomous branch initiation is just a modification of the mechanism that initiates lateral branches, then this mechanism should have the capacity to define lateral branching in hyphae of wild type strains as well. In wild type strains of N. crassa the vesicles arrive at the hyphal tip at a constant rate. The rate of vesicle migration depends on the rate of hyphal extension: the faster the hypha grows, the
higher the rate of vesicle migration (Collinge and Trinci, 1979). Abrupt vesicle accumulations that arise from cytoplasmic contractions at the hyphal tip during apical branching, as proposed to occur in the current model for the ccr-100 mutant of Neurospora, are not expected to happen. The formation of a new Spitzenkörper would occur at the site of the emergence of a new branch (the vesicle supply center according to Bartnicki-Garcia et al., 1989). A cue marking the site of a lateral would have to be left behind by the existing Spitzenkörper that continued moving at an approximately linear path past this site. The activation of this cue would then be dependent upon reaching a critical accumulation of vesicles in that region of the hypha, consistent with the model of Prosser and Trinci (1979). If the proposed model is valid, the factor initiating both apical and lateral branch formation is a component of the Spitzenkörper.

One of the main characteristics of mutant ccr-100 growth is the increase in the branching frequency through development. This observation implies that the regulation of the branching frequency is altered through time. The higher branching frequency of hyphae growing from an inoculum that consists of a mycelial fragment in the constant phase than the branching frequency of hyphae growing from an inoculum that consists of conidia supports this view. Other mutant strains of Neurospora crassa that show increased branching frequency, e.g. pk mutant (Perkins, 1959), show the increase from the earliest stages of mycelium growth. Because only one gene locus is mutated in the ccr-100 strain, it should be possible to explain both dichotomous branching and the increase in the branching frequency through time with the same mechanism. This would mean that the occurrence of abrupt accumulations of vesicles and the cytoplasmic contractions leading to these accumulations that are presumed to be a prerequisite for the
formation of apical branches have to increase through time. It is possible that the tension that the cytoskeleton and/or the cortical microfilament network have to resist is more pronounced in later stages of growth, and the events leading to the cytoplasmic contractions do not occur as often in the early stages of growth. Another possibility is the existence of a protein that stabilizes the cytoskeleton and/or cortical microfilament network produced at the early stages of growth and prevents frequent contractions of the cytoskeleton. This protein would not be present at the mature stage of the mycelium. When the contractions would occur more frequently the total amount of accumulated vesicles, although enough to induce the dispersal of the Spitzenkörper, would be less than is necessary to maintain the current growth rate, resulting in a growth rate reduction.

The next logical question is how does a new Spitzenkörper assemble the components that will make the emergence of a new branch possible? At sites where new branches emerge, a microfilament network is assembled (Heath, 1990), together with a tip-high gradient in cytoplasmic calcium concentration (Levina et al., 1995). The Spitzenkörper-derived initiating factor may attract microfilaments together with vesicles and other organelles, or it may act as a center that nucleates microfilaments. In both cases it would provide conditions for the attachment of microfilaments to the plasma membrane through actin and plasma membrane binding proteins. Both actin and actin binding proteins require calcium for their assembly and function (Yin et al., 1981) and could lower the cytoplasmic calcium concentration and induce a cell signaling reaction aimed to restore the calcium concentration in the cytoplasm. The calcium could then be released from adjacent organelles that store calcium, or from ion channels in the plasma membrane (Levina et al., 1995). The localized influx would initiate the calcium gradient
necessary for hyphal elongation. Increased cytoplasmic calcium concentration at the apex would facilitate fusion of vesicles with the plasma membrane and give the microfilament network properties that enable it to both resist turgor pressure and support cell wall extension as proposed initially by Picton and Steer for pollen tubes (1982). This would mean that although the cytoplasmic calcium gradient is imperative for the growth of hyphae, it is not the cause of branch formation. In experiments that induce branching by UV ray treatment a localized increase in cytoplasmic calcium levels was detected immediately after irradiation, and the branch inducing function was assigned to calcium (Grinberg and Heath, 1997). The increased calcium level was proposed to induce a contraction of the cytoskeleton followed by a precocious emergence of a branch. The authors of this study failed to explain what is inducing the rise in calcium levels in the absence of an artificial calcium gradient generator. According to the model based on the ccr-100 strain, the cytoplasmic calcium gradient is generated after the branch is initiated by an initiation factor derived from the Spitzenkörper. The Spitzenkörper-derived cue is proposed to be responsible for the positioning of a new branch and generating the calcium gradient, but once the gradient is formed, the site would be “locked” at that position. If this were true, then any factor that can generate a calcium gradient similar to the one preceding the emergence of a branch in standard conditions would be able to induce branch formation without the presence of the Spitzenkörper-derived cue. This could explain why a variety of mutations in Neurospora crassa, e.g. pk, sp, the colonial or semicolonial mutant strains (Perkins et al., 1982), have an altered branching pattern and/or branching frequency. It is also possible that the targets for UV irradiation are not
only calcium channels in membranes of organelles that store calcium, but also some other cell components involved in the branch initiating process.

Raised levels of calcium in the medium do not increase the branching frequency of mutant ccr-100 indicating that the signal for calcium influx is actively controlled from within the hypha.

Hyphae of mutant ccr-100 formed small protrusions during growth, and were different in their morphology from the mainly smooth, linear wild type hyphae. The tests with cAMP showed that the ccr-100 phenotype was not a result of cAMP deficiency. However, because the morphology of some wild type hyphae is similar to mutant ccr-100 hyphae, it is possible that there is a slight increase in cAMP levels in this mutant strain. Even if this is true, these raised levels are not inducing branch formation but are rather a secondary effect. It is also important where cAMP levels are raised. Calcium and cAMP are secondary messengers in cell signaling pathways and can interact (Alberts et al., 1989). Reissig and Kinney (1983) proposed that such an interaction is taking place when high branching is induced by ionophores (see Chapter I). If this is true, and a high concentration of cytoplasmic calcium reduces the concentration of cAMP at the tip, a high concentration of cAMP would be expected to be present at low concentration of cytoplasmic calcium. The possible elevated cAMP concentration in mutant ccr-100 could then prevent the generation of calcium gradients at sites distant from the hyphal tip and the subsequent emergence of lateral branches even in the presence of a Spitzenkörper-derived cue.

The presented model corresponds with the group of models proposing that the origin of the branch initiating factor(s) is from within the mycelial body, and extends it. It
also corresponds with results obtained from studies of *Neurospora crassa* strains with an altered branching frequency and pattern. A *N. crassa* strain deficient in kinesin, a force-generating molecule implicated in the transportation of organelles, showed severe alterations in hyphal extension, morphology and branching, and the absence of a Spitzenkörper (Seiler *et al.*, 1997). The change in hyphal morphology and branching as a result of the impairment of vesicle transport is expected in the model based on the characteristics of mutant *ccr-100*. Disruption of protein phosphatase 2A in *Neurospora crassa* resulted in slower growth rates, an increase in branching frequency and the occurrence of dichotomous and multiple branches (Yatzkan *et al.*, 1998). In the light of the proposed Spitzenkörper based model, it is possible that the disruption in the function of this signalling protein is influencing branching processes through altering the influx and efflux of calcium through membranes. All these data demonstrate the complex nature of the branching process, with multiple steps being precisely and simultaneously regulated.

The fact that when two different mating types of strain *ccr-100* are crossed, no ascospores are produced suggests that the defective protein is involved in the sexual cycle. After fertilization the perithecia darken and enlarge, but beaks do not form. If the darkened perithecia are crushed, no ascospores can be found in them, indicating that there is a developmental block that prevents meiosis. Mutant strains that are defective in different steps in the sexual development pathway are characterized in *Neurospora crassa* (Springer, 1993). The position of the block in the developmental pathway in *ccr-100* could be determined by analyzing progeny from crosses of strain *ccr-100* with these mutants.
The loss of polarized growth at 7°C represents a change induced by hypothermic conditions that is not found in the wild type strains at the same temperature. Because the branching pattern is not altered, it is possible that the loss of orientation is a secondary effect of the mutation. Treatment of Candida albicans Robin germ tubes with F-actin and microtubule inhibitors showed that actin filaments were responsible for localized polarization of actin at the tip of the germ tube and that microtubules were controlling overall polarity (Akashi et al., 1994). In the ccr-100 strain of Neurospora crassa the swelling in the region of the hypha that changed direction indicates that a component related to the localized polarization was temporarily changed without preventing the incorporation of vesicles into the plasma membrane and cell wall. The change was presumably a result of the disassembly or disruption of F-actin function (e.g. by capping of the fast growing ends) leading to a halt in polarized extension. One of the ways by which this could happen is by a change in the binding-affinity of the existing F-actin filaments for actin monomers, nucleotides or divalent ions. A modified actin or actin binding protein could elicit such a reaction. After an adaptation period, polarized growth resumed at a random point which indicates that by longer exposure to cold conditions the mutant protein may lose its modifying capability, possibly by a further change in conformation and disassembly from F-actin. Another possibility is that new F-actin filaments that do not bind to the mutant protein assemble at cold temperatures. The lack of binding can be due to the incapability of the mutant protein to bind to the new actin filaments that may or may not be the same as the filaments assembled at 25°C. In yeast, the polarity of the cell during budding is determined by a set of proteins that are localized at the budding site (Chant, 1994). It is therefore also possible that an unidentified protein
that localizes only at the hypha tip is influenced by the modified ccr-100 protein directly or indirectly.

**5.2. Summary and discussion of cold-sensitive mutant strain characteristics.**

For a complete comprehensive understanding of cold-related changes it is first necessary to obtain more information on the reaction of the wild type strains to cold conditions, a topic in fungi that has not attracted attention until recently (M. K. Watters, 1999a, in press). However, by comparison to model systems in other groups of organisms it is possible to grasp at least the variety of options that exist (see Chapter I).

The locations of the mutated sites in the cold sensitive mutant strains obtained in the present study need to be mapped more precisely. It is necessary to cross these cold sensitive strains with strains containing markers in linkage group VII, or markers that are distant from al-1, cot-1, or ylo-1. Mutations causing hypothermic sensitivity in *Neurospora crassa* occur throughout the genome. This differs from some other organisms, namely *D. melanogaster* Morgan, in which cold sensitive mutations are limited to a certain region of the genome (Waldron and Roberds, 1974a), although this may be due to the type of selection for specific phenotypes.

Low temperatures change the characteristics of proteins and lipids resulting in altered activity, e.g. when the growth temperature of *N. crassa* is lowered a cold-induced increase in enzyme activity occurs (North, 1973). Having in mind the presence of a category of cold-stable microtubules (Wallin and Strömberg, 1995), it is also conceivable that there are actin molecules that have similar cold-resistant properties and can interact with cell components in a manner different from the regular actin molecules. The result of cold-induced changes of mutant strains on the cellular level that differ from wild type
with regards to branching can reveal components involved in branch initiation and
development.

5.3. **Future research.** The results of this thesis are just a beginning in the
research that will lead to a better understanding of branching in fungi. So far, basic
genetic analysis was applied to obtain a pool of mutations in *Neurospora crassa* that will
be helpful in identifying components of the branch initiating process, and some mutated
genes influencing branching were characterized. With all these prerequisites
accumulated, the terrain is set-up for molecular genetic analysis. Further research in this
field will include cloning, sequencing and characterization of the mutated loci. The
results from these studies will make manipulation with the genes possible. Tracing the
expression of these genes through development should pinpoint when components
involved in branch initiation are produced. The consequences of null mutations and over-
expressions of the genes will give information on the function of the gene products on the
cellular level. Mutations in selected regions of the genes can show which regions of the
genes are crucial for the activity of the gene products. To understand the function of the
putative proteins on the cellular level, the localization and interaction with other cellular
components can provide necessary information.

In parallel with genetic dissection, it is necessary to observe changes related to the
components involved in branch initiation both in wild type and the mutant strains at the
cytological level. These components should be visualized *in vivo* by light microscopy and
by electron microscopy, and mutant and wild type strains compared.

The main asset obtained during the course of this study are several mutants that
are cytochalasin A resistant, cold sensitive, or both. The results from the presented work
together with other research in this area provided a foundation from which the complete understanding of the branching process in fungi will emerge. The research area dealing with branch initiation and branch development is surprisingly under-investigated considering the unique nature of this process in the fungal kingdom and the applications of potential discoveries. This study contributes to the effort to explore different aspects of this fundamental, yet still mysterious process.
Figure 5.1. Model for apical branching in the cytochalasin A resistant ccr-100 strain. A. Tension bearing elements of the cytoskeleton are under stress (CS-cytoskeleton component, CW-plasma membrane and cell wall, SL-stress links, SK-Spitzenkörper, and V-vesicles). B. Some connections between cytoskeleton constituents break and the cytoskeleton with the surrounding cytoplasm and organelles contracts in the apical direction. The arrow indicates the direction of contraction. The accumulation of vesicles results in the dispersal of the Spitzenkörper. C. Two new Spitzenkörpers form from cues left by the original Spitzenkörper.


Table 2.1. Crosses of the wild type strain with existing cytochalasin A resistant strains. 
R-resistant; I-intermediate; NR-not resistant; NG-not growing. In the last two crosses (I) designates the first backcross and (II) designates the second backcross.

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Random</th>
<th>Octads</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>I</td>
<td>NR</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-4</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-5.2</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-5.4</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-9</td>
<td>3</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
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<td>0</td>
<td>6</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-29</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-35.1</td>
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<td>0</td>
<td>7</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-35.2</td>
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<td>3</td>
</tr>
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<td>74-OR8-1a X ccr-L3516</td>
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<td>0</td>
<td>3</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-50 (I)</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-50 (II)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.2. Crosses of the wild type strain with the new cytochalasin A resistant strains.

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Random</th>
<th>Octads</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Resistant</td>
<td>Not Resistant</td>
</tr>
<tr>
<td></td>
<td>Resistant</td>
<td>Not Resistant</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-100</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-101</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-119</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-120</td>
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<td>2</td>
</tr>
<tr>
<td>74-OR8-1a X ccr-128</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Number of randomly picked ascospores</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Viable ascospores</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Non-viable ascospores</td>
<td>74</td>
<td></td>
</tr>
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<table>
<thead>
<tr>
<th>Type</th>
<th>Parental</th>
<th>Recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ccr</td>
<td>pk</td>
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<tr>
<td>Total</td>
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<td>14</td>
</tr>
<tr>
<td>Type total</td>
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</tr>
<tr>
<td>Type total [%]</td>
<td>92.31</td>
<td>7.69</td>
</tr>
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</table>

**Table 2.3.** Progeny of cross *pk a X ccr-100 A.*

<table>
<thead>
<tr>
<th>Number of randomly picked ascospores</th>
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</thead>
<tbody>
<tr>
<td>Viable ascospores</td>
<td>110</td>
</tr>
<tr>
<td>Non-viable ascospores</td>
<td>90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type</th>
<th>Parental</th>
<th>Recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ccr</td>
<td>gran</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>47</td>
</tr>
<tr>
<td>Type total</td>
<td>103</td>
<td>7</td>
</tr>
<tr>
<td>Type total [%]</td>
<td>93.64</td>
<td>6.36</td>
</tr>
</tbody>
</table>

**Table 2.4.** Progeny of cross *gran a X ccr-100 A.*

<table>
<thead>
<tr>
<th>Number of randomly picked ascospores</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable ascospores</td>
<td>40</td>
</tr>
<tr>
<td>Non-viable ascospores</td>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type</th>
<th>Parental</th>
<th>Recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ccr</td>
<td>col-4</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Type total</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Type total [%]</td>
<td>47.5</td>
<td>52.5</td>
</tr>
</tbody>
</table>

**Table 2.5.** Progeny of cross *col-4 A X ccr-100 a.*
<table>
<thead>
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<th>Number of randomly picked ascospores</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Viable ascospores</td>
<td>10</td>
</tr>
<tr>
<td>Non-viable ascospores</td>
<td>90</td>
</tr>
<tr>
<td>Type</td>
<td>Parental</td>
</tr>
<tr>
<td>Phenotype</td>
<td>ccr</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
</tr>
<tr>
<td>Type total</td>
<td>4</td>
</tr>
<tr>
<td>Type total [%]</td>
<td>40</td>
</tr>
</tbody>
</table>

**Table 2.6.** Progeny of cross *ccr-100 a X col-4 A*.

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>Viable ascospores</td>
<td>85</td>
</tr>
<tr>
<td>Non-viable ascospores</td>
<td>15</td>
</tr>
<tr>
<td>Type</td>
<td>Parental</td>
</tr>
<tr>
<td>Phenotype</td>
<td>ccr</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
</tr>
<tr>
<td>Type total</td>
<td>56</td>
</tr>
<tr>
<td>Type total [%]</td>
<td>65.88</td>
</tr>
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</table>

**Table 2.7.** Progeny of cross *col-17 A X ccr-100 a*.

<table>
<thead>
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<th>Number of randomly picked ascospores</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable ascospores</td>
<td>35</td>
</tr>
<tr>
<td>Non-viable ascospores</td>
<td>65</td>
</tr>
<tr>
<td>Type</td>
<td>Parental</td>
</tr>
<tr>
<td>Phenotype</td>
<td>ccr</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
</tr>
<tr>
<td>Type total</td>
<td>28</td>
</tr>
<tr>
<td>Type total [%]</td>
<td>80</td>
</tr>
</tbody>
</table>

**Table 2.8.** Progeny of cross *ipa a X ccr-100 A*.
<table>
<thead>
<tr>
<th>Phenotype Type</th>
<th>al</th>
<th>ylo-1</th>
<th>cot-1</th>
<th>Linkage group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental [%]</td>
<td>Recombinant [%]</td>
<td>Parental [%]</td>
<td>Recombinant [%]</td>
</tr>
<tr>
<td>alcoy X ccr-100</td>
<td>40.85</td>
<td>59.15</td>
<td>42.25</td>
<td>57.75</td>
</tr>
<tr>
<td>alcoy X cs-24</td>
<td>48.48</td>
<td>51.52</td>
<td>70.71</td>
<td>29.29</td>
</tr>
<tr>
<td>alcoy X cs-43</td>
<td>48.42</td>
<td>51.53</td>
<td>52.63</td>
<td>47.37</td>
</tr>
<tr>
<td>alcoy X cs-245</td>
<td>57.14</td>
<td>42.86</td>
<td>54.40</td>
<td>45.60</td>
</tr>
<tr>
<td>alcoy X cs-460</td>
<td>46.93</td>
<td>53.07</td>
<td>50.84</td>
<td>49.16</td>
</tr>
<tr>
<td>alcoy X cs-466</td>
<td>52.57</td>
<td>47.43</td>
<td>51.43</td>
<td>48.57</td>
</tr>
<tr>
<td>alcoy X cs-566</td>
<td>57.53</td>
<td>42.47</td>
<td>41.10</td>
<td>58.90</td>
</tr>
<tr>
<td>alcoy X cs-611</td>
<td>58.59</td>
<td>41.41</td>
<td>68.75</td>
<td>31.25</td>
</tr>
</tbody>
</table>

Table 4.1. Phenotype distribution of progeny from crosses of the linkage tester strain *alcoy* with the *ccr* and *cs* mutants.
<table>
<thead>
<tr>
<th>Number of randomly picked ascospores</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Viable ascospores</td>
<td>255</td>
</tr>
<tr>
<td>Non-viable ascospores</td>
<td>45</td>
</tr>
<tr>
<td>Type</td>
<td>Parental</td>
</tr>
<tr>
<td>Phenotype</td>
<td>ccr</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
</tr>
<tr>
<td>Type total</td>
<td>235</td>
</tr>
<tr>
<td>Type total [%]</td>
<td>92.16</td>
</tr>
</tbody>
</table>

**Table 4.2.** Phenotype distribution of progeny from crosses of strain *V1 (cot-1; inl; aur) A* and strain *ccr-100 a.*
<table>
<thead>
<tr>
<th>Crosses</th>
<th>Number of octads</th>
<th>Number of complete octads</th>
<th>Number of incomplete octads</th>
<th>Parental ditype</th>
<th>Recombinant ditype</th>
<th>Tetratype</th>
</tr>
</thead>
<tbody>
<tr>
<td>cs-24 X ccr-100</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cs-43 X ccr-100</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>cs-245 X ccr-100</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>cs-259 X ccr-100</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cs-460 X ccr-100</td>
<td>6</td>
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<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>cs-566 X ccr-100</td>
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<td>3</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>cs-611 X ccr-100</td>
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<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.3. Distribution of progeny from crosses of mutant ccr-100 with the cs strains