CHARACTERISATION OF THE MATING-TYPE GENES *mt A-2* AND *mt A-3* OF *Neurospora crassa* AND REGULATION OF SEXUAL DEVELOPMENT BY MATING-TYPE.

by

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Abstract

The filamentous fungus *Neurospora crassa* has two mating-types, *A* and *a,* responsible for mating and vegetative heterokaryon incompatibility. The *mt* locus is composed of dissimilar DNA sequences termed idiomorphs. In *a* individuals, *mt a-1* is the gene responsible for regulation of all sexual and vegetative functions of the mating-type locus. In *A* strains, *mt A-1* is responsible for fertilisation and heterokaryon incompatibility functions. Two additional genes of the *A* idiomorph, *mt A-2* and *mt A-3*, were isolated and characterised in this study. The *mt A-2* and *mt A-3* genes code for proteins which have characteristics of transcription factors. Isolation of *mt A-2* and *mt A-3*-specific mutants indicated that mutations at these genes lead to arrest of the sexual development at about 3 days after fertilisation, but only if both *mt A-2* and *mt A-3* are affected. Six different mating-type mutants defective at *mt A-1*, *mt A-2*, *mt A-3* and *mt a-1* were used in transcriptional analyses of genes preferentially expressed during early sexual development (*sdv* genes). The results obtained with these analyses suggested the involvement of all four mating-type proteins in regulation of some of these genes. Regulation of different genes by *N. crassa* mating-type seems to depend on combinatorial interactions between the different mating-type proteins.

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1. General Introduction

1.1 RATIONALE AND OBJECTIVES

Neurospora crassa is an ideal model organism for genetic studies and great progress has been made towards understanding the regulation of genes involved in metabolism and development. However several questions remain regarding sexual development and the role of mating-type genes. The *mt A-1* and *mt a-1* mating-type genes have been characterised functionally and molecularly (Glass *et al.,* 1990a, Staben and Yanofsky, 1990; Philley and Staben, 1994; Chang and Staben, 1994; Saupe *et al.,* 1996). A 4 kb region in the *mt A* idiomorph, important for post-fertilisation functions (Glass and Lee, 1992), required further characterisation. The objectives of this thesis were: 1) the molecular and functional characterisation of genes present in the *mt A* idiomorph besides *mt A-1* and 2) determining the role of mating-type genes in the regulation of the sexual development of *N. crassa*. This study was aimed at understanding mechanisms that control *Neurospora* sexual development.

In this chapter an overview will be given on the following subjects:

1. Transcriptional control and development.
2. Fungal mating-types, highlighting the *Saccharomyces cerevisiae* system and briefly reviewing other ascomycetous and basidiomycetous fungi.
3. Description of *Neurospora crassa* life cycle and mating-type.

1.2 TRANSCRIPTIONAL CONTROL AND DEVELOPMENT

Development in eukaryotes relies on the concerted expression of genes in a temporal and spatial manner during the course of the cell cycle or in response to environmental changes. It is

known that regulation of gene expression is mostly dependent on the control of transcription and a substantial amount of information has been accumulated that has provided an understanding of gene regulation at the transcriptional level.

1.2.1 The transcription machinery

Three eukaryotic RNA polymerases exist and each has a distinct function: Pol I transcribes ribosomal DNA; Pol II is responsible for transcription into messenger RNA and small nuclear RNA; Pol III synthesises transfer RNA, 5S rRNA and other small cellular and viral RNAs (reviewed in Sentenac, 1985). All of the RNA polymerases require different general transcription factors to form a pre-initiation complex and for their specific function. Some common factors, however, may be part of the three enzyme complexes such as the TATA-binding protein (reviewed in White and Jackson, 1992).

Genes transcribed by Pol II are referred to as class II and typically contain a common core promoter element, recognised by general transcription factors, and gene-specific DNA elements that are recognised by regulatory factors (reviewed in Buratowski, 1994, Roeder, 1996). The most common core-promoter elements are consensus sequences known as the TATA box, located near position -30 to -25 and a pyrimidine rich initiator (Inl) located near the transcription start site. Among several well-characterised transcription factors (TFIIs) isolated so far, only TFIID is capable of sequence-specific binding to the promoter. TFIID is composed of the TATA-binding protein (TBP) and certain TBP-associated factors (TAFs). The pathway for the formation of the pre-initiation complex is well delineated (reviewed in Hori and Carey, 1994, Roeder, 1996, Burley and Roeder, 1996). The pathway involves binding of the TBP to the TATA box followed by association of TFIIA, TFIIB, TFIIF, TFIIE and TFIIH with Pol II in an ordered multistep manner (see Figure 1-1). In *in vitro* assays, the basal machinery can only maintain a relatively

low level of transcription without the aid of activators; in the absence of activators, expression of most genes *in vivo* is silenced by chromatin and other repressors (Verrijer and Tjian, 1996).

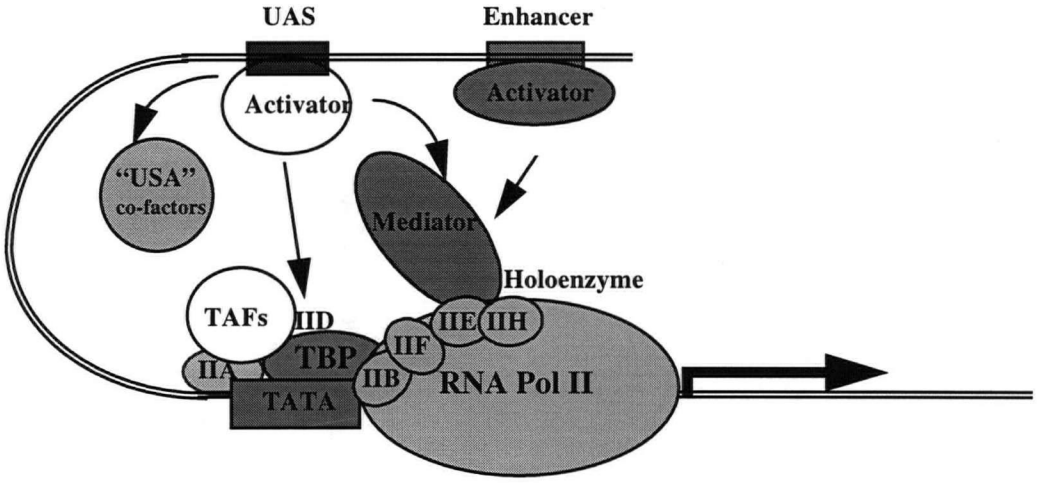


Figure 1-1 The transcription machinery. RNA polymerase II and general transcription factors (IIA, IIB, IID, IIE, IIF and IIH) involved in the assembly of the pre-initiation complex are shown. Different models for activation are represented: activators can interact with IID(TAFs + TBP), with polypeptides of the mediator or by interaction of other co-factors to regulate transcription (e.g. "USA"). Repression is not represented. TBP=TATA-binding protein; TAFs=TBP-associated proteins; USA=upstream stimulatory activity; UAS=upstream activation site (cis-acting element). See text for other abbreviations and details. The diagram is based on several reviews published in the special issue of Trends in Biochemical Sciences (Sept. 1996) on transcriptional control.

1.2.2 Regulators of Transcription

Even though there is increasing evidence that core promoters and the general transcription complex may play an important role in the temporal and spatial regulation of some genes (reviewed in Novina and Roy, 1996), it is known that transcription is modulated by a number of trans- and cis-acting regulatory elements. Trans-acting elements are factors that bind to sequences adjacent to the core promoter (cis-acting elements) (reviewed in Ptashne, 1992, Clark and Docherty, 1993 and Cowell, 1994). Enhancers are cis-acting elements that can promote transcription from more than 1 kilobase upstream or downstream from the site of transcription initiation. Transcriptional regulators are typically modular in nature and have separate DNA

binding and activation or repression domains (see below for details on domains). Repressors may act passively by competing for DNA binding sites of activators or actively by interacting directly with the transcription machinery (reviewed in Clark and Docherty, 1993; Hanna-Rose and Hansen, 1996). Activators promote transcription in general by stabilizing or increasing the number of initiation complexes (reviwed in Hori and Carey, 1994) but may also stimulate the rate of elongation (Yankulov *et al.,* 1994).

Until very recently it was thought that activators exerted their effects on transcription by direct binding to components of the trancriptional machinery. However, experimental evidence suggests the existence of intermediary factors that convey activation to the initiation complex. Three of these co-factors are: TAFs, mediator and the human USA (Upstream Stimulatory Activity) (reviewed in Verrijzer and Tjian, 1996, Björklund and Kim, 1996; Meisterernst *et al.,* 1991). In TAF-mediated activation, transcriptional activators work by directly contacting TAFs in the multiprotein TFIID complex (Figure 1-1). It has been shown that different classes of activation domains bind distinct TAFs (reviewed in Verrijzer and Tjian, 1996). Activation by the mediator has been studied in yeast. Mediator is a complex of polypeptides (20 have been identified) which associates with the carboxy terminal domain (CTD) of the Pol II to form what is termed the holoenzyme (Figure 1-1). Activators bind to mediator and this association allows or stimulates transcription. Several genes that were initially implicated in transcription regulation in yeast, for instances some *SSN-* genes involved in glucose regulation, are components of mediator (Song *et al.,* 1996). Transcriptional represssion may also involve mediator. Another group of co-factors, distinct from TAFs and mediator, was identified through biochemical activation studies with mammalian cellular extracts. This set of co-factors is termed upstream stimulatory activity (USA) and contains positive and negative factors. In contrast to TAFs this group is not associated

tightly with the general transcription machinery but instead forms a complex that stimulates transcription in conjunction with a complete set of general factors (Figure 1-1) (reviewed in Kaiser and Meisterernst, 1996). The USA contains positive and negative co-factors and includes previously known proteins such as HMG1 and HMG2. Although studied in different systems (Drosophila, yeast and humans) the three types of co-factors may not be mutually exclusive and may act together to allow efficient transcription by activator proteins.

1.2.3 Structural domains of transcriptional regulators

Recognition of DNA sequences or protein-protein interactions depends initially on the tertiary structure of the transcription factor and secondly on atomic contacts, which define specificity (reviewed in Johnson and Mcknight, 1989). Transcriptional regulators have a modular nature: a DNA-binding domain and an activation or repression domain. The large number of characterised transcriptional regulators allows classification according to conserved sequence and functional domains. Common examples of characterised domains are: zinc-fingers, helix-turn-helix, homeodomain, leucine-zipper, helix-loop-helix, HMG, steroid receptor and ETS (reviewed in Latchman, 1990, Johnson and Mcknight, 1989, Pabo and Sauer, 1992, Grosschedl *et al.*, 1994). A number of other different structures that mediate sequence-specific binding exists and new families of transcriptional regulators are discovered as novel genes are isolated (see for example Dhawale and Lane, 1993 for a list of genes involved in transcriptional control in fungi). As a rule of thumb, DNA binding domains of a particular class bind similar DNA target sequences. Some interactions are more stringent than others. The combination of affinity of the DNA-binding domain and activation domains to their sites predicts the efficiency of the transcriptional regulator and determines the levels of gene transcription.

While DNA-binding domains are well defined, activation and repression domains show poor conservation between factors (reviewed in Tjian and Maniatis, 1994, Clark and Docherty, 1993, Cowell, 1994, Hanna-Rose and Hansen, 1996). Some activation domains are acidic or glutamine-, proline- and isoleucine-rich. The importance of these regions for activation was demonstrated, but the structural relationships and mechanisms by which they act are unclear. Even more obscure is the classification of repression motifs, since composition, size and charge of the sequences can be important for function. Proline-, alanine- and glutamine-rich sequences were also found to act as repressors (reviewed in Cowell, 1994, Hanna-Rose and Hansen, 1996). As an example, *Krüppel*, a Drosophila developmental regulator, is normally a transcription repressor but can act as an activator depending on its concentration, as indicated by *in vitro* studies with cell lines (Sauer and Jackle, 1991). The p53 protein, which is an activator of many genes, can also act as a repressor when it is overexpressed (Liu *et al.,* 1993). It seems that many proteins have the ability to act as repressor or activator and the predominant function may depend on the promoter context (Cowell, 1994). Other unique repressor domains such as the Drosophila *Egr1* and *WT1* have been identified. The large number of varied repressor domains is consistent with models that postulate multiple targets for repression among the cellular transcription factors (reviewed in Clark and Docherty, 1993, Cowell, 1994, Hanna-Rose and Hansen, 1996).

Many of the fungal mating-type genes isolated so far encode products with characteristics of transcription factors (reviewed in Kües and Casselton, 1992; Kothe, 1996). The best characterised mating-type system is from the yeast *Saccharomyces cerevisiae* in which activation, repression and protein-protein interactions have been demonstrated biochemically and genetically (see section 1.3.1 below).

1.3 FUNGAL MATING-TYPES

The term mating type is used to differentiate individuals that are sexually compatible. Sexual dimorphism is almost non-existent in fungi and in most cases (as in *N. crassa,* for example) individuals are hermaphroditic, i.e. they produce male and female reproductive structures. The existence of mating types was first recognised in the beginning of this century in the fungus *Rhizopus* (Blakeslee, 1904) but it was not until the early eighties when the first molecular characterisation of mating type loci was achieved in the yeast *Saccharomyces cerevisiae* (Astell *et al.,* 1981).

The Ascomycotina and Basidiomycotina are two of the three major groups in the kingdom Fungi. The structures of mating type loci in these two groups differ greatly in number of genes and alternate alleles. Mating types are classified as bipolar or tetrapolar, depending on the existence of two or four mating determinants, respectively. In the ascomycetes, there is one mating-type locus with two alternate alleles, thus mating is bipolar. Mating-types in basidiomycetes can be extremely complex with both bipolar and tetrapolar mating systems (reviewed in Kües and Casselton, 1992 and Kothe, 1996). The number of mating-types varies, depending upon whether they are determined by two loci with two or more different alleles as in basidiomycetous yeasts or by several loci bearing hundreds of alleles which result in several thousands of mating-types as in the hymenobasidiomycetes. DNA sequence analyses of some mating type loci (Astell *et al.,* 1981; Strathern *et al.,* 1980; Kelly *et al.,* 1988; Glass *et al.,* 1988) indicated that the term allele was not appropriate to denote alternate mating-types since they consist of mainly dissimilar sequences that do not seem to have common evolutionary origins; the term idiomorph was proposed instead (Metzenberg and Glass, 1990).

1.3.1 *Saccharomyces cerevisiae* mating-type

The ascomycete *S. cerevisiae* has the best studied mating-type locus. Various functions in the *S. cerevisiae* life cycle are interconnected due to mating-type locus regulation. Mating-type genes determine the cell type as haploid or diploid and the choice between sexual or vegetative growth (reviewed in Herskowitz, 1989, Nasmyth and Shore, 1987). The two haploid cell types are denominated **a** and α and their identity is determined by sequences present at the MAT locus. Each **a** and α cell contains sequence determinants for both cell types but, by a process of internal recombination and transcription silencing, a haploid cell can switch to have the opposite mating-type (Herskowitz *et al.,* 1992). The diploid cell (**a**/α) results from pheromone-mediated fusion of two haploid cells. Under nutrient starvation the fate of the diploid cell is meiosis with the formation of four haploid cells, two of which are **a,** and two are α.

Each MATa and MATα idiomorph contains two divergently transcribed genes **a1**, **a2** and **α1** and **α2,** respectively; these genes, except for **a2** whose function is unknown, encode transcription factors which are involved in the determination of cell identity (Herskowitz, 1988). In haploid cells, **α1** regulates the expression of cell-type specific genes including α-specific pheromones and pheromone receptors but **a1** is not required for expression of any **a**-specific genes (Fields, 1990). Mating is triggered following binding of pheromones to receptors. One of the earliest responses in the mating process is the induction of cell surface agglutinins which facilitate mating (reviewed in Lipke and Kurjan, 1992). The cells then synchronise their cycles by arresting at the G1 phase, i.e. before DNA replication. A diploid cell cannot express genes needed for mating but instead drives the cellular process to meiosis and sporulation depending on environmental conditions (reviewed in Herkowitz, 1988, 1989). A novel regulatory factor is

formed by interaction of **a1** and **α2** proteins that represses **α1** and general haploid cell functions (Goutte and Johnson, 1988; Dranginis, 1990).

Cloning and characterisation of the **a** and **α** mating-type locus showed that these regions are composed of non-homologous sequences (Astell *et al.,* 1981; Strathern *et al.,* 1980). The **a1** and **α1** proteins are encoded by totally dissimilar sequences of 642 and 747 bp, respectively, while **a2** and **α2** sequences have partial homology (Nasmyth and Tatchell, 1980; Strathern *et al.,* 1980; Astell *et al.,* 1981). The **a1** and **α2** proteins are together transcriptional repressors and they both contain a homeodomain DNA-binding motif (Sheperd *et al.,* 1984). The **α1** protein was shown to be a transcription activator (Sprague *et al.,* 1983; Sengupta and Cochran, 1991) but its DNA binding domain (the α box) has yet to be characterised in detail.

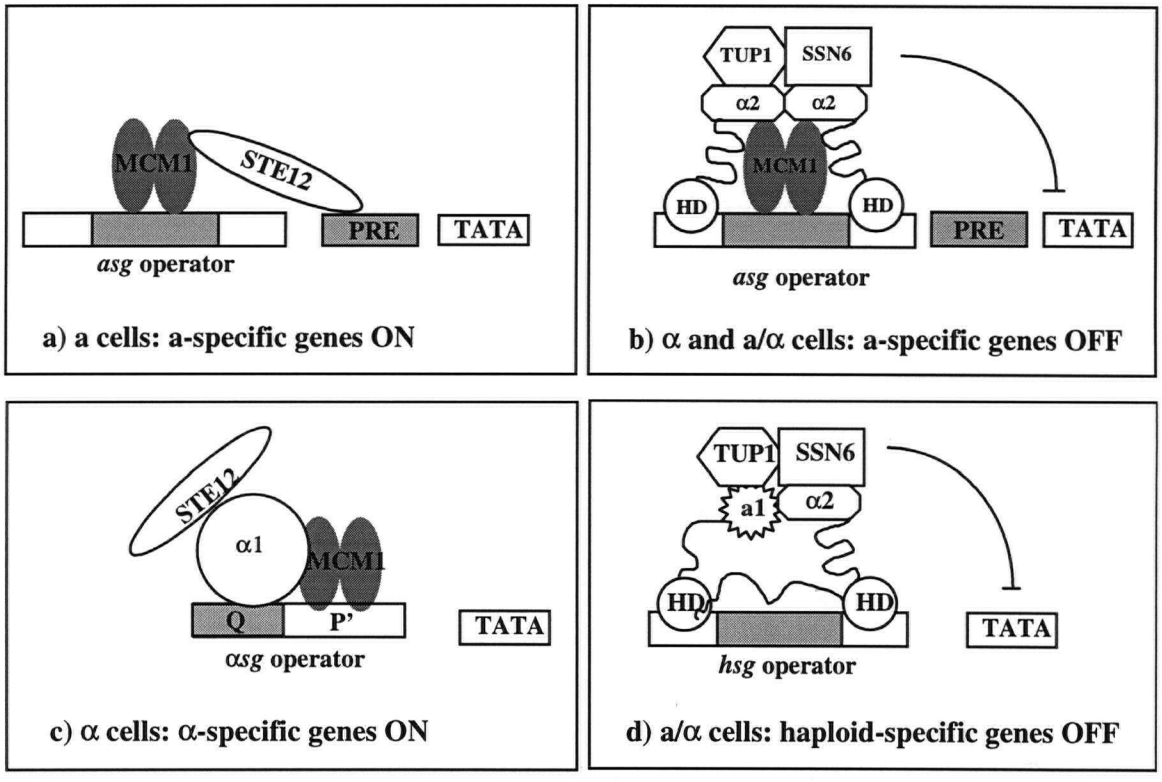


Figure 1-2 Cell-type gene regulation in *S. cerevisiae.* asg, αsg and hsg stand for a-specific, α-specific and haploid-specific genes, respectively. Refer to text for details. Adapted from Johnson, 1995.

Pheromone and pheromone-receptor genes are specific for each cell-type but haploid specific genes are equally expressed in **a** and α cells. Some of the haploid-specific genes are STE12, an activator of mating genes, and RME1, a repressor of meiosis. Sporulation genes and genes encoding components of the heterotrimeric G-protein (STE4, STE18 and GPA1) are also expressed in **a** and α cells (reviewed in Johnson, 1995).

In **a** cells, **a**-specific genes are constitutively expressed after co-operative binding of STE12 and a general transcription activator MCM1 to **a**-specific promoters (Figure 1-2a) (Primig *et al.,* 1991; Kirkman-Correia *et al.,* 1993). Because STE12 and MCM1 are also expressed in α cells, an obvious way to explain the absence of expression of **a**-specific genes in α and **a**/α cells is the presence of transcription repressors of **a**-specific genes (Figure 1-2b). This repression depends on a complex formed by α2, SSN6, TUP1, and MCM1 that binds to **a**-specific promoters (reviewed in Johnson, 1995). STE12 and MCM1, when in a complex with α1, positively regulate α-specific genes by binding to their promoter at sequences known as the QP' element (Figure 1-2c) (Primig *et al.,* 1991). In diploid cells the **a1**/α2 complex directs the TUP1/SSN6 repressor to the promoter of haploid specific genes (Figure 1-2d) (Tzamarias and Struhl, 1994; Komachi *et al.,* 1994).

While pheromone-mediated intercellular communication permits the recognition of **a** and α cells prior to fusion, processes thereafter depend on a G-protein signalling pathway (reviewed in Marsh *et al.,* 1991, Konopka and Fields, 1992). The yeast G-protein consists of three subunits (α, β, γ) and many genes that code for components of the signal transduction pathway have been identified, such as *STE5, STE7, STE11 STE12, FUS3* and *KSS1* (reviewed in Marsh *et al.,* 1991). Mutations in these genes lead to inability to respond to pheromone. Binding of pheromone to

receptors causes the trimeric G-protein to dissociate, which activates a cascade of events (through kinases and transcription factors) that ultimately arrest the cell cycle at G1. The decision to enter mitotic or meiotic division depends on nutritional factors. A cAMP-dependent signal transduction pathway is involved in sensing nitrogen and glucose levels and this cascade exerts its effects on a gene responsible for meiosis induction, *IME1* (reviewed in Klein *et al.,* 1994). *IME1* is under the repressive control of *RME1* (repressor of meiosis). Transcription of *RME1* is repressed in diploid MATa/ MATα cells by the action of the heterodimer **a1**/α2, so meiosis and sporulation can progress (Mitchell and Herskowitz, 1986). Genes involved in the control of sporulation and meiosis have been well documented (Klein *et al.,* 1994).

1.3.2 Mating-type in Basidiomycetes

Understanding of Basidiomycete mating-type regulation comes mainly from studies conducted on *Coprinus cinereus, Schizophyllum commune* and *Ustilago maydis* (reviewed in Kües and Casselton, 1992, Kothe, 1996 and Banuett, 1992). Of the three fungi, the heterobasidiomycete *U. maydis* has the simplest mating-type locus structure: two unlinked loci, *a* with two alleles and *b* with multiple alleles. The hymenobasidiomycetes *C. cinereus* and *S. commune* mating-type locus structure is similar and is composed of two (*A* and *B*) multiallelic loci.

U. maydis exists as either haploid or diploid cells; fusion of two yeast-like haploid cells leads to formation of a filamentous dikaryon that is pathogenic on corn plants. Karyogamy occurs in tumours formed in several tissues of the plant but meiosis only occurs after the diploid spores have germinated (reviewed in Banuett, 1992). Haploid mating partners must have different specificities at the *a* and *b* loci to fuse and form a stable dikaryon. The *a* locus contains genes that code for mating-specific pheromones (*mfa1* and *mfa2*) and their receptors (*pra1* and *pra2*).

The *a* locus genes are the key regulators of cell fusion in *a1* and *a2* cell types and, together with the *b* locus are required for maintenance of filamentous growth (Bolker *et al.,* 1992; Spellig *et al.,* 1994). Pheromone and pheromone receptors in *U. maydis* exhibit similarity to components of *S. cerevisiae* pheromone system. The *b* locus controls filamentous growth of the dikaryon and pathogenicity. A compatible reaction between two different partners is necessary for pathogenicity. The number of alternative alleles at the b locus is estimated to be at least 25 (Puhalla, 1970). Each *b* locus has genes that code for two divergently transcribed homeodomain proteins, bE and bW (Kronstad and Leong, 1990; Schulz *et al.,* 1990; Gillissen *et al.,* 1992). It appears that specificity at the *b* locus is mediated by variable N-terminal regions of the bE and bW polypeptides (Yee and Kronstad, 1993; Kronstad *et al.,* 1995). By using chimeric alleles of bE and bW it was possible to define the regions of specificity. Small changes in amino acid sequence on the borders of recognition regions can alter specificity, at least for the two studied alleles (*b1* and *b2*). A gene (*Prf1*) was isolated recently that mediates the pheromone response and might be the intermediary in the interplay of *a* and *b* loci during development (Hartmann *et al.,* 1996). Learning how specificity is regulated is the first step towards understanding the control of pathogenicity and could lead to identification of target genes involved in the process.

S. commune and *C. cinereus* have an asexual monokaryotic mycelia and fusion of hyphae from two different mating types gives rise to a fertile dikaryon. The *A* and *B* mating-type loci determine compatibility, but unlike in other fungi, cell fusion occurs independently of the identity of mating-type genes (reviewed in Kües and Casselton, 1992, Casselton *et al.,* 1995, Kothe, 1996). The *A* locus controls cell division and formation of the clamp cell(cell developed on the side of the tip cell, seen before nuclei exchange) while the *B* locus governs nuclear migration. Recombination and molecular analyses showed that the *A* and *B* loci are very complex

and are composed of two closely linked loci with multiple alleles at each (Day, 1960; Stamberg and Koltin ,1973). A fertile dikaryon will only form if both *A* and *B* loci contain different alleles. The number of alleles is so large in both *C. cinereus* and *S. commune* that over 12,000 and 20,000 mating types, respectively, could be generated (Whitehouse, 1949; Raper, 1966). The *A* loci ($A\alpha$ and $A\beta$) in *C. cinereus* are composed of four pairs of homeodomain proteins that are encoded by the two *A* loci (Kües *et al.,* 1992). The $A\alpha$ locus of *S. commune* has been cloned and like the *C. cinereus* counterpart, it codes for multiallelic homeodomain proteins (Giasson *et al.,* 1989; Stankis *et al.,* 1992). In a manner similar to *U. maydis,* homeodomain proteins originating from different nuclei form active heterodimers that regulate specificity in *S. commune* and *C. cireneus* (Kothe, 1996). In *S. commune,* the *B* locus complex ($B\alpha$ and $B\beta$) have also been cloned (Specht, 1995) and have been shown to encode elements of a pheromone system (Wendland *et al.,* 1995; L. Vaillacourt and C. Raper, pers. comm. cited in Kothe, 1996). Because attraction of partners is not necessary in these homobasidiomycetes the existence of a pheromone receptor system has to account for other mating functions. It has been proposed that a signal prior to the fast nuclear migration process would allow a prompt use of the dikaryotic cell machinery upon nuclei entry (Kothe, 1996).

The identification of components of pheromone and signal transduction systems in basidiomycetes indicate similarities between some mating strategies in this group and Ascomycotina. In contrast to the Basidiomycotina, the Ascomycotina exhibit specialised mating structures (male and female gametes) and the dikaryotic phase is very short compared to the prolonged vegetative dikaryotic stage of basidiomycetous fungi. Although some aspects of mating are essentially different (cell fusion, cell differentiation and multicellularity), it seems that the basic processes through which mating and meiosis occurs may be conserved in fungi.

1.3.3 Mating-type in filamentous Ascomycetes

In the following sections, an overview of *N. crassa* including life cycle characteristics will be described, before presenting a description of mating-type in the genus *Neurospora.*

1.3.3.1 *Neurospora crassa*, the organism and the genetic model

The genus *Neurospora* is classified within the Ascomycotina. In the acomycetes group, the sexual progeny are enclosed in sac-like structures, the ascus. The genus itself is defined by the grooved wall of its sexual spores, the ascospores (Shear and Dodge, 1927).

A systematic sampling of *Neurospora* from all over the world showed that *Neurospora* species are found naturally in moist tropical and sub-tropical areas and are less frequently encountered where the hot season is dry and the winter is very cold; *N. crassa* specifically is more often found in samples from the Gulf of Mexico area, the Caribbean, Africa and India (Perkins and Turner, 1988).

Two mating-types exist in *Neurospora* species, termed *A* and *a* (see section 1.3.3.3). *Neurospora* species can exhibit three types of mating strategies: heterothallism, homothallism or pseudohomothallism (or secondary homothallism). In heterothallic species, two compatible individuals of different mating-types are required for mating and an equal number of progeny of both *A* and *a* mating-types are produced. Heterothallic *Neurospora* species are *N. crassa, N. discreta, N. intermedia and N. sitophila* and, with the exception of *N. discreta,* they are sufficiently related to give low levels of viable progeny in interspecific crosses (Perkins *et al.,* 1976; Perkins and Raju, 1986). Homothallic species are self-fertile and there is no genetic difference in the nuclear content of the mycelia or of the progeny. The homothallic species are separated in two groups based on the characteristics of the mating-type locus (see section 1.3.3.4). One group of homothallic species consists of *N. terricola and N. pannonica* and a

second group includes four species: *N. africana, N. lineolata, N. dodgei and N. galapagosensis.* Only one pseudohomothallic *Neurospora* species, *N. tetrasperma,* has been identified. Self-fertility of pseudohomothallic species arises from the heterokaryotic nature of the mycelia and results in progeny that enclose nuclei of both mating-types. A few phenotypic characteristics distinguish species within the three groups. Only the non-homothallic species produce conidia, rendering the colonies bright orange, due to deposition of carotenoids. A total of 8 progeny are enclosed in the ascus, but the pseudohomothallic *N. tetrasperma* has only four-spored asci containing two nuclei of opposite mating-types. (Perkins and Turner,1988).

Several features of *Neurospora* were advantageous for initial genetic studies. *Neurospora* has a long haploid phase with short generation time and it grows on simple, defined media . All four products of meiosis can be readily recovered and this facilitates the mapping of genes and centromeres. Heterokaryons can be formed and complementation tests are easily done. Stocks can be preserved, conferring immortality to individual strains (Perkins, 1992).

Among the *Neurospora* species, *N. crassa* was chosen as the most suitable for genetic work. One of the advantagesof *N. crassa* is that it is a heterothallic strain and crosses can be performed easily between individuals of opposite mating-type. Several researchers in the first half of the century developed *N. crassa* as a model organism. Dodge (1927) performed the first tetrad analysis in *N. crassa* and showed a 4:4 segregation of mating type. The isolation of mutants containing auxotrophic markers allowed the identification of the first linkage groups (reviewed in Lindegren, 1973). The first nutritional mutants brought about Beadle and Tatum's "one gene-one enzyme" theory (Beadle and Tatum,1941) which contributed to the science of biochemistry and molecular genetics.

Numerous tools and techniques have been developed that allowed molecular biology studies on *Neurospora*. *N. crassa* has a small haploid genome for an eukaryote (~47 million bp), with very few repetitive DNA sequences and has seven chromosomes (reviewed in Mishra, 1991). Demonstration of plasmid DNA mediated transformation (Case *et al.,* 1979) has allowed efficient cloning and disruption of *N. crassa* genes (reviewed in Fincham, 1989). A phenomenon unique to *Neurospora*, termed RIP (Repeat Induced Point mutation), allows directed mutagenesis to specific DNA sequences -see Chapter 3 (Selker *et al.,* 1987; Cambareri *et al.,* 1989). *N. crassa* populations also show molecular polymorphism in DNA sequences (RFLP) which is of great interest, especially for population and evolution studies.

1.3.3.2 The life cycle of *N. crassa*

Figure 1-3 shows the *N. crassa* life cycle. *N. crassa* hyphae have chitinous cell walls and grow linearly by the addition of new material at the tips. Hyphae have partial septa and multiple nuclei are present in the cells which can move through the hyphae. The haploid individual produces two types of vegetative conidia: macroconidia and microconidia (reviewed in Springer, 1993). Macroconidiation is induced mainly by environmental factors such as carbon starvation and desiccation (Ricci *et al.,* 1991; Nelson *et al.,* 1975). Microscopic and genetic studies throughout the years have focused on different aspects of conidiation in *N. crassa*. The earliest sign of conidiation is the apical buds that are formed from the tip of aerial hyphae (conidiophore). A chain of macroconidia that resembles beads on a string, form on the conidiophore (Turian and Bianchi, 1972). Multinucleate conidia result from septation which is achieved by deposition of several layers of cell wall material in the budding cells. Microconidia are not formed by budding at the ends of aerial hyphae, but originate within vegetative hyphae and rupture out through the hyphal cell wall (Grigg, 1960). Free microconidia differ from the

more abundant macroconidia in that they are smaller, uninucleate and lack carotenoids (Lowry *et al.,* 1967). Several mutants have been isolated which are blocked in various developmental steps of macroconidiation, such as *fl (fluffy), acon-2 (aconidiate-2)* and *csp-2 (conidial-separation-2)* (Lindegren, 1933; Springer and Yanofsky,1989; Selitrennikoff *et al.,* 1974). Cloning of genes involved in vegetative development, however, is still under way and will be crucial for the understanding of the regulatory mechanisms of conidiation pathways.

Several heterokaryon incompatibility loci are present in *N. crassa* which inhibit growth after fusion of individual hyphae containing different alleles (reviewed in Glass and Kuldau, 1992). Mating-type associated heterokaryon incompatibility is observed during the vegetative phase and is dependent on at least one other unlinked locus, *tol (tolerant)* (Newmeyer, 1970; Vellani *et al.,* 1994). During the sexual cycle, a mechanism may interfere with heterokaryon incompatibility to allow cellular and nuclear fusion between vegetatively incompatible individuals.

In general, the selective advantages of having a sexual phase in the life cycle are the production of diversity through recombination, the loss of deleterious mutations and acquisition of new gene combinations. In *N. crassa,* an additional advantage of the sexual cycle is the production of progeny that will survive environmental adversity. *Neurospora* sexual spores are dormant and can be activated by a period of heating or exposure to certain chemicals (Emerson, 1954).

The sexual phase of *N. crassa* life cycle is detailed in Figure 1-3. Sexual development is induced partially by nutrient starvation, especially nitrogen depletion (Westergaard and Mitchell, 1947). Under these conditions the female organ, the ascogonium starts to develop and gives rise to a protoperithecium. The ascogonium initiates as lateral buds from vegetative hyphae and grow

asymmetrically into hooks and then tight coils (Turian, 1975). The ascogonium is then surrounded by sheath hyphae which delimits the protoperithecium. Protoperithecia can reach from 10 to 50 µm in diameter. Specialised hyphae called trichogynes emanate from the protoperithecium in search of male fertilising cells of the opposite mating-type. The trichogyne was shown to grow towards the male cell by the action of a diffusible pheromone-like factor (Bistis, 1981, 1983) after which cell fusion occurs between the tip of the trichogyne and the male cell. The male nucleus then migrates to the ascogonium via the trichogyne. Approximately three days after fertilisation, paraphysoidal hyphae can be seen within the central cavity of the enlarging perithecium. Paraphyses are hyphae from maternal origin thought to help in the expansion of the perithecium after fertilisation (Luttrell, 1951). A time course of the main events after fertilisation is shown in Table 1-1. After migration to the ascogonium, the two haploid nuclei from the opposite mating-types do not fuse immediately but instead, proliferate through multiple cycles of mitosis that give rise to numerous ascogenous cells (Raju, 1980). There is evidence that an association between the spindle pole body and nuclear DNA may be important

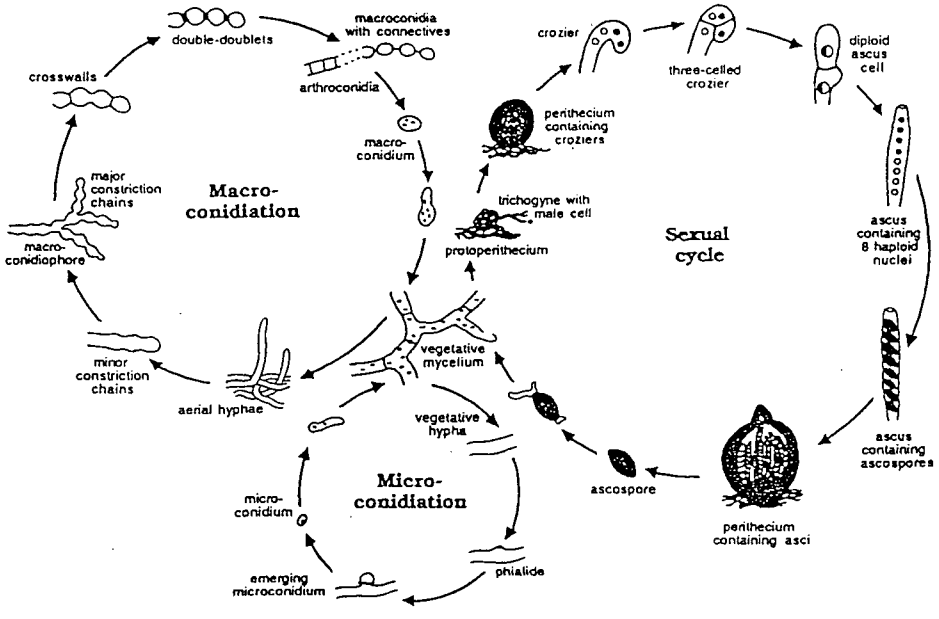


Figure 1-3 Life cycle of *N. crassa*. Refer to text for details. Reproduced from Springer, 1993.

in the recognition of opposite mating-type nuclei in the ascogenous hyphae (Thompson-Coffe and Zickler, 1994). Asci are formed from the penultimate cells of the ascogenous hyphae that bend to form hook-shaped cells which are called croziers. A pair nuclei (of opposite mating-type) is enclosed in the crozier and undergo one round of conjugate mitosis. In a very regulated manner, the penultimate cell of the crozier delimits two opposite mating-type nuclei that undergo fusion. This constitutes the only diploid stage of *N. crassa* life cycle. Meiosis immediately takes place giving rise to four products of meiosis. A final round of mitosis yields the ascus with 8 ascospores. Ascospores are delimited and chitinous cell wall is deposited around each of them by the fifth day after fertilisation (reviewed in Read and Beckett, 1996). Ascospores become black after melanin deposition and maturation is achieved within 10 days post-fertilisation. Asci develop asynchronously (Singleton, 1953) and over 200 may be present in a normal perithecium (Raju, 1980). At about 5 days post-fertilisation, the perithecia are already full-sized (300 μm) and show beaks with an ostiole through which ascospores are discharged.

DAYS AFTER FERTILIZATION	EVENTS
3 Days	First croziers can be observed
4 Days	Karyogamy and prophase I
5 Days	Meiotic and first mitotic divisions
From 5 Days On	Ascospores delimited in asci
7-8 Days	Ascospores become black
From 8 Days On	Ascospore discharge starts

Table 1-1 Progression of normal sexual development in *N. crassa*. Compiled from Raju and Perkins, 1978; Raju, 1980; Raju, 1992.

1.3.3.3 *N. crassa* mating-type

The history of *N. crassa* mating-type studies starts with Dodge in the early 1920's when *N. crassa* was shown to be a heterothallic species with two mating types termed *A* and *a*. During vegetative growth, the *mt* locus has a heterokaryon-incompatibility function (Beadle and

Coonradt, 1944; Sansome, 1946) such that nuclei from opposite mating type cells cannot coexist in the same cell (forced heterokaryons between *A* and *a* strains are inhibited in their growth). Both the *A* and *a* idiomorphs have been cloned and characterised (Glass *et al.,* 1988; Glass *et al.,* 1990a; Staben and Yanofsky, 1990).

The *N. crassa A* mating type was cloned based on complementation of a mutation linked to *mt* (Vollmer and Yanosfsky,1986; Glass *et al.,* 1988). An indirect selection method was utilised since no phenotype for the mating-type genes could be selected in a transformation experiment. The selection was for the *un-3*$^{+}$ gene which confers the ability to grow at restrictive temperature to a *un-3*$^{-}$ strain (Glass *et al.,* 1988). A 50 kb *un-3*$^{+}$ cosmid conferred *A* mating behaviour when introduced into sterile strains, as assayed by crossing to an *a* strain. The *a* mating-type was cloned based on hybridisation of a phage library to sequences flanking the *A* region in the cosmid (Staben and Yanofsky, 1990). It was shown that the two alternate forms do not share sequence identity (Glass *et al.,* 1988) and were thus termed idiomorphs (Metzenberg and Glass, 1990).

The *a* idiomorph is a 3.2 kb DNA segment (Figure 1-4) and contains a 747 bp ORF corresponding to the *mt a-1* gene (Staben and Yanofsky, 1990). The MT a-1 polypeptide contains a HMG (High Mobility Group) DNA-binding motif which is present in the *Schizosaccharomyces pombe* Mc mating-type protein (Kelly *et al.,* 1988). Mutations in *mt a-1* abolish *a* mating identity and heterokaryon-incompatibility (Griffiths and De Lange, 1978; Staben and Yanofsky, 1990). MT a-1 synthesised in *E. coli* was shown to specifically bind *in vitro* the sequence 5'-CTTTG-3' through its HMG domain (Philley and Staben, 1994). The same sequence is also bound by other related HMG proteins (Bianchi *et al.,* 1992; Griess *et al.,* 1993). This site is present within and around both the *A* and *a* idiomorphic sequences. In the *a* idiomorph, two regions 5' of the *mt a-1*

coding sequence were protected in the binding assay with *mt a-1* (and contained CTTTG or CTTCG); four other CTTTG sequences are found in the *mt a* centromere-distal flank. In the *A* sequences the same five nucleotide sequence appears 21 times, and seven of them cluster in a segment important for ascospore formation (see below) (Glass and Lee, 1992). Mutations in the HMG binding domain of MT a-1 abolish mating but the importance of binding of MT a-1 to the CTTTG repeat *in vivo* has not been addressed. It was also determined that heterokaryon incompatibility function of MT a-1 is independent of the HMG binding properties (Philley and Staben, 1994). It was shown that the *mt a-1* gene is sufficient to confer all *a* functions by a gene replacement of the *A* idiomorph with the whole *a* idiomorph or the *mt a-1* ORF only (Chang and Staben, 1994).

The *A* idiomorph is a 5.3 kb region (Figure 1-4) that has very little DNA sequence similarity to the *a* idiomorph (Glass *et al.,* 1990a). Only one gene, *mt A-1,* was initially characterised from the *A* idiomorph. The *mt A*-1 ORF has a region of amino acid similarity to the *S. cerevisiae* **α1** polypeptide in the α-box (Sprague *et al.,* 1983) suggesting that *mt A-1* is also a transcriptional activator. The MT A-1 protein exhibits separate functional domains: female-fertility, male-fertility and heterokaryon incompatibility (Saupe *et al.,* 1996). As in *mt a-1*, null mutations in the *mt A-1* gene simultaneously compromise mating identity and heterokaryon incompatibility (Griffiths, 1982; Glass *et al.,* 1990a; Glass and Lee, 1992). One exceptional *mt A-1* mutant (A^{m99}) was obtained in which heterokaryon incompatibility and male-fertility were lost, but when the mutant was used as a female, a small number of progeny was produced (Saupe *et al.,* 1996). The region of greatest similarity between the *S. cerevisiae* **α1** is retained in A^{m99} MT A-1 polypeptide. The α-box region in *S. cerevisiae* is thought to be important for interaction of

α1 with MCM1 (Hagen *et al.,* 1993; Yuan *et al.,* 1993) which suggests MT A-1 may have a MCM1-like counterpart.

Mutational analyses have shown that the *A* idiomorph contains a second functional region, in addition to that encoding *mt A-1,* which is important for post-fertilisation events (Glass and Lee, 1992). A single strain (A^{IIRIP}) mutated in this region (corresponding to a 3 kb *Sst* II fragment - Figure 1-4) is phenotypically normal during the vegetative and early sexual phase. The A^{IIRIP} mutant mates as male or female but produces very few ascospores. Two ORFs derived from this region have similarities to two *Podospora anserina* genes in the *mat-* locus that are involved in sporulation- see section 1.3.3.6 (Debuchy *et al.,* 1993). As with other *N. crassa* mating-type mutants, the A^{IIRIP} mutant cannot be fully complemented to normal fertility with introduced ectopic copies of mating-type sequences (Glass *et al.,* 1988; Arnaise *et al.,* 1993;

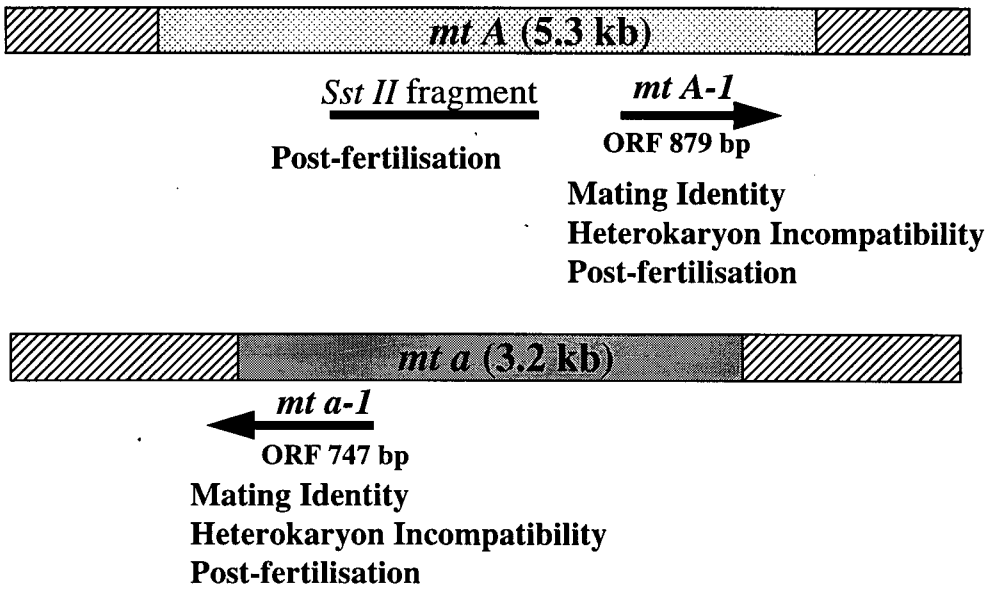


Figure 1-4 *N. crassa* mating-type locus structure. Relative positions of *mt A-1* and *mt a-1* genes and the 3 kb fragment utilised for mutagenesis (*Sst II*) of the A^{IIRIP} mutant are shown. *mt A- 1 is* transcribed towards the centromere, while *mt a-1 is* transcribed in the opposite direction in linkage group I.

Glass and Lee, 1992) making it difficult to study post-fertilisation functions in *N. crassa*.

Signal transduction is involved in the mating-type response in yeast (see section 1.3.1) and is likely to be an important part of the mating response in *N. crassa*. Analyses of *N. crassa* signal transduction led to the isolation of a gene (*gna-1*) that codes for a G-protein (subunit α_i) (Turner and Borkovich, 1993). A *gna-1* deletion strain is defective in multiple differentiation pathways during both vegetative and sexual growth, including conidiogenesis and protoperithecial development (Ivey *et al.,* 1996). No $G\alpha_i$ is found in budding and fission yeasts, which suggests that in *N. crassa* and other higher eukaryotes this type of G-protein can regulate processes such as multicellularity and cell differentiation (Borkovich, 1996).

1.3.3.4 Mating-type in other *Neurospora* species and mating-type flanking sequences

The cloning of *N. crassa* idiomorphs has allowed investigation of mating-type structure in other *Neurospora* species (Glass *et al.,* 1988; Glass *et al.,* 1990b; Beatty *et al.,* 1994; Glass and Smith, 1994; Randall and Metzenberg, 1994). In all other heterothallic species of *Neurospora* (*N. sitophila, N. intermedia and N. discreta*), the mating-type idiomorph sequences are quite conserved (Glass *et al.,* 1988). At least for *N. sitophila* and *N. intermedia,* there is evidence that the *mt* locus is linked to genes whose functional analogs in *N. crassa* are in the same linkage group (Perkins *et al.,* 1976; Perkins, 1977). The heterokaryotic mycelia of the pseudohomothallic *N. tetrasperma* can be resolved into *A* and *a* homokaryons (Raju, 1994). Homokaryotic mycelia derived from *N. tetrasperma* have DNA that hybridise with either *A* or *a* sequences but never with both (Glass *et al.,* 1988). The group of homothallic species can be subdivided into two groups from which *N. terricola* and *N. africana* are the best studied representatives. In *N. terricola* (and also in the other member *N. pannonica*) *A* and *a* sequences are both present and are closely linked (~20kb) (Glass *et al.,* 1988; Beatty *et al.,* 1994). In contrast, only sequences

corresponding to the *A* idiomorph are present in species of the second homothallic group (Glass *et al.,* 1988). No homothallic species was observed to contain *a* sequences only (Glass *et al.,* 1990b). It is not known if a third alternative idiomorph is present in the *A*-only homothallic species or if these species can progress through the sexual cycle with only the *A* idiomorph.

Although sexual behaviour of the homothallic species is different from the heterothallic species, the post-fertilisation events that lead to meiosis and ascospore formation seem to be similar among the *Neurospora* species. The *mt A-1* and *mt a-1* genes are highly conserved among several of the homothallic *Neurospora* species (88-98%, Beatty *et al.,* 1994; Glass and Smith, 1994; Randall and Metzenberg,1995; T. Vellani, pers. comm.). The *N. terricola* and *N. africana* idiomorphs are able to confer mating identity and heterokaryon incompatibility when introduced into *N. crassa* sterile mutants which suggested definition of sexual behaviour is due to differences elsewhere in the genome (Beatty, 1993; T. Vellani, pers. comm.; Glass and Smith, 1994).

Analyses of sequences flanking *A* and *a* idiomorphs in several heterothallic and homothallic *Neurospora* species showed defined regions of similarities and differences that characterise species-specific and/or mating-type specific sequences (Randall and Metzenberg, 1995). Three to five kb of the centromere-proximal flank showed high levels of dissimilarity between species, although analyses of the centromere-distal flanks did not reveal substantial differences between the species or mating types. Representatives from heterothallic, pseudohomothallic and homothallic species contained species-specific and mating-type specific variable sequences. The variable regions were bordered by regions that are highly homologous in all species. In addition, there are specific regions of 57-59 or ~140 bp, designated *A*- and *a*-common region, respectively, which are mating-type specific (Randall and Metzenberg, 1995). In

the 4 kb of centromere-proximal flank adjacent to the idiomorphs there are ORFs that give rise to two pairs of mRNAs with different sizes in *A* (A-R1 and A-R2) or *a* (a-R1 and a-R2) strains, suggesting functional differences outside the idiomorph (Metzenberg and Randall, 1995; Randall and Metzenberg,1995). Accumulation of A-R1 transcripts is greater under crossing conditions and seems to be regulated by *mt A-1* (Randall and Metzenberg, 1995). One *A* strain containing a mutation in A-R2 mates and forms perithecia but no ascospores are produced (Metzenberg and Randall, 1995). Although these data suggest mating type-specific and sexual cycle-related roles for the ORFs in this region, a strain in which the *A* idiomorph sequences have been replaced with *a* sequences seems to be unaffected in any vegetative or sexual functions (Chang and Staben , 1994).

1.3.3.5 Models for action of mating-type genes in *Neurospora*

All models for action of the *N. crassa* mating type genes, proposed up to now, are based mainly on what is known about *mt A-1* and *mt a-1* regulatory characteristics and, consequently have similarities with the well studied yeast mating-type system. Two models described here account for the vegetative and sexual functions of the mating-type genes and imply that a modified regulator would be formed between *A* and *a* products during formation of the ascogenous cells. In the first model, in which the mating-type structure of pseudohomothallic and true homothallic species was considered, it was postulated that the mating type products would be transcribed at a higher rate after nutrient starvation leading to sexual development (Metzenberg and Glass, 1990); higher expression of mating-type genes would then activate transcription of other target genes, necessary for completion of ascosporogenesis. The second model (Glass and Staben, 1990) postulates that, in a manner similar to yeast, *A* and *a* mating-types would regulate the production of cell-specific products during vegetative growth. In the

sexual cycle, nitrogen deprivation induces female differentiation genes and female specific genes. Fusion of opposite nuclei would then allow the formation of a novel regulatory element which would, in turn, activate perithecium differentiation genes and genes that regulate meiosis and ascospore formation. Mating type-associated incompatibility would be triggered during vegetative growth if a complex between *A* and *a* proteins activates incompatibility genes such as *tol* (Newmeyer, 1970). The elements of mating and heterokaryon incompatibility in *N. crassa* need to be investigated further before a more detailed model can be formulated.

1.3.3.6 Mating-type in *Podospora anserina*

P. anserina is closely related to *Neurospora* and exists as pseudohomothallic or heterothallic mycelia (Esser, 1974). Crosses produce ascospores with two opposite mating-type nuclei or with only a single nucleus. The *mat-* was cloned by cross-hybridisation with *N. crassa A* sequences; the opposite mating-type, *mat+*, was isolated by hybridisation between the *P. anserina mat-* flanking sequences (Picard *et al.,* 1991). Sequencing analyses showed structural and sequence similarities between genes encoded by the *N. crassa* and *P. anserina* idiomorphs (Figure 1-5 and Figure 1-6) (Debuchy and Coppin, 1992). Unlike *N. crassa*, *P. anserina* mating-type genes do not confer heterokaryon incompatibility even if transformed into *N. crassa* (Arnaise *et al.,* 1993). A gene from the *mat+* idiomorph, *FPR1* (coding for an HMG-protein), was shown to be 56% identical to *mt a-1* while an *mt A-1* homolog (*FMR1*) has 62% identity to the *N. crassa* gene (Debuchy and Coppin, 1992). A more detailed study of the *mat-* idiomorph revealed the presence of two additional genes, *SMR1* and *SMR2* that are involved in post-fertilisation functions such as sporulation (Debuchy *et al.,* 1993). Data base searches with *SMR1* sequences did not show any significant similarity to known genes or protein domains; however, analyses of the *N. crassa A* idiomorph revealed a sequence 5' of the *mt A-1* coding region that

had homology with *SMR1*. *SMR2* encodes an HMG-domain protein. Analyses using the two-hybrid system showed that the carboxy terminal moiety of FMR1 interacts with SMR2 (R. Debuchy, pers. comm.).

A study involving the reciprocal introduction of mating-type genes in *P. anserina* and *N. crassa* was undertaken to test whether structural similarities in mating-types also reflect functional correspondence (Arnaise *et al.*, 1993). These results indicated that the *N. crassa* and *P. anserina* mating-type idiomorphs are interchangeable in relation to mating reactions but not for heterokaryon incompatibility or post-fertilisation functions. Post-fertilisation functions can be restored in the *P. anserina mat+* deletion strain even with ectopic copies of *mat+* or *mat-* (Coppin *et al.*, 1993). It is possible that ectopically positioned *N. crassa A* and *a* idiomorphs can

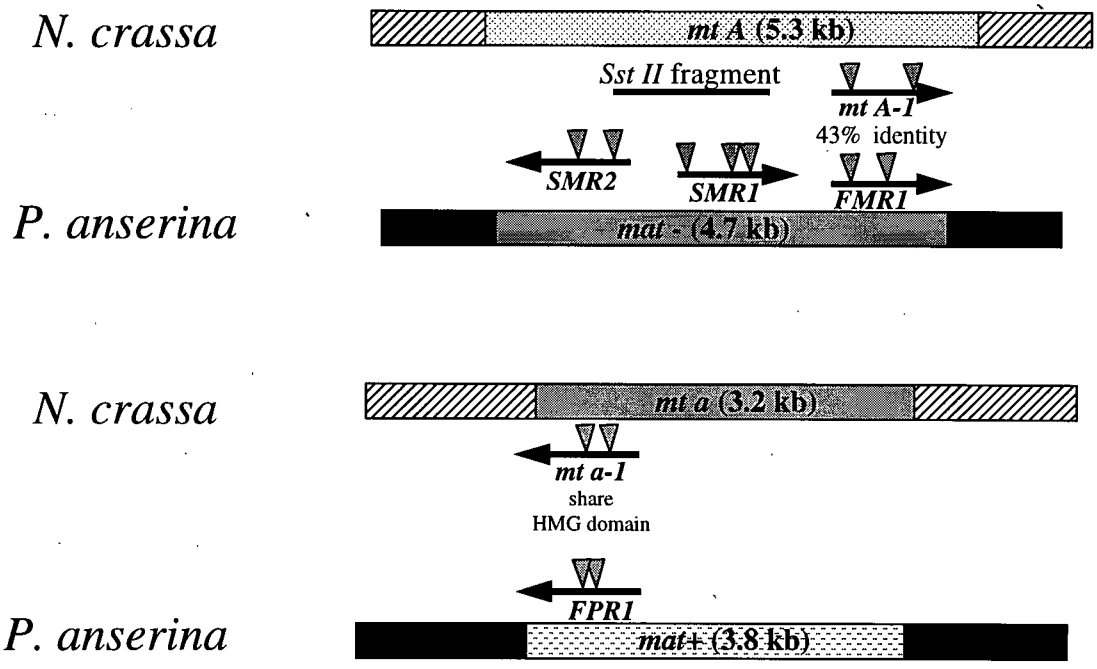


Figure 1-5 *N. crassa* and *P. anserina* mating-type structure. Arrows represent genes identified and orientation of transcription. Triangles represent intron positions. Position of the *Sst* II fragment in the *mt A* is represented by a bar on the left of *mt A-1*.

not rescue post-fertilisation functions in *P. anserina* as do *mat-* and *mat+* (Arnaise *et al.,* 1993).

Transformation of a strain deleted for *mat+* (Coppin *et al.,* 1993) with clones containing *mat-* or *mat+* idiomorphs mutated *in vitro* at each gene, provided important information about the function of *P. anserina* mating-type genes (Zickler *et al.,* 1995). In crosses of each mutant to the wild-type strain, reduced numbers of ascospores were observed including the presence of uniparental progeny and uninuclear croziers. It was suggested from this study that mating-type genes in *P. anserina* are essential for distinguishing self from non-self during early steps of sexual reproduction; after cellularisation of two opposite mating-type nuclei the *mat* information seems to be dispensable.

1.3.3.7 Mating-type in *Cochliobolus heterostrophus*

Cochliobolus heterostrophus is a pathogenic ascomycete that infects corn leaves. *C. heterostrophus* is heterothallic and mating is controlled by the MAT-1 and MAT-2 idiomorphs (Yoder, 1980). Unlike *P. anserina* mating-type sequences, *C. heterostrophus* mating-type genes do not hybridise to *N. crassa* idiomorphs even under low stringency conditions (Turgeon *et al.,* 1993). Isolation of MAT-1 genes was based on the change from the uni-mating phenotype to dual-mating upon transformation of a MAT-2 strain with a cosmid library from a MAT-1 strain. The opposite mating-type was isolated after screening of a cosmid library with the MAT-1 clone. The *C. heterostrophus* idiomorphs are considerably smaller than the *P. anserina* and *N. crassa* counterparts. A single ORF was characterised in each idiomorph, *MAT-1* and *MAT-2* (Turgeon *et al.,* 1993). The predicted MAT-1 and MAT-2 proteins have regions of similarity with MT A-1/FMR1 and MT a-1/FPR1 (Figure 1-6), corresponding to the α-box and HMG domains, respectively. Furthermore, *MAT*-specific homologs are functionally interchangeable in relation to

mating identity among species of *Cochliobolus, Neurospora* and *Podospora* (Christiansen, 1993; Arnaise *et al.,* 1993). By hybridisation with the *C. heterostrophus* mating-type sequences, it was shown that several other heterothallic *Cochiobolus sp.* have either MAT-1 or MAT-2 homologs and two homothallic species hybridised to both idiomorphs (Turgeon *et al.,* 1995). Interestingly, an asexual species, *Bipolaris sacchari,* related to *C. heterostrophus*, contains a *MAT-2* homolog that confered dual mating when transformed into a MAT-1 *C. heterostrophus* strain (Sharon *et al.,* 1996). It seems that *B. sacchari* lacks genes other than *MAT* genes that are important for the sexual cycle because it fails to cross even when carrying transgenic *MAT-1* or *MAT-2* from *C. heterostrophus.* The *MAT-1* and *MAT-2* genes of *C. heterostrophus* are believed to be required for fertilisation but it is not known if they are needed for post-fertilisation events (Turgeon *et al.,* 1993). Idiomorphic regions that correspond to the sporulation control region of *P. anserina* (and *N. crassa*) are absent in *C. heterostrophus*; thus if genes controlling post-fertilisation functions work the same way in this ascomycete, they must be present in locations other than at the mating-type locus.

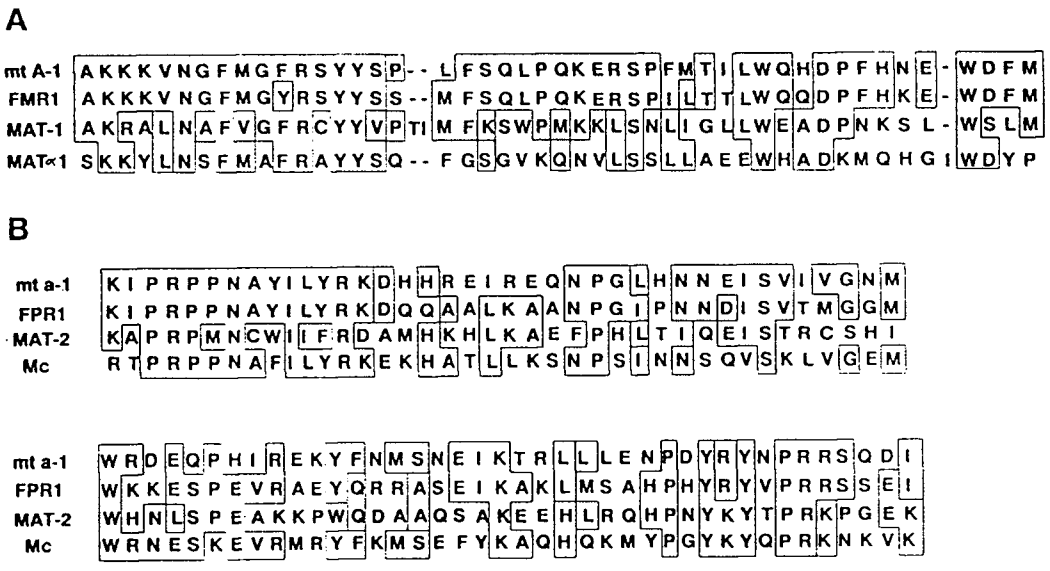


Figure 1-6 Comparison of regions of similar amino acid sequence between mating-type proteins of ascomycetes. Panel A shows *N.crassa* MT A-1, *P. anserina* FMR1 and *C. heterostrophus* MAT-1 as compared to the *S. cerevisiae* α-box. Panel B shows a comparison of four HMG-domain mating-type proteins from *N. crassa* (MTa-1), *P. anserina* (FPR1), *C. heterostrophus* (MAT-2) and S. pombe (Mc). Regions of amino acid identity are boxed. Reproduced from Glass and Nelson, 1994.

1.4 THESIS ORGANISATION

Chapter 2 describes the molecular characterisation of *mt A-2* and *mt A-3,* two additional genes of the *N. crassa mt A* idiomorph.

In Chapter 3, I describe the isolation of *mt A-2* and *mt A-3* specific mutants and their functional characterisation.

In Chapter 4 the involvement of the four *N. crassa* mating-type genes in sexual development is investigated by the analyses of genes that are preferentially expressed under crossing conditions.

2. Characterisation of *mt A-2* and *mt A-3* mating-type genes of *Neurospora crassa*

Some experiments in this chapter were done in collaboration with Dr. Sven Saupe (presently at Institut de Biologie, CNRS, Mont Pellier, France) as denoted in the Materials and Methods section. Most of the results described herein are published in Ferreira *et al.* (1996).

2.1 INTRODUCTION

Initial mutational analyses of the *Neurospora crassa A* and *a* idiomorphs identified mutants that had lost mating identity and heterokaryon incompatibility, simultaneously (Griffiths and De Lange, 1978; Griffiths, 1982). Cloning and molecular analyses of the mating-type locus showed that the regions mutated in the *A* and *a* idiomorphs previously, contained two genes, *mt A-1* and *mt a-1,* respectively, of approximately 1 kb each (Glass *et al.,* 1990; Staben and Yanofsky, 1990) that conferred both mating identity and heterokaryon incompatibility.

By a gene replacement of the *A* idiomorph with *mt a-1* sequences, it was shown that *mt a-1* is necessary and sufficient to confer all *a* functions during vegetative and sexual phases (Chang and Staben, 1994). Subsequent mutational analyses of the *A* idiomorph showed that there was a second functional region in addition to that encoded by *mt A-1* (Glass and Lee, 1992). A 3 kb *Sst* II fragment in the 4 kb region centromere distal to the *mt A-1* ORF (see Figure 1-4, Chapter 1) was transformed into *N. crassa A* spheroplasts. Progeny from transformants containing duplicated sequences are prone to G:C to A:T changes due to repeat-induced point mutation (RIP) (Selker *et al.,* 1989-see Chapter 3). A single mutant was isolated by this method and it showed methylation and RFLP changes typical of RIP in southern blot analyses using the 3 kb *SstII* fragment as a probe. This mutant (A^{IIRIP}) was morphologically indistinguishable from wild-type during vegetative growth and could participate in a cross as either a female or male parent. However, the development of perithecia was arrested 3-4 days post-fertilisation. A very limited

number of croziers was observed and only a small number of perithecia contained a few mature ascospores. Analyses of A^{IIRIP} progeny showed that *a* and the A^{IIRIP} mutant phenotype segregated 1:1, demonstrating that there was at least one gene in addition to *mt A-1* in the *A* idiomorph that had a post-fertilisation function (Glass and Lee, 1992). In addition to the genetic data suggesting the existence of additional genes in the *A* idiomorph, two mRNAs from the 4 kb region were detected from a strain grown in crossing media (Glass and Lee, 1992).

N. crassa and *P. anserina* are very closely related but yet maintain differences in vegetative growth, mating strategies and spore formation. It will be useful to investigate the similarities and differences between mating-type structures of the various filamentous ascomycetes in order to understand evolution of mating strategies. The conservation of structure and function of mating-type genes in different groups would bring about answers to questions such as the role of sexual reproduction, especially in pseudohomothallic and homothallic species which are self-fertile. The *mt A-1* and *mt a-1* mating-type genes of *N. crassa* seem to be conserved, at least functionally, within ascomycetes (Debuchy *et al.,* 1993; Turgeon *et al.,* 1993), however it is not known whether other regions of the mating-type are conserved. Characterisation of the 4 kb centromere-distal region *N. crassa* will help to answer this question. In this chapter, I describe the structural and transcriptional characterisation of two genes, *mt A-2* and *mt A-3,* in the *N. crassa A* idiomorph which are involved in the ascosporogenesis.

2.2 MATERIALS AND METHODS

2.2.1 Strains and media

The *N. crassa* strain 74-OR23-1V *A* (Fungal Genetics Stock Center -FGSC 2489) and *inl, fl A* (FGSC 3631) were used for RNA isolation. They were grown on solid vegetative (Vogel's) and crossing media (Westergaard's) as described in Davis and De Serres (1970) in Petri dishes layered with Miracloth (Calbiochem, La Jolla, CA) to facilitate removal of mycelia and perithecia. Strain OR 8-1 *a* (FGSC 532) was used as the male in crosses. *Escherichia coli* DH5α (Hanahan, 1983) was used as the host strain for plasmid amplification.

2.2.2 Transcript Analyses

2.2.2.1 Northern Blotting

Total RNA was prepared according to Logemann *et al.* (1987) a method based on guanidine-HCl and phenol: chloroform extraction. The OligoTex mRNA kit (QIAGEN, Chatsworth, CA) was used to enrich poly(A)$^+$ RNA for Northern Blots. Gel electrophoresis and transfer of RNA to nylon filters (Nytran+, Schleicher & Schuell, Keene, NH) was done according to Fourney *et al.* (1988). Two μg of poly(A)$^+$ RNA were loaded per lane. DNA probes were labelled with [^{32}P]dCTP using the T7 Quickprime kit (Pharmacia, Baie d'Urfe, PQ).

2.2.2.2 Reverse Transcriptase-PCR

For the Reverse Transcriptase-PCR (RT-PCR), cDNAs were synthesised from 10 μg total RNA using random hexamers as primers (First Strand cDNA Synthesis kit, Pharmacia). One to 5 μl of the reverse transcriptase reaction was used in a 100 μl PCR reaction containing 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 200 μM each dNTP, 2.5U Taq polymerase (Perkin & Elmer, Mississauga, ON) and 0.4 μM each primer (designed based on previous sequencing of *mt* A-

Glass *et al.,* 1990). For cDNA amplifications, primers rI.1 and rI.2 were used for *mt A-2* and, rII.1 and rII.2 for *mt A-3* (see Appendix 1 for primer sequences). PCR was performed in a Perkin-Elmer model 480 DNA thermocycler using 3 cycles of 95°C for 1.5 min., 55°C for 1min., 72°C for 1min followed by 30 cycles of 95°C for 1 min., 60°C for 1min., 72°C for 1min, with 10 sec. of auto extension. PCR products were gel purified and cloned into pGEM-3Zf(-) (Promega, Madison, WI) cut with *Sma* I and T-tailed with *Taq* polymerase as described by Marchuk *et al.,* (1991).

2.2.2.3 RACE (Random Amplified cDNA Ends)

RACE experiments were performed by Dr. Sven Saupe as described by Frohmann *et al.,* (1988). In brief, cDNA was synthesised from 1µg of poly(A)$^+$ RNA using a *NotI*-d(T)18 primer (First-Strand cDNA Synthesis Kit, Pharmacia) and gene specific primers for 3' and 5' RACE experiments, respectively (see Appendix 1 for primer sequences). One µl of a 10-fold dilution of the RT reaction was used in PCR amplifications. Re-amplifications contained 1 µl of a 100-fold dilution of the first PCR.

The 3' RACE PCR was done with an 18 base adapter primer (identical to a region in the 5' part of the *Not* I-d(T)18 primer) and a gene specific primer, 2126-2142 for *mt A-2* and 1454-1433 for *mt A-3* (numbered according to *mt A* sequence deposited in Gen Bank, accession number M33876). Reamplification was performed using the 18 base adapter primer and a second gene-specific primer, 3105-3121 for *mt A-2* and 34-18 for *mt A-3* .

Gene-specific primers for the 5' RT reaction were 3003-2984 for *mt A-2* and 233-249 for *mt A-3*. After purification of cDNA in Centrisep spin columns (Princeton Separations, Adelphia, NJ) a poly(A) tail was added using terminal transferase (Boehringer Mannheim, Laval, PQ). PCR

was done with the *NotI*-d(T)18 primer and a second nested primer, 2423-2406 for *mt A-2* and 630-650 for *mt A-3* . PCR reamplification was with the 18 base adapter primer and a third nested gene-specific primer, 2196-2179 for *mt A-2* and 1417-1434 for *mt A-3*.

A second polyadenylation site for the *mt A-3* transcript was identified by a modification of the RACE protocol. The first 3' RACE PCR reaction was incubated for 16 hours at 65°C in 1 ml of hybridisation buffer (5X SSC, 0.5% SDS, 5X Denhardt's solution and 0.1 mg/ml of salmon sperm DNA) with a $5mm^2$ nylon membrane on which 0.5 µg of plasmid DNA containing genomic mating-type sequence had been spotted. The membrane was washed 2 times in 0.1X SSC, 0.1% SDS at 65°C and 2 times with 0.1X SSC at 65°C. Mating-type specific products were recovered by washing the membrane with 100 µl of water for 5 min. at 95° C. Five µl of this reaction was amplified using the 858-842 *mt A-3* primer and the 18 base adapter primer.

RACE products were cloned into the pCRII vector (Invitrogen, San Diego, CA). For each 5' RACE experiment (except for the distal *mt A-2* start site) the DNA sequence of 2 to 3 individual clones was determined. In some experiments (the distal *mt A-3* and the proximal *mt A-2* start sites), different clones showed slightly different 5' ends (not more than 10 bp apart).

2.2.3 DNA Sequence Analyses

Automated DNA sequencing was performed using the ABI Taq DyeDeoxy Terminator cycle method (Mississauga, ON) at the NAPS Unit, Biotech Lab, UBC. Computer sequence analyses were done using the GCG package (Genetics Computer Group, University of Wisconsin, WI, Devereaux *et al.,* 1984).

2.2.4 Molecular Biology Techniques

Standard molecular biology procedures were as in Sambrook *et al.* (1989).

2.3 RESULTS

2.3.1 Isolation of *mt A-2* and *mt A-3* cDNAs

Two uncharacterised transcripts within the *A* idiomorph in addition to *mt A-1* mRNA were previously reported (Glass and Lee, 1992). The A^{IIRIP} mutant, which produces a very small number of ascospores, was mutated in the region containing these two transcripts. This result suggested that the 4 kb region of the *A* idiomorph centromere distal to *mt A-1* encoded additional gene products that are important to sexual reproduction in *N. crassa*. Based on transcript analyses of the *A* idiomorph and DNA sequence comparisons with *SMR1* and *SMR2,* (Debuchy *et al.,* 1993) DNA primers were designed to amplify cDNAs by RT-PCR from the 4.0 kb centromere-distal region of the *N. crassa A* idiomorph. Two cDNAs, *mt A-2* and *mt A-3*, of approximately 1.1 kb were recovered by RT-PCR amplification with primer pairs rI.1/rI.2 and rII.1/rII.2, respectively (see sections 2.3.3 and 2.3.7 for cDNA sequence analyses). The *mt A-2* and *mt A-3* cDNAs differed in size from their PCR-amplified genomic DNA counterparts by approximately 200 bp, corresponding to introns. DNA sequence analysis of the *mt A-2* and *mt A-3* cDNAs showed that the direction of transcription of *mt A-2* is towards the centromere (and in the same direction as *mt A-1*), while the direction of transcription of *mt A-3* is towards the telomere (see section 2.3.5 and Figure 2-4).

2.3.2 Expression analyses of *mt A-2*

The expression of *mt A-2* was examined during both vegetative and sexual reproduction in *N. crassa* by Northern analyses and RT-PCR. A 1.6 kb *mt A-2* transcript could be detected in poly(A)$^+$ RNA from an *A* strain grown in vegetative media, crossing media and from perithecia five days post-fertilisation (Figure 2-1). In a cross, five days after fertilisation, perithecia contain numerous croziers and developing asci with immature ascospores. A 1.5 kb mRNA was also

detected in some *mt A-2* Northern blots (data not shown) that may correspond to transcripts originating from the proximal transcriptional start site (see section 2.2.2.3). The *mt A-2* cDNA was detected by RT-PCR from RNA isolated from a 10 day-old vegetative culture of an *A* strain as well as from RNA isolated from a cross 2, 4, 6, 8 and 10 days post-fertilisation (data not shown).

2.3.3 Analyses of *mt A-2* cDNA

The sequence of the entire *mt A-2* cDNA was determined and compared to the genomic sequence; the *mt A-2* DNA and translated protein sequences are shown in Figure 2-2. The two possible initiator codons of MT A-2 had 7 out of 11 nucleotides that matched the *N. crassa* consensus for translation initiation site - CAC/AC/AATGGCT--C (Edelman and Staben, 1994); the ATG that yielded the longest ORF was chosen. Four introns are spliced from the *mt A-2* mRNA. Three introns cluster close to the 3' end of *mt A-2* and exhibit 5', 3' and internal branch

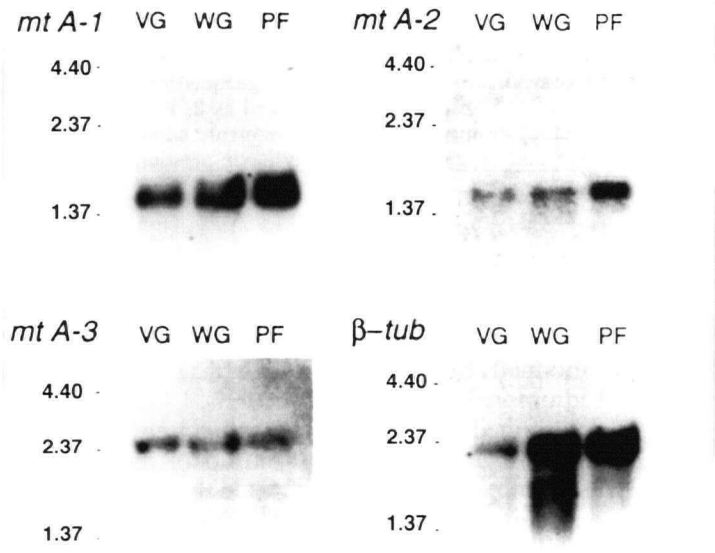


Figure 2-1 Northern Blot analyses showing expression of *mt A-2* and *mt A-3*. Poly(A)$^+$ RNA was isolated from cultures grown for 5 days on solid vegetative (VG for Vogel's) and crossing (WG for Westergaard's) media and from perithecia 5 days after fertilisation (PF for post-fertilisation). Probes were synthesised from cDNA PCR fragments for *mt A-2* and *mt A-3*. RNA size standards are given in kb to the left of each panel. Probes synthesised from cloned *mt A-1* and β–tubulin genes were used as controls for amount of RNA/lane.

point splicing sequences that correspond to those described for other *N. crassa* genes (Edelman and Staben, 1994). The first intron of *mt A-2* is an exception to the consensus as its 5' donor site is 5'G/GTAGCA 3'. The *mt A-2* cDNA would encode a polypeptide of 373 amino acids. The isoelectric point of the polypeptide was calculated to be 6.6 with an N-terminal basic region (from aa 1 to 250, pHi 9.6) and an acidic C-terminal region (aa 251 to 373, pHi 4.5). Database searches with the translated *mt A-2* ORF using BLAST (Altschul *et al.*, 1990) failed to show significant similarities between the *mt A-2* putative peptide and any protein domain, with the exception of the *SMR1* ORF of *P. anserina*.

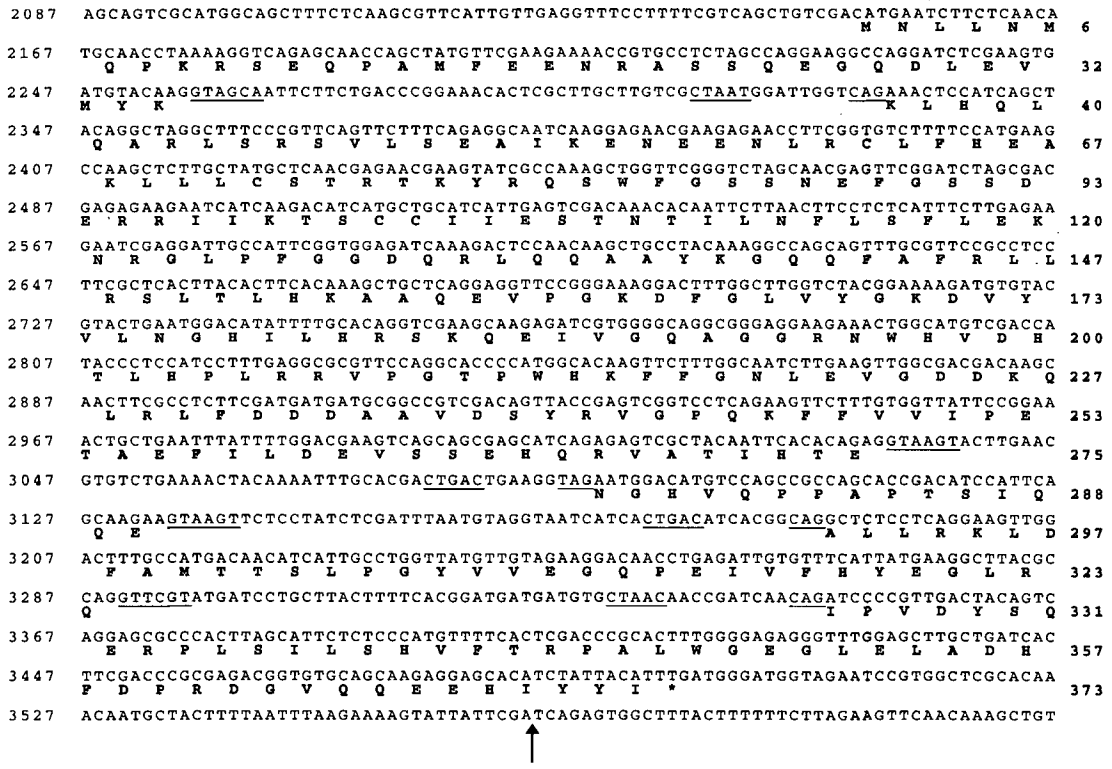


Figure 2-2 DNA sequence of the *mt A-2* gene. The DNA sequence is numbered on the left, according to the revised sequence deposited in GenBank (Accession no. M33876). The deducted polypeptide sequence (numbered on the right) is shown in bold under the DNA sequence. Intron splicing consensus sequences are underlined. Arrow indicates polyadenylation site.

The *mt A-2* and *SMR1* ORFs show 24% amino acid identity and 45% similarity (Figure 2-3) with five gaps in the alignment of the two ORFs. A six amino acid gap in the alignment (aa 79

to 85 in MT A-2) corresponds to a 18 bp duplication in the *mt A-2* DNA sequence (nt 2454-2489). Two of the intron locations are conserved between *mt A-2* and *SMR1*. There is a 18 aa region of 82% identity between the two polypeptides (amino acids 203-220, MT A-2; Figure 2-3). This region has been proposed to be a novel DNA-binding domain and a second region of 20 amino acids, that can potentially form an acidic amphipathic α-helix (aa 156-176 in SMR1), was proposed to act as the transactivation domain (Debuchy *et al.,* 1993). Acidic amphipathic α-helices are common feature of transcriptional activators (Giniger and Ptashne, 1987).

The region proposed as an amphipathic α-helix in SMR1 (aa 156-176) (Debuchy *et al.,* 1993) corresponds to a gap in the alignment with MT A-2 (Figure 2-3). However, this amino acid motif was found to be 47% similar to an amino acid sequence in the amino terminal end of MT A-2 (aa 24-42), which is contained in the first gap in the comparison of the two ORFs. A sequence in the carboxyl terminus of MT A-2 (310-329) is 60% similar to the 20 aa region. Both sequences in the MT A-2 polypeptide can be drawn as amphipathic α-helices (PCGene, Intelligenetics Inc.) and may correspond to transactivation domains of MT A-2, even though they do not have a strong acidic character. Alternatively, it is possible that MT A-2 lacks this putative transactivation domain and is functionally different from SMR1.

An alternative possibility is that MT A-2 and SMR1 may function as transcriptional repressors as opposed to activators. Repressor motifs are, as with activation domains, difficult to categorise. Some repression regions appear to be unique and repression may depend on multiple targets (reviewed in Hanna-Rose and Hansen, 1996). MT A-2 and SMR1 both have a glutamine- and alanine-rich region (aa 130-157 and 87-125, respectively). The alanine- and glutamine-rich motif is a non-DNA-binding domain that is well characterised in the Dr1 protein of *Drosophila* (Yeung *et al.,* 1994) which prevents formation of the transcription initiation complex.

2.3.4 Characterisation of the *mt A-2* transcript ends

To determine the transcriptional start and stop sites of *mt A-2*, 5' and 3' RACE experiments were performed with mRNA isolated from mycelia from an *A* mating-type strain grown on crossing medium.

Two different transcriptional start sites were identified for *mt A-2*, at positions 1852 and 2084 of the *mt A* sequence (Figure 2-5). The polyadenylation site of the *mt A-2* transcript was mapped to position 3566, 69 bp 3' of the *mt A-2* stop codon (Figure 2-2). A putative polyadenylation signal sequence AAGAAA is found 15 bp 5' to the polyadenylation site. Poly(A) tails are typically 25 to 75 bp in *N. crassa* (Sachs and Yanofsky, 1991) and therefore the

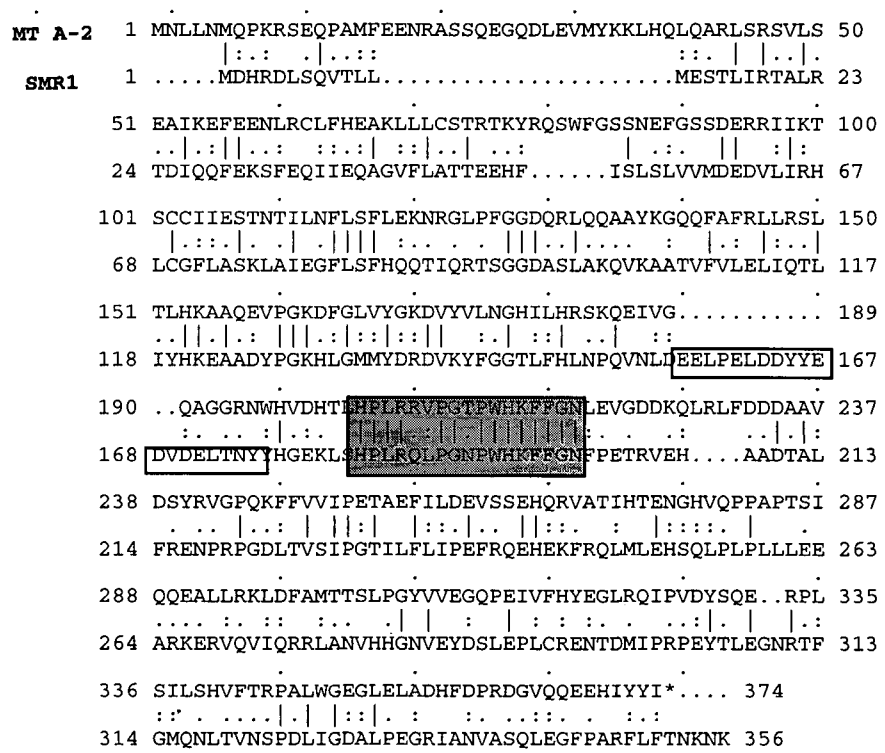


Figure 2-3 Comparison of the deduced amino acid sequence of MT A-2 and SMR1. The alignment was done with the GAP program of the GCG package (Devereux *et al.*, 1984). The region of greatest similarity between the two polypeptides is shaded. The hypothetical acidic amphipathic alpha-helix region of SMR1 polypeptide is boxed.

calculated sizes of the two *mt A-2* transcripts are approximately 1540 bp for the distal start site and 1310 bp for the proximal start site. Based on the size of the mRNA transcript detected in Northern analyses (1.6 kb, Figure 2-1), the distal transcriptional start site for *mt A-2* is preferred. A 1.5 kb mRNA has been detected on some *mt A-2* Northern blots (not shown) that may correspond to transcripts originating from the proximal transcriptional start site.

The distal transcriptional start site for *mt A-2* (1852) has a CAAT box (5'-CAAAT-3'), (Bruchez *et al.,* 1993), 82 bp 5' to the start site (Figure 2-4 and Figure 2-5). Three small autonomous ORFs (coding for peptides of 22, 29 and 9 amino acids in length - Figure 2-5 and Figure 2-6) are present in the 5' leader region before the proposed ATG for *mt A-2*. *P. anserina SMR1* also contains 3 ORFs 5' of the proposed translation start (Figure 2-6). One of the *P. anserina* upstream ORFs (uORFs) is out-of-frame with the proposed translation site and overlaps with the main ORF. Comparison of the translated *N. crassa* and *P. anserina* uORFs do not show similarity (Figure 2-6) except for a few amino acid repeats, such as a pair of serines that appears in one of the *P. anserina* uORFs and in all uORFs of *mt A-2*. As in one of the *mt A-2* uORFs, the first methionine of the shortest *SMR1* uORF is followed by an asparagine. Sequences corresponding to transcriptional start sites such as CAAT or TATA boxes were not identified in the region near the proximal start site (2084). An ATG is located 15 bp from the proximal start site, but is unlikely to function as a translational start site; ATGs located less than 40 nucleotides from transcriptional start sites are not thought to be used as translational start sites (Kozak, 1991).

2.3.5 Promoter features

The distal start sites for the *mt A-2* and *mt A-3* transcripts (see sections 2.3.7 and 2.3.8) are separated by only 70 bp and therefore *mt A-2* and *mt A-3* may have overlapping promoters (Figure 2-4 and Figure 2-5). The 70 bp fragment that separates the two start sites contains repeated sequences. A 50 bp sequence is repeated three times with 64% identity, with a region of 15 bp that is perfectly conserved (Figure 2-5). The first complete repeated unit is located exactly between the distal start sites of *mt A-3* (see section 2.3.8) and *mt A-2* (see above). Seven additional repeats of part of the 15 bp unit are present in the region between *mt A-2, mt A-3* and also in the *mt A-2* ORF (not shown).

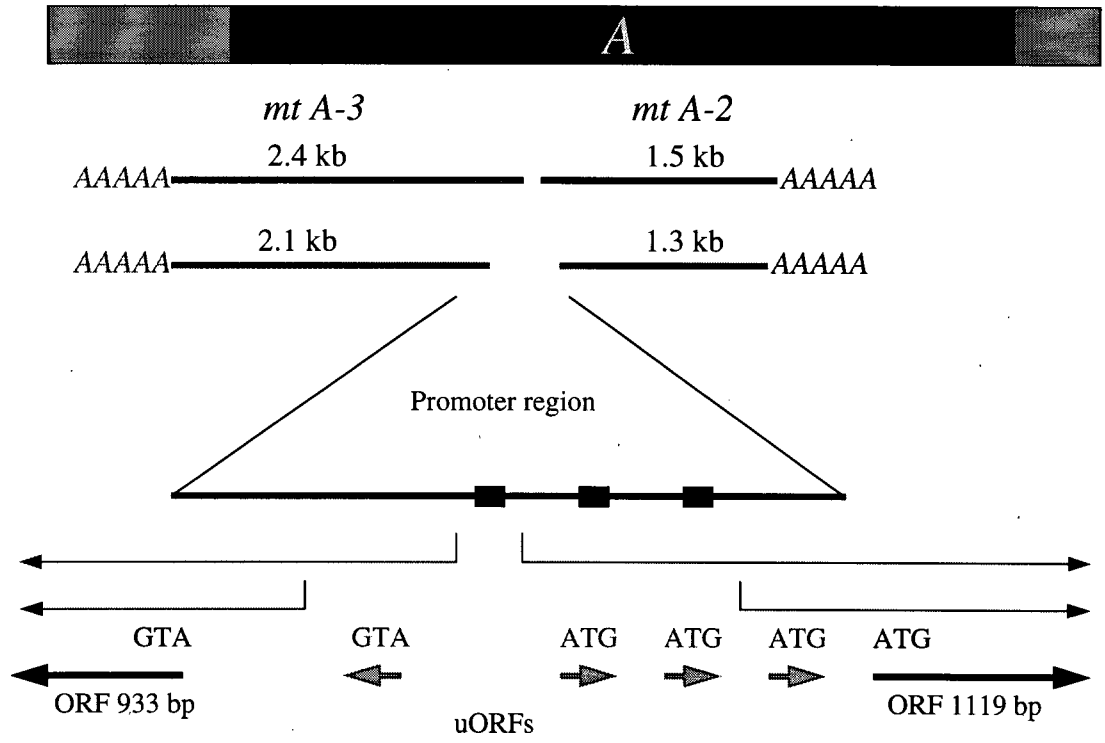


Figure 2-4 Diagrammatic representation of the *mt A-2* and *mt A-3* transcripts. Promoter region with repeats (boxes) and positions of uORFs (grey arrows) is shown in detail.

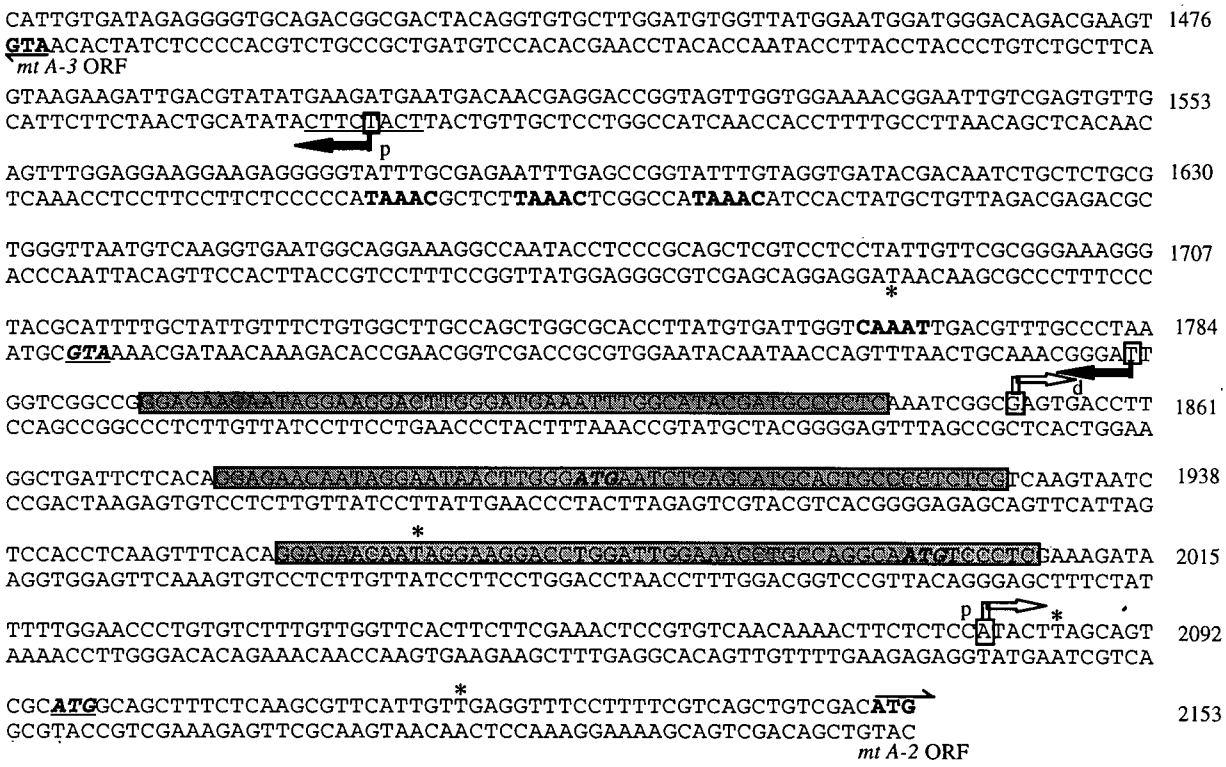


Figure 2-5 Positions of the transcriptional start sites of *mt A-2* and *mt A-3*. The DNA sequence between the proposed start codons of *mt A-2* and *mt A-3* is given. The arrows (in grey for *mt A-2* and black for *mt A-3*) indicate the positions of the proximal (p) and distal (d) transcriptional start sites. The putative CAAT box sequences are shown in bold type. The repeated sequences are shown by the stippled boxes. ATG codons upstream of the proposed translational sites are shown as bold italic characters and asterisk indicate stop codons.

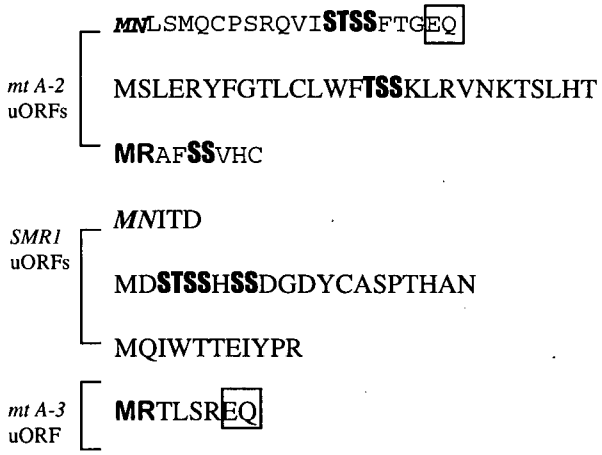


Figure 2-6 uORFs of *mt A-2* and mt A-3. Two or more adjacent amino acids common in the uORFs are represent by different fonts or they are boxed.

2.3.6 Expression analyses of *mt A-3*

Northern and RT-PCR analyses were carried out to examine the expression of *mt A-3* during both vegetative and sexual reproduction. A 2.4 kb *mt A-3* mRNA could be detected in poly(A)$^+$ from an *A* strain grown in vegetative and crossing media and in perithecial RNA five days post-fertilisation (Figure 2-1). As with the *mt A-2,* the *mt A-3* cDNAs were detected by RT-PCR from RNA isolated from a 10 day-old vegetative culture of an *A* strain as well as from RNA isolated from a cross 2, 4, 6, 8 and 10 days post-fertilisation (data not shown)

2.3.7 Analyses of *mt A-3* cDNA

The *mt A-3* DNA sequence and predicted 311 amino acid polypeptide are shown in Figure 2-7. The proposed initiator codon matches the *N. crassa* consensus in 9 out of 11 nucleotides. Three introns were identified by sequencing of the *mt A-3* cDNA; all three introns show consensus splice sites typical of *N. crassa* genes. The isoelectric point of the putative MT A-3 is 4.3, although the polypeptide contains a stretch of basic amino acids (aa 146-215) that corresponds to an HMG DNA-binding domain (Figure 2-8 and Figure 2-10) (Grosschedl *et al.,* 1994). The carboxyl terminus of MT A-3 is rich in acidic amino acids and proline residues (Figure 2-8). A sequence of amino acids (93 to 105) in the *mt A-3* ORF contains a "PEST" domain (sequence of proline, glutamic acid, serine and threonine); proteins containing these sequences have very short half-lives and are usually rapidly degraded (within 30 minutes) (Rechsteiner *et al.,* 1987).

Database searches identified similarity to many HMG polypeptides including the MT a-1 of *N. crassa* (Staben and Yanofsky, 1990), the *P. anserina* SMR2 and FPR1 mating-type proteins (Debuchy *et al.,* 1993), the *Schizosaccharomyces pombe* Mc polypeptide (Kelly *et al.,* 1988) and the mouse *SRY* gene product (Sinclair *et al.,* 1990). Comparison of the predicted amino acid

sequence of MT A-3 with the *P. anserina* SMR2 showed 22% identity and 46% similarity (Figure 2-8) with 7 gaps in the alignment, mostly very small. The region of significant similarity is restricted to the HMG domain. Amino acid comparisons of MT A-3 with MT a-1 and FPR1 showed ~ 50% amino acid similarity but the number of gaps were greater than in comparisons with the SMR2 polypeptide (see comparison between MT A-3 and MT a-1 in Figure 2-9). The region of greatest similarity between MT A-3, MT a-1, FMR1 and SMR2 is restricted to the HMG box. Figure 2-10 shows HMG sequences of the mating-type proteins as compared to the HMG consensus sequence. In MT A-3 and SMR2, an intron is present in exactly the same location within the DNA sequence encoding the HMG domain (after the sequences for the conserved isoleucine and serine) while an intron occurs in the middle of these sequences in *mt a-1* and *FPR1*, suggesting two different origins for the two HMG-domain mating-type polypeptides. A leucine is inserted in the MT A-3 sequence at position 69 of the 70 aa consensus HMG domain sequence (Figure 2-10). Another difference in the MT A-3 sequence compared to the other HMG proteins is the proline rich fragment which appears after the HMG domain (Figure 2-8) which is absent in the other polypeptides. Sequence-specific HMG domain proteins generally contain conserved asparagine and histidine residues at positions 7 and 62 of the HMG consensus, respectively and a proline at position 67, but not at position 2 (Grosschedl *et al.*, 1994). An asparagine is present in position 7 and a proline at position 67 in all four HMG proteins of *N. crassa* and *P. anserina* suggesting they may all be sequence-specific HMG proteins. *E. coli* synthesised MT A-3 polypeptide is capable of binding specifically to the same DNA sequences that MT a-1 binds *in vitro* (Philley *et al.*, 1995; Philley and Staben, 1994).

Figure 2-7 DNA sequence of the *mt A-3* gene. The DNA sequence is numbered on the left, according to the revised sequence deposited in GenBank (Accession no. M33876). The deduced polypeptide sequence (numbered on the right) is shown in bold under the DNA sequence. Intron splicing consensus sequences are underlined.

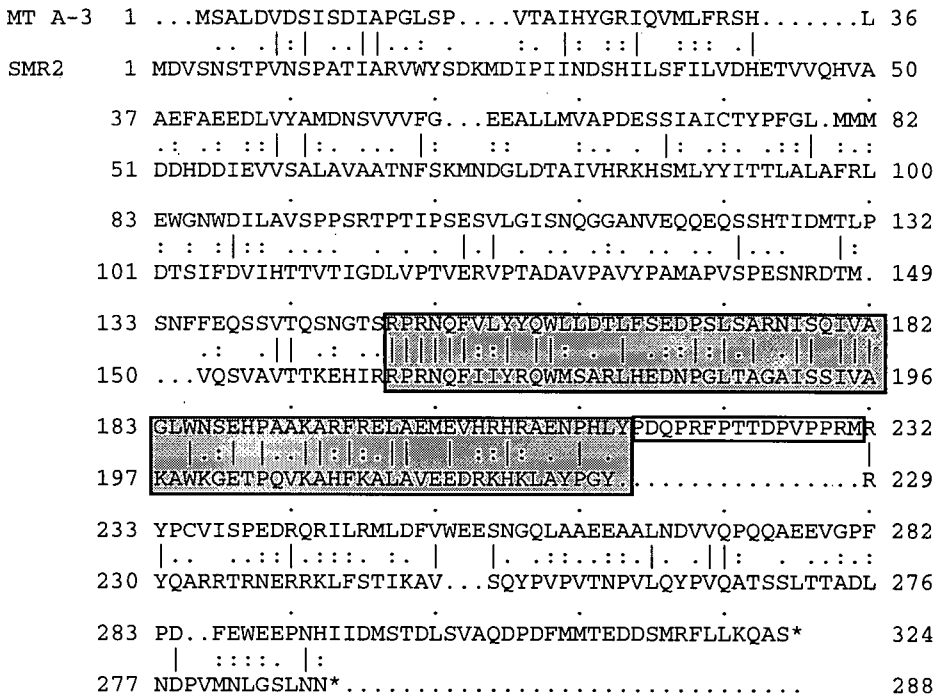


Figure 2-8 Comparison of the deduced amino acid sequence of MT A-3 and SMR2. The alignment was done with the GAP command of the GCG program (Devereux *et al.*, 1984). The HMG domain is shaded. A proline-rich stretch absent in SMR2 polypeptide is boxed.

2.3.8 Characterisation of the *mt A-3* transcript ends

The transcriptional start and stop sites for *mt A-3* mRNA were determined by 5' and 3' RACE experiments. As in *mt A-2*, two different transcriptional start sites were identified for *mt A-3* , at positions 1782 (distal) and 1501 (proximal) (Figure 2-5). Sequences corresponding to CAAT or TATA boxes or transcriptional start site consensus sequences were not present for the distal start site. In *mt A-3* , a short autonomous uORF of 9 amino acids is also present in the 5' leader sequence of the distal transcript (Figure 2-5). The translation of this short uORF shows that there are two pairs of amino acids (methionine followed by arginine in the beginning and glutamic acid followed by glutamine at the end) that are also present in *mt A-2* uORFs (Figure 2-6).

The proximal start site lies within a 5'TCATCTTC 3' sequence that matches the TCATCANC consensus sequence for transcriptional start sites in *N. crassa* (Bruchez *et al.*, 1993). Three CAAT boxes were found 76, 86 and 98 bp upstream of the proximal start site (Figure 2-5). The polyadenylation site of the *mt A-3* transcript was mapped at position -771 in the flank of the *A* idiomorph, 1075 bp from the stop codon of the *mt A-3* ORF (see diagram, Figure 2-4); no sequence matching the polyadenylation signal consensus sequence for *N. crassa* was present. The 3'UTR of the *mt A-3* transcript extends into the centromere-distal flanking sequences of the *A* idiomorph (Figure 2-4), which are homologous between *A* and *a* strains. Long 3' UTRs are not common and the mean length of 3' UTR for most mRNAs in *N. crassa* is approximately 200 bases (Bruchez *et al.*, 1993). The *mt a-1* transcript also has a 3' UTR that extends into the centromere distal flank sequence by approximately 1 kb (C. Staben, pers. comm.).

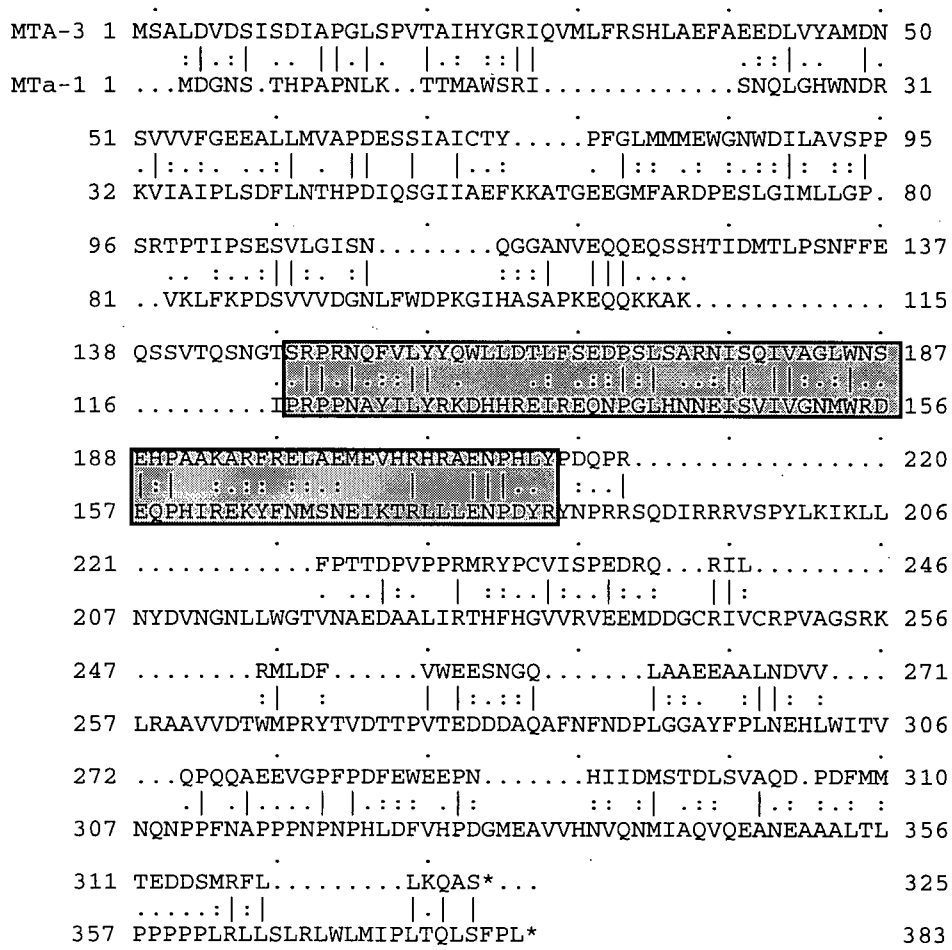


Figure 2-9 Amino acid comparison between MT A-3 and MT a-1. Proteins have 52% similarity and 25% identity with 16 gaps. HMG domain is shaded.

The calculated sizes of the *mt A-3* transcripts are 2395 (distal start site) and 2114 bases (proximal start site) from start to polyadenylation site. The size of the *mt A-3* transcript based on Northern analyses is 2.4 kb (see Figure 2-1). Therefore, assuming a normal poly(A) tail length of approximately 50 bases (Sachs and Yanofsky, 1991) for the *mt A-3* mRNA, the distal site is the preferred start for transcription.

Because of the presence of the unusually long 3' UTR of the *mt A-3* transcripts a 3' RACE experiment was designed to identify an alternate polyadenylation site for *mt A-3* (see section

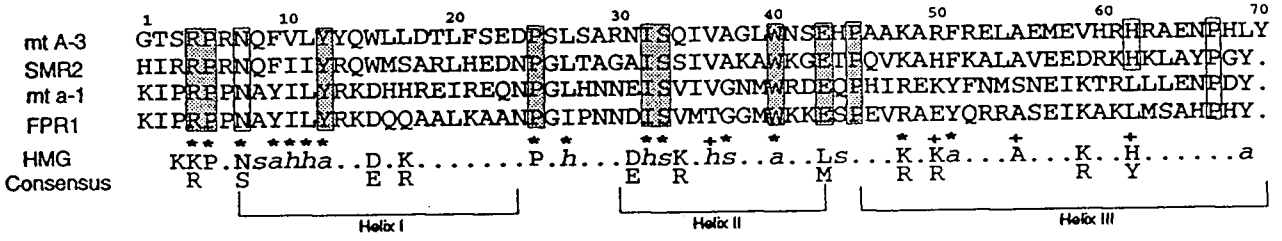


Figure 2-10 Comparison of HMG domains of four mating-type proteins. The *N. crassa* MT A-3 and mt a-1 and *P. anserina* SMR2 and FPR1 mating-type proteins contain very similar HMG boxes. The HMG consensus compiled from Bianchi *et al.,* (1992) and Grosschedl *et al.*, (1994) is shown in the bottom line. Amino acids that matched the consensus were marked with an asterisk while (+) indicates accordance of the MT A-3 sequence to the consensus. The italicised lower cases in the consensus sequences stands for: a- F, W, Y; h- M, V, L, I and s- P, A, G, S, T. Highlighted boxes represent highly conserved amino acids between the four mating-type sequences. Amino acids in empty boxes at positions 7, 62 and 67 are indicative of sequence-specific DNA binding HMG proteins.

2.2.2.3). DNA sequencing of the RACE product revealed a polyadenylation site at position 741 within the third intron of *mt A-3* (Figure 2-7). A putative polyadenylation signal sequence (AACAAA) was found 9 bp upstream of that site. Using a primer located within the third intron, a cDNA was amplified by RT-PCR in which the first two introns were spliced out. In this cDNA, the *mt A-3* ORF was interrupted by a stop codon in the beginning of the last intron. It encodes a putative polypeptide of 179 amino acids which is truncated in the middle of the HMG domain. The size of the mispliced mRNA from the distal and proximal start sites is 984 and 703 bases, respectively. A mRNA of this size was not detected in vegetative nor perithecial RNA by Northern analyses.

2.4 DISCUSSION

In this chapter, the characterisation of *mt A-2* and *mt A-3*, two genes encoded by the *A* mating-type locus of *N. crassa* was described. The structural features of the *mt A-2* and *mt A-3* gene products together with the phenotype of the A^{IIRIP} mutant, suggest they are transcriptional regulators that control the expression of genes necessary for post-fertilisation events leading to meiosis and ascospore formation. The *mt A-2* and *mt A-3* genes were found to be similar to the *P. anserina SMR1* and *SMR2* mating-type genes which have been shown to be both required for post-fertilisation events (Debuchy *et al.,* 1993).

The similarity between MT A-2 and SMR1 protein sequences suggests that *mt A-2* encodes an activator of transcription. The *P. anserina* SMR1 has been proposed to contain a novel DNA binding motif and a transactivating domain (Debuchy *et al.,* 1993). It is possible, however that MT A-2 and SMR1 may function as transcriptional repressors as opposed to activators based on the fact that they have a small glutamine- and alanine-rich region. Some transcription repressors contain this type of motif (reviewed in Hanna-Rose and Hansen, 1996). However, the confirmation of the regulatory function for MT A-2 or SMR2 will only come from transcription analyses of target genes. MT A-3 has an HMG domain and was shown to bind DNA specifically (Philley *et al.,* 1995). It is not known if MT A-3 has a repressor or activator function. HMG proteins are known for their ability to bind and bend DNA to facilitate the formation of higher-order nucleoprotein complexes; in addition, sequence-specific HMG proteins can also regulate gene expression by contacting other enhancer-bound proteins (reviewed in Grosschedl *et al.,* 1994). Observations from studies on mammalian HMG proteins indicate that a single HMG protein, by interacting with one or more accessory proteins, could regulate distinct promoters or regulate the same promoter in different directions (reviewed in Alexander-Bridges *et al.,* 1994).

A Drosophila HMG-like protein (DSP1) can convert at least two transcription activators that contain a negative regulatory element (NRE) into repressors (Lehming *et al.,* 1994).

The characterisation of the *mt A-1*, *mt A-2* and *mt A-3* transcripts by RACE experiments has revealed that nearly the entire *A* idiomorph is transcribed and that the intergenic regions are remarkably small (Saupe *et al.,* 1996; this study). The *mt A-3* transcript extends 1 kb outside of the *A* idiomorph while the *mt A-1* transcript stops only 23 bp from the border of the idiomorph (Saupe *et al.,* 1996). The *mt A-2* and *mt A-3* distal transcription start points are only 70 bp apart while the *mt A-1* start site is only 259 bp from the polyadenylation site of *mt A-2*. In contrast, only a portion of the 3.2 kb *a* idiomorph is transcribed and it has been proposed to contain 1.7 kb of non-essential DNA (Chang and Staben, 1994). It is possible that non-essential sequences have been eliminated from the *A* idiomorph but maintained in the *a* idiomorph to keep the size difference between the two idiomorphs to a minimum, perhaps to allow efficient recombination around the mating-type locus.

Northern and RT-PCR analyses showed that *mt A-2* and *mt A-3* transcripts are present in RNA extracted from cultures grown under vegetative and crossing conditions. The *mt A-1* transcript is also present in both vegetative and sexual phases (see control on Northern analyses and Saupe *et al.,* 1996). MT A-1 has obvious roles in both phases of *N. crassa* life cycle, however there is no apparent role for *mt A-2* and *mt A-3* in vegetative phase because the A^{IIRIP} mutant is identical phenotypically to wild-type during vegetative growth and during mating (Glass and Lee, 1992). These data suggest that *mt A-2* and *mt A-3* are either necessary for some subtle vegetative function or they might be regulated at the post-transcriptional level. The *mt A-2* and *mt A-3* genes seem to share promoter sequences. If the two mating type genes work together as a complex with other regulatory proteins the regulation of transcription and translation of both

genes may be somehow coupled. However, if they have antagonistic effects on genes or are needed at a slightly different time during the sexual process, it would be more economical for the cell to regulate them differentially only at the translational level.

Some characteristics of the *mt A-2* and *mt A-3* transcripts suggest they can be regulated at the translational level. Both *mt A-2* and *mt A-3* are expressed as transcripts with either longer or shorter 5' leader sequences. The long transcripts of *mt A-2* and *mt A-3* appear to be the main transcriptional products based in Northern analyses. These transcripts contain one and three uORFs upstream of the proposed ATG for *mt A-2* and *mt A-3*, respectively. In addition, the 3' end of the *mt A-3* transcript is especially long and extends beyond the idiomorph boundary. Translation of mRNA is emerging as an important regulator of gene function. Although still not well understood, translational regulation is known to be dependent on the 5' and 3' untranslated regions (UTRs) of the transcript which seem to control translation *per se* and stability of mRNA, respectively (reviewed in Sonenberg, 1994). The major point of control exerted by the 5' UTRs is binding of mRNA to the ribosomes (Hershey, 1991) which can be accomplished by a translation suppressive effect of uORFs or binding of activator or repressor proteins to the transcript.

In eukaryotes, mRNAs with ATG codons upstream of the major ATG are rare (less than 10 % in vertebrates) and this population of mRNA is strongly biased toward products of genes with a regulatory function such as proto-oncogenes and developmental genes (reviewed in Geballe and Morris, 1994). The uORFs can be categorised by their possible arrangements: 1) autonomous, uORFs that begin and end within the 5' leader; 2) contiguous, uORFs that are within the reading frame of the major cistron, resulting in a protein with an extended amino terminus; and 3) overlapping, out-of-frame uORFs (in relation to the main ATG) that overlap and end within the major cistron (Geballe and Morris, 1994). Translation of the *S. cerevisiae GCN4*

transcription factor is modulated by amino acid levels in the cell and it is an example of regulation by 4 autonomous uORFs (Thireos *et al.,* 1984). Regulation is achieved by interference with the translational apparatus and although independent of the sequence of uORFs reinitiation is strongly affected by sequences 5' of the first uORF (Grant *et al.,* 1995). In *N. crassa*, regulatory uORFs that affect translation have been described in *cpc-1* (Paluh *et al.,* 1988) and in *arg-2* (Orbach *et al.,* 1990), two genes which are homologous to the *S. cerevisiae GCN4* and *CPA1* (Davis, 1986), respectively. In contrast to *GCN4*, the amino acid sequence of the uORFs of the *S. cerevisiae CPA1, N. crassa cpc-1* and *arg-2* is important for the regulatory function of these genes (Luo *et al.,* 1995; M. Sachs, pers. comm.).

The proposed SMR1 transcript (the *P. anserina* homolog of MT A-2) also contains three uORFs upstream of the proposed major ATG (Debuchy *et al.,* 1993). Two of the *P. anserina* uORFs have two pairs of amino acids that are also consistently present in the uORFs of *mt A-2*. The presence of uORFs in *P. anserina* and *N. crassa* mating-type transcripts and the motif similarity among them, suggests similar mechanisms of post-transcriptional regulation for *SMR1* and *mt A-2*. However, the overall organisation of the uORFs in *N. crassa* transcripts is very different from that of *P. anserina*. One of the *P. anserina* uORFs has an ATG out-of-frame with the main transcript which overlaps with the *SMR1* sequence. The other uORFs in the *P. anserina* gene are contiguous with the main ORF possibly resulting in a protein with extended 5' end. The *N. crassa* uORFs in *mt A*-2 and *mt A-3* are of the autonomous type and could give rise to small peptides. However, up to this moment there are no rules to distinguish between regulatory uORFs and others that may not have a significant role in translation. In addition, deletion of seven nucleotides of the *P. anserina* out-of-frame uORF indicated that its presence may be of little importance since an altered phenotype was not observed (R. Debuchy, pers. comm.).

Another means of translational regulation is the control of mRNA stability. The 3' UTR and the poly(A) tail are involved with stability and control of translation of mRNA (reviewed in Sonnenberg, 1994). Control of translation efficiency by 3' UTR occurs particularly during development and differentiation. The 3'UTR of the *mt A-3* transcript is unusually long (1075 bases) since the mean length of 3' UTR in *N. crassa* is about 200 bases (Bruchez *et al.,* 1993). The *mt A-3* 3' UTR corresponds mainly to sequences flanking the *A* idiomorph and this region also corresponds to a 3' UTR of the *mt a-1* transcript (C. Staben, pers. comm.). The significance of the long 3' UTR in the control of *mt A-3* remains a question since the levels of *mt A-3* mRNA in the Northern analyses during the *N. crassa* life cycle (Figure 2-1) were not quantitatively measured.

The cell must possess mechanisms to counteract translation inhibition when a repressed transcript is to be translated. For example, the suppressive effect of uORFs on translation can be overcome if alternative promoters are used or the structure of the mRNA is changed during pre-mRNA processing (Geballe and Morris, 1994; Sonenberg, 1994). Although the *N. crassa A* mating type genes are constitutively expressed during the life cycle, only *mt A-1* has a role during both vegetative and sexual phases. So it is plausible that regulation of *mt A-2* and *mt A-3* occurs at the translational level and the proteins are expressed only during sexual development. Similar to regulation of *cpc-1*and *arg-2* by amino acid levels (Luo *et al.,* 1995) high levels of carbon and nitrogen sources might affect translation of *mt A*-2 and *mt A-3* by action of the uORFs. The *mt A-2* and *mt A-3* transcripts starting at the proximal start site would escape the suppressive effect of uORFs and could be preferentially translated when a change occurred in the environment.

During RACE experiments, mispliced *mt A-1* transcripts (Saupe *et al.,* 1996) and *mt A-3* were characterised. Detection of mis-spliced and aberrantly terminated transcripts by RT-PCR

has been reported. The defect can result from a mutational event (Horowitz and Berg, 1994) or can occur naturally. For example, it has been shown that a certain proportion of transcripts of the murine tumour necrosis factor retain the third intron and are transported to the cytoplasm (Neel *et al.,* 1993). It has also been shown that the splicing of the last intron of pre-mRNAs is dependent on the poly(A) tail (Niwa and Berget, 1991). It is possible that the splicing defect in *mt A-3* is correlated with the extremely long 3' UTR. It is unknown if the presence of the mis-spliced *mt A-3* and *mt A-1* transcripts is of biological consequence to mating and meiosis in *N. crassa* or whether the existence of a proportion of incorrectly spliced polyadenylated mRNAs is a general phenomenon uncovered by the high sensitivity of the RT-PCR technique.

Models of mating type regulation in *N. crassa* have been proposed (Glass and Staben, 1990; Metzenberg and Glass, 1990; Glass and Lorimer, 1991) in which a heterodimer formed between MT A-1 and MT a-1 controls the transcription of post-fertilisation genes including those encoded by the mating-type locus (see Chapter 1 section 1.3.3.5). It was also proposed that MT a-1 might regulate transcription of a gene expressed in the *A* idiomorph because seven copies of DNA sequences (CTTTG) bound *in vitro* by *E. coli* synthesised MT a-1 are clustered in the *A* idiomorph, within the *mt A-2* ORF (Philley and Staben, 1994). Because Northern and RT-PCR analyses revealed that the three *A* mating- type genes are transcribed throughout *N. crassa* life cycle, the previous models need to be reviewed. A new model has to accomodate the facts that MT A-1 and MT a-1 do not activate the transcription of *mt A-2* and *mt A-3* and that these genes may be regulated at the translational level.

If *mt A-2* and *mt A-3* only regulate post-fertilisation events, translational control of *mt A-2* and *mt A-3* may play an important role in the regulation of the sexual phase. A model can be drawn in which *mt A-2* and *mt A-3* transcripts would only be efficiently translated into

polypeptides in the ascogenous hyphae, where they would promote post-fertilisation events. Alternatively, even though MT A-2 and MT A-3 polypeptides are expressed during the vegetative and sexual phases, sporulation does not occur because MT A-2 and MT A-3 function as a complex with accessory regulatory proteins that are expressed only in perithecia (and/or MT a-1). Either idea is supported by the fact that the A^{IIRIP} mutant is phenotypically normal during vegetative growth.

The *mt A* idiomorph is similar in organisation to the *mat-* idiomorph of *P. anserina.* However, the levels of amino acid sequence identity between SMR1 and MT A-2 (24%) and SMR2 and MT A-3 (22%) are less than the identity between MT A-1 and FMR1 (43%). This difference in conservation suggests functional constraints to maintain FMR1/MT A-1 structure versus SMR1/MT A-2 and SMR2/MT A-3. Although *mt A-1* will complement a *FMR1* mutant to confer *mat-* mating activity, sequences encompassing *mt A-2* and *mt A-3* do not complement *SMR1* and *SMR2* mutants for post-fertilisation events (Arnaise *et al.,* 1993). Progeny from the A^{IIRIP} mutant segregate 1:1 for mating-type in a cross with a wild type *a* strain (Glass and Lee,1992) while mutations in *SMR1* lead to production of 100% *mat-* uniparental progeny (Zickler *et al.,* 1995). Together, these results suggest that mating-type function after fertilisation may involve slightly different mechanisms in the heterothallic *N. crassa* and the pseudohomothallic *P. anserina.* However, further DNA sequence analyses of the A^{IIRIP} mutant are necessary before function can be specifically assigned to *mt A-2* and *mt A-3* (see Chapter 3). Since the A^{IIRIP} mutant is able to produce at least a few normal eight-spored asci, a question arises about how important the *mt A-2* and *mt A-3* genes are for sexual reproduction in *N. crassa.* The differences and similarities in mating strategies in the ascomycetes could shed some light on the evolution and importance of the various mating-type genes in filamentous fungi. Further

functional characterisation of *mt A-2* and *mt A-3* genes and their roles in regulating sexual development genes will be described in Chapter 3 and 4.

3. Isolation of *mt A-2* and *mt A-3* mutants

3.1 INTRODUCTION

3.1.1 Functional analyses of *mt A-2* and *mt A-3*

In chapter 2, I described the characterisation of *mt A-2* and *mt A-3*, two genes that are contained in the 4 kb centromere proximal region of the *N. crassa A* idiomorph (Figure 1-3, Chapter 1). Deduced amino acid sequences of both *mt A-2* and *mt A-3* suggest that they are transcription factors.

A single mutant in the region coding for both *mt A*-2 and *mt A-3* was recovered by RIP mutagenesis (see below) (Glass and Lee, 1992). It had not been determined, however, if the phenotype observed was due to mutations in either *mt A-2* or *mt A-3* or if A^{IIRIP} was a *mt A*-2/*mt A-3* double mutant.

MT A-2 and MT A-3 show about 20% identity to the *P. anserina* SMR1 and SMR2, respectively (Chapter 2). The *FPR1*, *FMR1*, *SMR1* and *SMR2* genes of *P. anserina* have been mutated *in vitro* (all frame-shift mutations, except for *FPR1*, which was truncated at the 3' end) and each of the four different constructs were introduced into mating-type deletion spheroplasts (Zickler *et al.,* 1995). Four transformants containing each, a mutated gene were crossed to a wild-type strain of the opposite mating-type. In all mutant x WT crosses, differences were observed when compared to WT x WT crosses: 1) All crosses with mutant strains showed a mixture of large and small perithecia instead of the uniform size of wild-type perithecia; 2) Mainly single haploid nuclei were observed in the croziers, in all mutant crosses, as opposed to the binucleate cell in normal croziers; 3) Asci with abnormal spores, resulting from haploid meiosis were observed only in crosses with mutants; 4) Uniparental progeny were recovered from all mutant

crosses; 5) Biparental progeny were not observed in *SMR1* mutant x wild-type crosses; 6) The number of ascospores produced was drastically reduced (20-80% of the wild-type, depending on the mutant).

N. crassa sterile mutants, affected at *mt A-1* or *mt a-1*, can be complemented for mating with the opposite mating-type by ectopically introduced mating-type sequences. However, most transformants cannot complete the sexual cycle, i.e. they do not produce ascospores (Glass *et al.*, 1988, Glass and Lee, 1992). Fully fertile transformants appear occasionally as a result of a gene replacement event at the mating-type locus (Glass *et al.*, 1988). It has been suggested that cis-acting elements to the *mt* locus may be important for conferring full mating-type function. In contrast, *P. anserina* and *C. heterostrophus* mating-type mutants can be fully complemented with ectopic copies of the respective mating-type sequences even though ascospore production is not as abundant as in wild-type crosses (Debuchy *et al.*, 1993; Turgeon *et al.*, 1993).

A *N. crassa* strain was constructed in which the entire *A* idiomorph was replaced by an unrelated gene, ade-5 from *Schizophyllum commune* (R. Metzenberg, pers. comm.). Mating-type deletion transformants containing ectopic copies of either *A* or *a* mating-type sequences can mate with the specificity of the introduced sequence but only form barren perithecia. The A^{IIRIP} mutant which can mate normally as an *A* strain but produces few ascospores, also cannot be complemented to full fertility with ectopically-placed *A* mating-type sequences (Glass and Lee, 1992). The lack of complementation of the *N. crassa* mating-type mutants with ectopic copies of mating-type sequences has made it difficult to study post-fertilisation functions since these functions can only be assayed at the resident locus. Therefore, studies of the function of *mt A-2* and *mt A-3* genes individually, required the isolation of null mutants at the *A* mating-type locus.

3.1.2 Mutagenesis Techniques

Mutagenesis using UV light and chemicals has been extensively used in the past and is a powerful means of generating mutations in different loci. The first *N. crassa* mating-type mutants were obtained after UV mutagenesis (Griffiths and De Lange, 1978; Griffiths, 1982). Advances in molecular biology techniques has allowed the introduction of cloned DNA into *N. crassa* mutants to complement the wild-type phenotype and recover the complementing sequences (Case *et al.,* 1979). Numerous genes from *N. crassa* have since been cloned through such complementation techniques (see FGSC 1996 Catalogue).

Molecular biology techniques have made it simpler to obtain gene-specific mutants, especially when the gene sequence has been determined. Such strategies make use of gene targeting via homologous recombination, for example. In *N. crassa,* a phenomenon called RIP (Repeat-Induced Point mutation) is another efficient process of mutagenesis (Cambareri *et al.,* 1989; reviewed in Selker, 1990). RIP causes A:T to G:C transitions in duplicated sequences and has been used to characterise gene function in diverse *N. crassa* genes (e.g. Jarai and Marzluf, 1991; Nelson and Metzenberg, 1992; Ferea and Bowman, 1996) including the mating-type genes (Glass and Lee, 1992).

3.1.3 Gene disruption in *N. crassa*

In *N. crassa,* obtaining mutants by gene replacement is widely utilised. Unlike in *S. cerevisiae*, transforming DNA is generally randomly integrated into the genome of *N. crassa* (Rine and Carlson, 1985; reviewed in Fincham, 1989). Integration at homologous sites through recombination is also observed at lower rates on the order of 1% (reviewed in Rambosek and Leach, 1987) but frequencies as high as 30% were observed for the *N. crassa am* gene (Asch and Kinsey, 1990). The frequency of homologous recombination varies depending on the strain

utilised (Kim and Marzluf, 1988), the location of the targeted locus (Irelan *et al.,* 1994) and length of DNA sequence homology to target sequences (Asch and Kinsey,1990). Transcriptional activity at the site of recombination was also shown to influence the rate of homologous recombination in mammalian systems (Nickoloff, 1992) and *S. cerevisiae* (Kotani *et al.,* 1994), and may also be an important factor for recombination in *N. crassa.*

A method for gene disruption of the *N. crassa ccg-1* locus was developed based on previous reports in mammalian systems and allowed a fast screening of putative gene replacements (Aronson *et al.,* 1994b). The protocol involved an enrichment of putative gene replacements by using two selectable markers: one internal to the homologous sequences and another flanking the disrupted gene. The gene for hygromicin B resistance (*hph*) was used to disrupt the *ccg-1* gene; the benomyl resistance gene was the second selectable marker. About 5 kb of *ccg-1* homologous sequences were present in the plasmid, 4 kb on the 5' end of the gene and 1 kb on the 3' end. Hygromycin B resistant ($HygB^R$) and benomyl sensitive (Bml^S) transformants were likely to be gene replacement strains. In their experiment, 129 $HygB^R$ transformants were screened and 17 were also Bml^S. Using primers specific for gene replacement at the *ccg-1* locus, PCR products were observed in 3 of the 17 transformants. Homologous recombination at the *ccg*-1 was estimated to be 3% using this method. This method was also efficient for disruption of the *frq* gene where homologous events are even less frequent, approximately 1 in 300 (Aronson *et al.,* 1994a). Other methods for gene replacement targeted to the *his-3* and *am* loci were developed based on homologous recombination (Aramayo and Metzenberg, 1995; Miao *et al.,* 1994). In both of these methods homologous sequences in the constructs were usually greater than 5 kb.

Recombination frequency at the mating-type locus was estimated to be 0.05 to 0.25 % (1-5 in 2000 transformants) (Chang and Staben, 1994), which is much lower than reported rates for other genes in *N. crassa* (see above). Gene replacement at the *mt* locus was obtained by transforming an *A* strain with the *mt a* idiomorph containing 1 kb of flanking sequences on each side. The method relied on the counter-selection of ectopic integrants due to the vegetative incompatibility reaction promoted by *mt A-1* and *mt a-1*.

Even though low recombination rates make attempts for gene replacement at the mating-type locus a time consuming task, this alternative was worthwhile as an attempt to obtain *mt A*-2 and *mt A-3* mutants. Furthermore, the availability of *N. crassa* mating-type sequences and restriction maps of regions surrounding the *mt* locus facilitated distinguishing between ectopic and gene replacement transformants using PCR and Southern blots.

3.1.4 RIP (Repeat-Induced Point mutation)

RIP is a phenomenon unique to *N. crassa* and is widely used as a means of obtaining gene-specific mutants. RIP occurs during the mitotic divisions that precede karyogamy in the ascogenous cells (Cambareri *et al.,* 1989; reviewed in Selker, 1990). Initially observed as instability and methylation of duplicated sequences introduced by transformation, RIP was thought to be due mainly to rearrangements that occurred between fertilisation and karyogamy (Selker *et al.,* 1987a; Cambareri *et al.,* 1989). Further analyses showed that the introduced sequences as well as the resident copy are altered: cytosine residues are methylated and G:C to A:T mutations occur, especially at sites where adenine is preceded by cytosine (CpA) (Cambareri *et al.,* 1989).

The *N. crassa* genome is mostly devoid of methylation with only about 1.5% of cytosines being methylated (Foss *et al.,* 1993; Russel *et al.,*1985). Some chromosomal loci however are

heavily methylated such as the ribosomal DNA zeta-eta (ξ–η) and psi-63 (ψ63) regions and they exhibit portable signals for *de novo* methylation (Selker *et al.,* 1987b; Cambareri *et al.,* 1991). These regions appear to be relics of a natural occurrence of RIP and account for one of the models in which DNA sequences and structures created by RIP are responsible for signalling methylation directly (reviewed in Selker, 1990; Singer *et al.,* 1995b). Foreign DNA sequences and some *N. crassa* sequences can also be methylated in the absence of RIP during vegetative growth; however this methylation seems to be position dependent and apparently only occurs sporadically (reviewed in Singer and Selker, 1995).

RIP has been reported to occur in unlinked duplicated sequences as short as 1 kb (Selker *et al.,* 1989). Linked duplications of as little as 383 bp can also trigger RIP (Stadler *et al.,* 1991). The frequency of alterations in unlinked duplications can vary between 10-70%, depending on the gene and size of duplicated fragment (Selker and Garret 1988; Irelan *et al.,* 1994). Transition mutations tend to be less frequent around the boundaries of the duplication (Cambareri *et al.,* 1989; Grayburn and Selker, 1989) but can extend beyond the duplicated sequences into unique regions of at least 900 base pairs (Foss *et al.,* 1991; Irelan *et al.,* 1994).

The A^{IIRIP} mutant was obtained through RIP by duplication of the region coding for both *mt A-2* and *mt A-3.* A 3 kb *Sst* II fragment from the *A* mating-type idiomorph was co-transformed with a *qa-2*$^+$ marker vector into an *A* strain (Glass and Lee, 1992). The *Sst*II fragment encompasses the entire *mt A-2* ORF and more than 700 bp of the *mt A-3* ORF (See Chapter 2 and Figure 1-2). The A^{IIRIP} mutant was the only mutant isolated among 157 *A* progeny that had an altered sexual phenotype (Glass and Lee, 1992).

Functional analyses of *mt A-2* and *mt A-3* will help to uncover the role of these mating-type genes on the regulation of *N. crassa* sexual development. This chapter describes the use of

both gene replacement and RIP techniques in an attempt to isolate *mt A-2* and *mt A-3* specific mutants. The molecular characterisation of such mutants and their phenotypic description will also be described.

3.2 MATERIALS AND METHODS

3.2.1 Strains and Media

Table 3-1 shows *N. crassa* strains used in different experiments. Media were the same as in Chapter 2 except that sorbose containing solutions (FIGS or BdeS sugar preparations, Davis and DeSerres, 1970) were added when colonial growth was desired. *E. coli* strains and growth were the same as Chapter 2.

Genotype of *A* strains	Reference	Genotype of *a* strains	Reference
qa-2 aro-9	RLM 52-22	*OR 8-1 wt*	FGSC 532
inl fl	FGSC 3631	*inl fl*	FGSC 4347
thr-2 het-6^{PA}	NLG C2(2)-9	*un-3 ad-3A nic-2*	NLG R1-51
A-2^{m1} ad-3B	This study	*qa-2 aro-9*	NLG R2-54
ad-3B arg-1	I-20-26	*ad-3B*	NLG R1-42
A-3^{m1} pyr-4	This study	*pyr-4 arg-5 inl pan-2*	RLM 57-26
A-3^{m2} thr-2	This study	*R al-1*	FGSC 2088
pyr-4 cyh-1	RLM 57-30	*Mauriceville 1c*	FGSC 2226

Table 3-1 List of *N. crassa* strains used in this study. FGSC= Fungal Genetics Stock Center; RLM= R. Metzenberg's Lab; NLG= L. Glass' Lab; I=T. Griffiths' Lab

3.2.2 Transformation

Spheroplasts were prepared by the digestion of germinated conidial walls with Novozyme 234 (Akins and Lambowitz, 1985). Transformation was according to Glass *et al.* (1988). Typically 1 µg of DNA per transformation reaction was used; selection was done in FIGS minimal media to allow colonial growth, plus required supplements. For transformation using *hph,* the level of hygromycin resistance was initially tested for each strain and 250 U/ml was used

for transformation experiments with NLG C(2)2-9. Spheroplasts of RLM 52-22 were selected for aromatic amino acid prototrophy when transformed with the *qa-2*$^{+}$ gene.

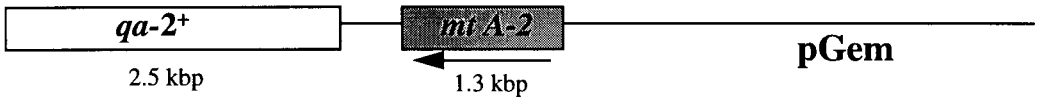
3.2.2.1 Gene replacement

A diagram of plasmids used in transformation experiments is shown in Figure 3-1. Plasmid pGq1595 contained a 1.6 *Pvu* II/*Eco*RV *mt A-3* fragment in a *qa-2*$^{+}$ vector (constructed by J. Grotelueschen, University of Wisconsin). For gene replacement experiments plasmid pDA-3 was initially constructed by introducing a 1.3 kb *Bam*HI *hph* fragment (from pCSN44, Staben *et al.,* 1989) into the *Bam*HI site of pGq1595, thus disrupting the beginning of the *mt A-3* ORF. For construction of plasmid pHA-3 the disrupted *mt A-3* fragment from pDA-3 was removed and ligated to the 6.5 kb *Kpn*I fragment of TLP-1-50-8 that contains the *het-6*^{OR} gene (gift of M. Smith, Universtity of British Columbia). pAL2 contains most of *mt A-3* ORF and more than 1 kb of its 3' end (2.5 kb *Eco*RV fragment of *mt A*) interrupted by the1.3 kb *Bam*HI *hph* fragment (gift of S. Saupe).

Another selection method was attempted by transforming NLG C(2)2-9 spheroplasts with a plasmid containing the *het-6*^{OR} gene as the second marker (Smith *et al.,* 1996) and the *mt A-3* gene, interrupted by the *hph* gene (pHA3, Figure 3-1). Transformants containing both alternate alleles (*het-6*^{OR} and *het-6*^{PA}) will not grow (Smith *et al.,* 1996; M. Smith, pers. comm.). This stategy is similar to the use of Bml^{R} as an indicator of gene replacement.

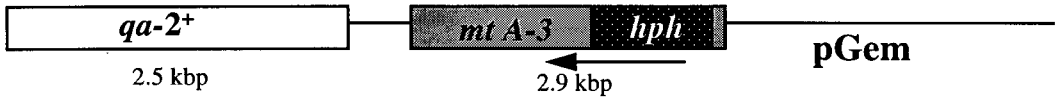
PCR was performed with oligonucleotides specific for the *hph* gene (HYG) and *mt A-3* sequences (primer rII.1 or Tango, see Appendix 1 for primer sequences). Sequences corresponding to both rII.1 and Tango primers were absent from the introduced plasmid (pDA-3 and pHA-3). DNA template was prepared according to Chow and Käffer (1993) or by

microwaving of dry conidia (Ferreira and Glass, 1995). PCR of individual or pooled samples was performed. Samples were pooled in groups of 5 or 10 transformants per PCR. PCR was

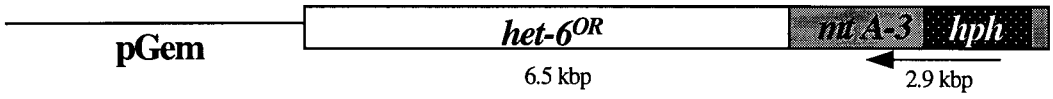


A - pGq1310

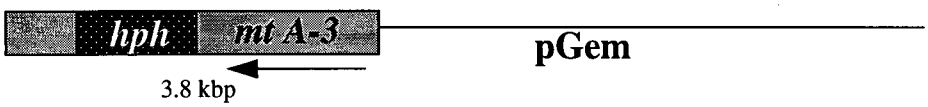
B-pGq1595



C - pDA-3



D - pHA-3



E - pAL2

Figure 3-1 Plasmids used in transformation experiments. Arrows represent direction of transcription of *mt* genes. The *hph* cassete is 1.3 kbp. See text for detail on construction of plasmids.

performed essentially as described in Chapter 2 (section 2.2.2.2) or according to Ferreira and Glass (1995). A PCR control was performed by using 9 parts of a DNA solution from an *a* strain and 1 part of DNA from an *A* strain. About 100 ng of DNA was used per PCR.

3.2.3 RIP of *mt A-2* and *mt A-3*

3.2.3.1 Isolation of homokaryotic transformants

Plasmids pAL2 and pGq1310 (Figure 3-1) were introduced into the *thr-2 het-6*^{PA} *A* (NLG C2(2)-9) and *qa-2 aro-9 A* (RLM 52-22), respectively. Transformants obtained from these experiments were randomly selected (about 25 from each transformation) and grown under restrictive conditions (presence of hygromycin and minimal medium, for pAL-2 and pGq1310, respectively). Genomic DNA was isolated, digested with *Hind* III, electrophoresed in 1% agarose gels and transferred to Nylon membranes according to the manufacturer (Nytran^{+} - Schleicher & Schuell, Keene, NH). Southern blotting was performed to determine the presence of a single copy of integrated plasmid. A gel-purified 1.1 kbp *mt A*-2 PCR fragment (primers rI.1/rI.2) was used for probing *mt A*-2 transformants. For *mt A-3* transformants a 1.3 kb *Bam*HI *hph* fragment was used in Southern blots. The membranes were probed with fragments labelled with α-^{32}P-dCTP using the T7 Quickprime kit (Pharmacia, Baie d'Urfe,PQ) and washed under stringent conditions. Two transformants from each transformation experiment were selected for isolation of homokaryons.

N. crassa transformants are usually heterokaryotic and contain both transformed and untransformed nuclei. To isolate homokaryons, selected transformants were plated several times under selective conditions. Homokaryon isolation was performed by first plating conidial suspensions under selective conditions in BdeS media (hygromycin for pAL-2 transformants and minimal media for pGqa1310 transformants); single conidial isolates were then subcultured

under selective conditions in Vogel's medium. Conidial suspensions were then plated on selective and non-selective media to evaluate the homokaryon purification process. This procedure was repeated 4 times until the number of conidia in non-selective medium was approximately the same as under selective conditions, indicating that untransformed nuclei were eliminated. The homokaryons were then kept under selective conditions.

3.2.3.2 Crosses and Screening of progeny

The homokaryotic transformants were used as females in crosses with either wild type *a* or *un-3 ad-3A nic-2 a* (see Table 3-1 for list of strains). Ascospores produced in the crosses were collected from the Petri dish lids, streaked on BDeS plates and heat-shocked in a 65°C oven for 30 to 60 min. Plates were placed at 30°C overnight and germinated ascospores were picked from plates under a microscope and transferred to 1 ml of minimal media plus required supplements. Progeny were tested for fertility on a lawn of *inl fl A* and *inl fl a* tester strains (Table 3-1). The fertility of *A* progeny x *fl a* was scored by performing perithecial squashes or by observation of the number of ascospores deposited on filter paper laid on top of the crosses.

3.2.3.3 Methylation and RFLP analyses

Homokaryotic transformants and selected first and second generation progeny were grown in liquid media for DNA extraction (Raeder and Broda, 1982). Genomic DNA from the transformants and from first and second generation progeny was digested with two isoschizomers, *MboI* (methylation insensitive) and *Sau3A*I (C methylation sensitive). Southern blot analyses were as described in section 3.2.3.1.

PCR amplification of *mt A-2* and *mt A-3* was performed as described in Chapter 2. The amplification products were ethanol-precipitated and subjected to digestion with different restriction enzymes (see results) and subjected to electrophoresis in 2% agarose gels.

3.2.4 Sequencing of mutants

The resident copies of *mt A-2* and *mt A-3* from putative mutants and from the A^{IIRIP} mutant (Glass and Lee, 1992) were amplified by PCR with the same primers used for amplification of the *mt A-2* and *mt A-3* cDNAs (see Chapter 2). PCR products were purified from low-melting point agarose by freezing and thawing for direct DNA sequencing or cloned into the pCRII vector (Invitrogen, San Diego, CA). Automated DNA sequencing was performed as described (see Chapter 2 section 2.2.3). Sequencing analyses and software were as described in Chapter 2.

3.2.5 Phenotypic characterisation of mutants

The mating-type mutants were crossed to WT *a* (Oak Ridge and Mauriceville background). Crosses were done to characterise the mutants with respect to quantity of ascospores and the presence of abnormal asci and ascospores and/or uniparental progeny. Pictures of perithecial squashes were taken at 8 and 11 days after fertilisation and development of mutant crosses was compared to crosses with the original non-transformed and transformed strains.

3.2.5.1 Ascospore production

The number of ascospores was quantified in the following manner: at 8 and 11 days post-fertilisation 10 perithecia were picked from a crossing plate and transferred to an Eppendorf tube containing 25 µl of water. Perithecia were lightly squashed in the tube using a fitting plastic pestle. A sample of the supernatant was counted in a hematocytometer. A total of 5 samples from each cross was counted. Statistical analyses were applied using ANOVA and Student's *t*-Test using two-tailed distribution and considering data as two-sample of unequal variance. Calculations were done using Microsoft Excel 5.0 software.

3.2.5.2 Uniparental progeny

Analyses for the presence of uniparental progeny were done by crossing the A-2^{m1}, A-3^{m1}, A-3^{m2} and A^{IIRIP} mutants to the *R al-1 a* strain (Table 3-1). *R* (*round spore*) is a dominant mutation which leads to formation of asci containing eight round ascospores when *R* nuclei are present in the ascogenous hyphae (Mitchell, 1966). If asci containing ellipsoidal ascospores were observed, an *R a* nucleus was not present in the crozier and *A* uniparental progeny were produced. Over 50 perithecia from each cross were squashed at 8 days post-fertilisation and observed for the ratio of round to spindle-shaped ascospores.

3.2.5.3 Heterokaryon incompatibility tests

Heterokaryon incompatibility tests were performed with the *mt A-2* and *mt A-3* mutants. Because the original A-2^{m1} and A-3^{m1} mutants were prototrophic, they were crossed to introgress auxotrophic markers. For heterokaryon tests, 25 µl of conidial suspensions from two auxotrophic strains containing approximately the same number of conidia (4 X 10^6/ml) were mixed. The combinations of strains that were used (refer to Table 3-1 for strain genotypes) is as follows: for A-2^{m1} heterokaryon tests, the compatible control (*a/a*) was a heterokaryon formed between strains NLG 7 and NLG R2-54; *A/a* incompatible control was I-20-26 and RLM 52-22. For heterokaryon tests of the A-3^{m1} and A-3^{m2} mutants, the compatible control (*a/a*) was a heterokaryon formed between strains RLM 57-26 and NLG R2-54. The *A/a* incompatible control was RLM 57-30 and RLM 52-22. Heterokaryons were forced between the A-2^{m1}, A-3^{m1} and A-3^{m2} mutants and strains bearing *qa-2 aro-9* markers from the two mating-types (RLM 52-22 and NLG R2-54, respectively). Heterokaryons were forced to grow on Vogel's minimal media in long race tubes (Davis and DeSerres, 1970) so linear growth could be measured. The heterokaryon tests were done in triplicate.

3.2.6 Expression analyses of *mt A-2* and *mt A-3* in the mating-type mutants

Transcription of *mt A-2* and *mt A-3* in the mutants was assessed using RT-PCR. RT-PCR was performed essentially as described in Chapter 2 (see Material and Methods section 2.2.2.2). As a control, RT-PCR was also performed with primers 2043 and 3194 which amplify the *mt A-1* cDNA. (see Appendix 1 for primer sequences).

3.2.7 Molecular biology techniques

Standard molecular biology techniques were performed according to Sambrook *et al.* (1989) or as suggested by manufacturers.

3.3 RESULTS

3.3.1 DNA sequence analyses of the A^{IIRIP} mutant

Because the *Sst* II fragment used for RIP in the A^{IIRIP} mutant (Glass and Lee, 1992) spanned all of *mt A-2* and part of *mt A-3* sequences it was likely that mutations had occurred in both ORFs. The DNA sequence of the A^{IIRIP} mutant was determined to identify any mutations within *mt A-2* and *mt A-3* that contributed to the mutant phenotype. Fifty-two A-T to G-C transition mutations characteristic of RIP were detected in *mt A-2* (Figure 3-8). Similar to other RIP events about two thirds of these mutations (30 out of 52) occur in 5'CpA 3' dinucleotides. These 52 mutations create 32 amino acid substitutions and 5 premature stop codons in the *mt A-2* ORF (Figure 3-9). The first stop codon in the ORF (a CAA to TAA) results in a truncated MT A-2 polypeptide of only 129 aa. Six RIP mutations were identified in the *mt A-3* ORF (Figure 3-13). They occur in the first 51 codons of the *mt A-3* ORF and in 5'CpA 3'dinucleotides. These mutations lead to only three amino acid substitutions: serine to leucine in positions 8 and 51 of the polypeptide; glutamic acid to lysine in position 41 (sequence not shown).

The promoter region of *mt A-2* and *mt A-3* was also sequenced in the A^{IIRIP} mutant. Thirty-six base pair changes were detected in the approximately 700 base pairs that separate *mt A-2* and *mt A-3* translational start codons (Figure 3-3). No mutations were found in sites known to be critical to general transcription such as CAAT boxes. However, a few G:C to A:T substitutions occurred in the three 50 bp repeats located in between the two genes which may be important for their regulation. In addition, mutations were also found in the uORFs of *mt A-2* and *mt A-3* (Chapter 2). As in the coding region, most of the mutations occurred in 5'CpA 3' dinucleotides (75%).

```
WT      1 MNLLNMQPKRSEQPAMFEENRASSQEGQDLEVMYKKLHQLQARLSRSVLS 50
          |||||:|||||||||||||||||||||||||:||||||||||||||||
A^IIRIP 1 MNLLNIQPKRSEQPAMFEENRASSQEGQDLEVIYKKLHQLQARLSRSVLL 50
                .         .         .         .         .
       51 EAIKEFEENLRCLFHEAKLLLCSTRTKYRQSWFGSSNEFGSSDERRIIKT 100
          ||||||||||||||||||||:|||||| ||||| ||||| |||||||||
       51 EAIKEFEENLRCLFHEAKLLLYSTRTKYYQSWFGFSNEFGFSDERRIIKT 100
                .         .         .         .         .
      101 SCCIIESTNTILNFLSFLEKNRGLPFGGDQRLQQAAYKGQQFAFRLLRSL 150
          |||||||.|||||| |||||||| |||| --------------------
      101 SCCIIESINTILNFLLFLEKNRGLLFGGD*KFQQIAYKG**FAFRLLRSF 150
                .         .         .         .         .
      151 TLHKAAQEVPGKDFGLVYGKDVYVLNGHILHRSKQEIVGQAGGRNWHVDH 200
          ----------------------------------------------------
      151 ILYKAA*EVPGKDFGLVYGKDVYVLNRYILYRSKQEIVGQAGGRNWHVDH 200
                .         .         .         .         .
      201 TLHPLRRVPGTPWHKFFGNLEVGDDKQLRLFDDDAAVDSYRVGPQKFFVV 250
          ----------------------------------------------------
      201 TLHPLRRVPGTP*YKFFNNLKIGDDKQLRLFDDNAAVDSYRVGPQKFFVV 250
                .         .         .         .         .
      251 IPETAEFILDEVSSEHQRVATIHTENGHVQPPAPTSIQQEALLRKLDFAM 300
          ----------------------------------------------------
      251 IPETTEFILDKVSSEHQRVATIHTENRYVQPPVPTSIQQEALLRKLDFAM 300
                .         .         .         .         .
      301 TTSLPGYVVEGQPEIVFHYEGLRQIPVDYSQERPLSILSHVFTRPALWGE 350
          ----------------------------------------------------
      301 TTSLPGYIVEGQPEIVFHYEGLRQIPVDYSQERPLSILSHVFTRPALWGE 350
                .         .         .         .         .
      351 LELADHFDPRDGVQQEEHIYYI*                           373
          ----------------------
      351 LELADHFDPRDGVQQEEHIYYI*                           373
```

Figure 3-2 Amino acid changes in the *MT A-2* protein in the A^{IIRIP} mutant. Changes are shown in bold. Sequences presumably absent in the translated protein are indicated with horizontal bars. Asterisks indicate stop codons.

The A^{IIRIP} mutant is not completely sterile and a few ascospores can be recovered from a cross and they segregate 1:1 for mating type; the *a* progeny are completely normal while the *A* progeny exhibit the A^{IIRIP} phenotype (Glass and Lee, 1992). Some of these *A* progeny show a more severe phenotype when crossed again with an *a* strain, with no ascospores recovered. DNA sequence analyses of two of these second generation A^{IIRIP} progeny did not reveal any additional mutations within *mt A-2* or *mt A-3* ORF sequences.

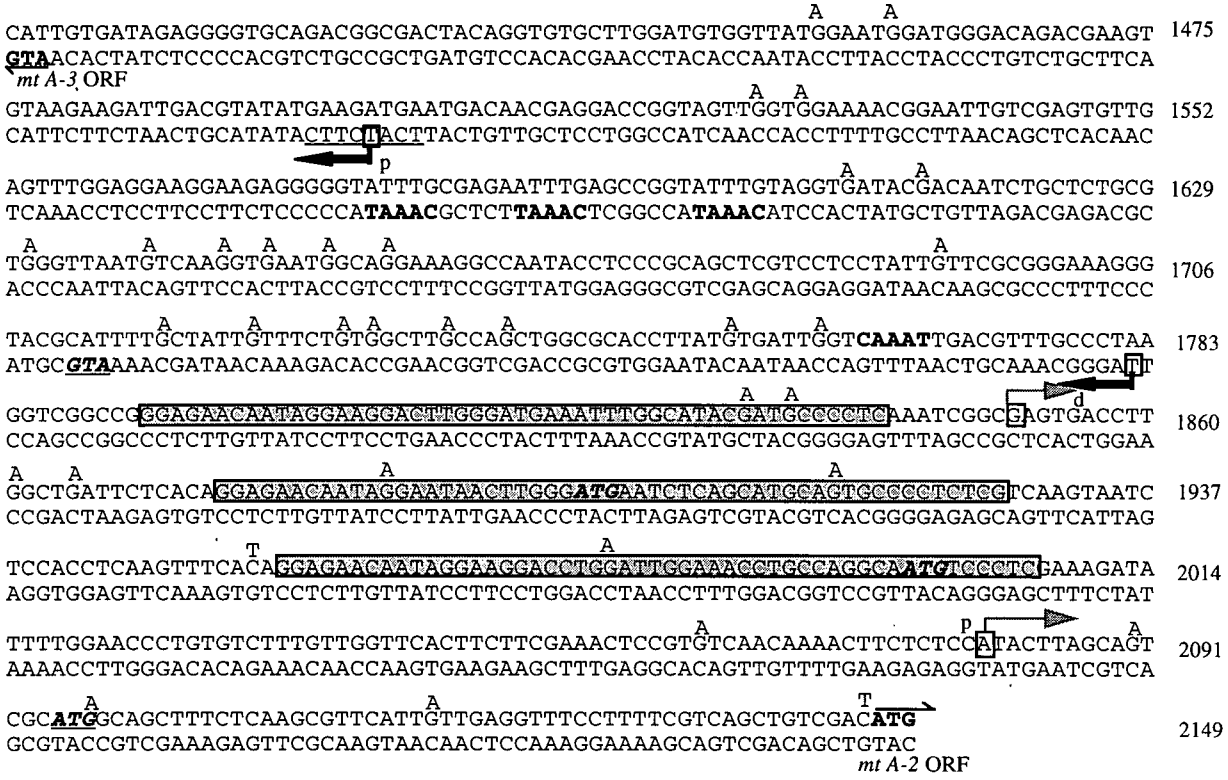


Figure 3-3 DNA sequence changes in the promoter region of *mt A-2* and *mt A-3* in the A^{IIRIP} mutant. Transcriptional start sites and relevant motifs and repeats are shown as in Figure 2-5, Chapter 2. Nucleotide base changes are shown above promoter sequences. Most of the mutations were polarised G to A transitions in one strand; only two C to T mutations appear on the same strand.

3.3.2 Gene replacement

Initially, pDA-3, a *qa-2*$^+$ plasmid containing *mt A-3* sequences disrupted by the hygromycin resistance gene (Figure 3-1) was used for gene replacement experiments of *mt A-3*. The plasmid was introduced into *qa-2 aro-9A* spheroplasts and transformants were selected on hygromycin. Transformants were picked from transformation plates and grown individually on media containing hygromycin supplemented with aromatic amino acids. Transformants were further tested for loss of *qa-2*$^+$ on BDeS drop out plates. This protocol allowed for selection of one- or two-step gene replacements. One-step homologous recombination events are dependent on the occurrence of a double crossover spanning the homologous sequences during

transformation. Two-step gene replacement involves the insertion of the plasmid by a single crossover followed by mitotic or meiotic loss of sequences. Transformants that had lost the aromatic amino acid prototrophy were then selected for PCR amplification. Initially, 1270 $HygB^R$ *mt A-3* transformants were screened for aromatic amino acid auxotrophy (aro^-). Only 37 of the transformants did not grow or grew poorly on minimal media, suggesting loss of the $qa\text{-}2^+$ marker. Genomic DNA from the aro^- transformants was then used in PCR to identify homologous recombination events. Primers used were such that size of products would differentiate between disrupted and resident gene (primers rII.1/rII.2) or only gene replacement products would be amplified (primers HYG/rII.1)-(See Appendix 1 for primer sequences). Bands of expected sizes for gene replacement events were not observed in any of the transformants. This strategy proved to be very time-consuming since a great number of transformants had to be initially screened for loss of the aromatic amino acid prototrophy in drop out plates. The existence of heterokaryotic transformants in *N. crassa* makes the analyses even more difficult because the desirable phenotype (hyg^R aro^-) may be have been masked by the presence of a second transformed nucleus in the heterokaryon containing other combinations of the two markers.

The presence of a marker that could be negatively selected upon or after transformation would be desirable for gene replacement strategies. Negative selection of ectopic transformants would decrease the number of transformants to be screened. The incompatibility reaction triggered by two different *het-6* alleles (OR and PA) leads to death of heterokaryons or of nuclei heterozygotic for *het-6* in transformation experiments (Smith *et al.*, 1996). Transformation of a $het\text{-}6^{PA}$ strain with the $het\text{-}6^{OR}$ gene was used for selecting against ectopic integration events. Figure 3-4 shows the strategy of gene replacement using plasmid pHA-3.

Plasmid pHA-3 was digested with *Asp*718 as depicted in Figure 3-1 to bias for gene replacement of mating-type sequences and integrity of *het-6*^{OR} upon transformation. The control experiment using pHA-3 cut in the middle of the *het-6*^{OR} gene with *Nsi* I gave a transformation efficiency of 40 transformants/μg DNA. This number was used to calculate the number of transformants that would have to be screened in the absence of *het-6*^{OR}. The average frequency for pHA-3 cut in the flanking sequences (keeping *het-6*^{OR} intact) was 3.5 transformants/μg. Two hundred and twenty transformants were selected as HygB^{R} and tested for gene replacement in PCR amplifications. The 220 HygB^{R} transformants corresponded to more than 2500 transformants tested for gene replacement if *het-6*^{OR} was not present in the transforming plasmid. The sizes of the PCR products expected from a gene replacement strain were 1.1 kb with primers HYG/rII.2 and 2.5 kb with primers rII.1/rII.2. The 1.1 kb band would only be amplified in gene replacement strains. Amplification with primers rII.1/rII.2 could yield two fragments (2.5 and 1.1 kb) depending whether gene replacement did or did not occur, respectively. In the PCR amplifications of the 220 HygB^{R} transformants, a specific band corresponding to a gene replacement event was not observed. Analyses were complicated by the fact that non-specific bands were observed in some cases with the specific HYG primer and rII.1, resulting in the identification of false positives. PCR artefacts were also observed during screening of *ccg-1* disruptions (Aronson *et al.*, 1994b).

To determine if a gene replacement-specific band was overlooked, a different PCR strategy was also used. After an initial amplification of a resident sequence that would span a possible gene replacement (rII.1/Pep), a second round of gene-replacement specific PCR (HYG/Pep) was done. Although a large number of bands appeared in the second round of PCR

amplification, a band corresponding to a gene replacement event at the *mt A-3* gene was not observed (not shown).

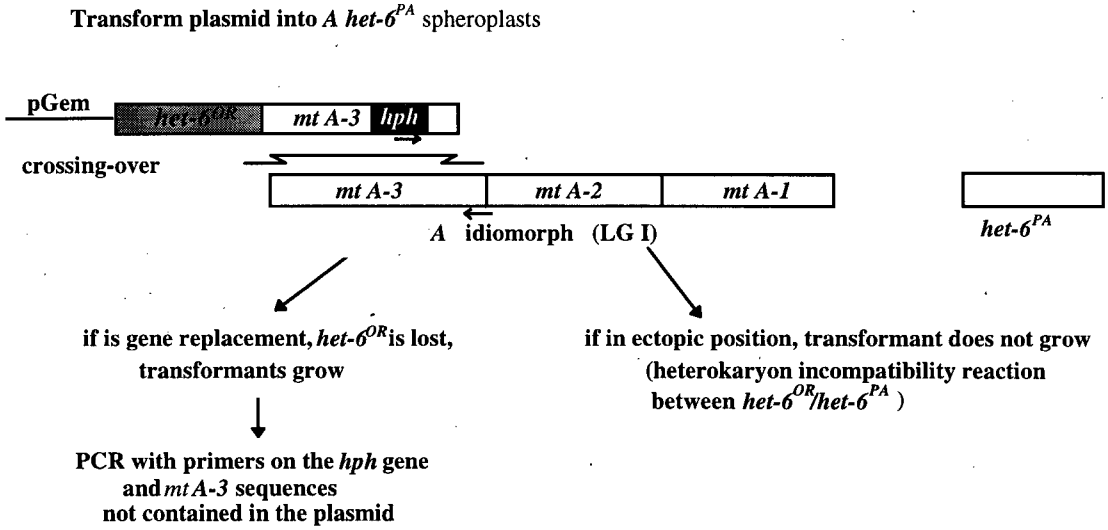


Figure 3-4 Strategy for isolation of gene replacement mutants. Position of gene replacement-specific primers are shown as arrows under *hph* and *mt A-3* sequences. See text for more details.

3.3.3 Isolation of *mt A-2* through RIP

3.3.3.1 Isolation of *mt A-2* homokaryotic transformants

The RIP process is more efficient when only one extra copy of the gene to be mutated is present in a homokaryotic transformant (Fincham *et al.*, 1989). Plasmid pGq1310 was introduced into *qa-2 aro-9 A* spheroplasts (RLM 52-22). About 25 aro^+ transformants were randomly selected and grown on minimal media. The *mt A*-2 transformants were probed with a 1.1 kb *mt A*-2 PCR fragment using primers rI.1/rI.2 (Figure 3-5). The recipient strain contains one band corresponding to the resident *mt A*-2. The *mt A-2* transformants G11 and G12 showed the least complex pattern and were likely to contain only one extra copy of *mt A-2*. Homokaryotic transformants were then obtained after 4 subcultures of single conidial isolations on minimal media.

3.3.3.2 Isolation of *mt A-2* RIP progeny

The G11 and G12 homokaryotic transformants were crossed to the WT OR *a* strain. The fertility of the progeny from the RIP crosses was tested on both *fl A* and *fl a* tester strains. The ratio of *A/a* progeny were approximately 1:1 in the two crosses.

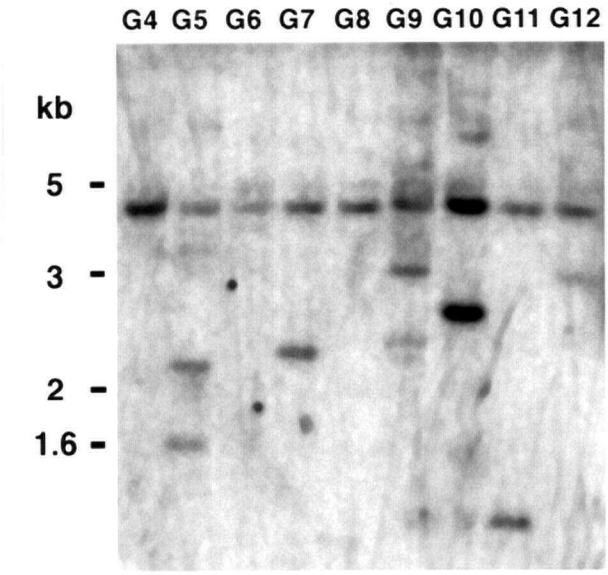


Figure 3-5 Southern Blot of genomic DNA of *mt A-2* transformants. DNA of transformants containing different number of integrated copies was digested with *Hin*dIII and hybridised with a 1.1 kb *mt A-2* probe.

Table 3-2 shows the number of progeny from G11 and G12 crosses to OR *a* that were tested for fertility. Ascospore formation was scored at 14 days after fertilisation by observation of deposition of ascospores on filter paper that was laid on top of the crosses. This method was previously used for screening mutants in the region containing *mt A-3* and *mt A-2* and identified the A^{IIRIP} mutant (Glass and Lee, 1992). Fertility of the *A* progeny originated from the crosses of

G11 and G12 did not seem to be affected. All of the approximately 90 *A* progeny produced perithecia and ascospores in crosses to *fl a* and were indistinguishable from wild-type crosses. A few progeny were generated (see section 3.3.3.3) by crossing first generation progeny with OR *a*. Second generation progeny (more than 20 from different parents) were also tested for the sexual development phenotype on tester plates, but as with their parents, an altered sexual development phenotype was not observed.

Homokaryotic transformant	**Total progeny isolated from cross to *OR a***	**Total *A* progeny tested for fertility on *fl a***
G11	185	~ 90
G12	225	~ 110

Table 3-2 Progeny of G11 and G12 homokaryotic transformants tested for fertility on *fl* tester plates.

The frequency of RIP can vary substantially depending on several factors. These factors include length of duplication, strain background and number of pre-meiotic mitosis (reviewed in Selker, 1990; Singer *et al.*, 1995b). It has been shown that RIP frequency can vary from 2 to 28% depending upon whether the ascospores were harvested 9-10 or 12-15 days after fertilisation, respectively (Singer *et al.*,1995a). Progeny from RIP crosses G11 and G12 were isolated 15 days after fertilisation. Mutant phenotypes, however, were not observed even in progeny from late ascospores. The inability to find a phenotype associated with *mt A-2* in these experiments suggested the following possibilities: 1) Transformants were not homokaryotic; 2) The size of the introduced fragment was not suitable for triggering RIP; 3) The *mt A-2* gene did not suffer RIP because of some type of protection associated with the mating-type sequences; 4) *mt A-2* specific mutants do not exhibit a sexual development phenotype (or the phenotype is very subtle). Some of these possibilities were investigated further.

The G11 and G12 strains resulted from introduction of plasmid pGq1310 that contained 2.5 kb of sequences bearing the *qa-2*$^+$ gene into RLM 52-22. Although the fragment corresponding to the *qa-2*$^+$ gene was larger than that of the *mt A*-2 sequences, the introduced *qa-2*$^+$ gene was, in principle, subjected to RIP as well as was *mt A-2*. To determine if RIP occurred in the G11 and G12 crosses, these transformants were crossed to a *qa-2 aro-9 a* strain. Ascospores resulting from a cross homozygous for *qa-2* are white. If RIP did not occur, ascospores from a *qa-2 aro-9 a* strain with G11 and G12 should be all black because the *qa-2*$^+$ allele was introduced by transformation. When G11 and G12 crosses to *qa-2 aro-9 a* were performed, the ratio of white:black ascospores was approximately 2:1 indicating that RIP occurred at a high frequency at least for the *qa-2* locus.

The idea of protection associated with mating-type sequences comes largely from the fact that previous attempts to RIP *mt A* proved to be difficult in the region coding for *mt A*-2 and *mt A-3* (Glass and Lee, 1992). Alternatively, the possibility existed that *mt A-2* mutants would only exhibit a drastic sexual phenotype when both *mt A*-2 and *mt A-3* genes were mutated, as in the A^{IIRIP} mutant.

3.3.3.3 DNA Methylation and RFLP Analyses of *mt A-2* RIP progeny

To determine if RIP occurred in *mt A*-2 without an obvious phenotype in *A* progeny, the first and second generation progeny (Table 3-3) were selected for RFLP, DNA methylation and sequence analyses. Some first generation progeny from crosses with G11 and G12 were crossed to OR *a* to produce second generation progeny. G11 second generation progeny 57-1, 57-3, 57-4 and 57-5 were from crosses of E57 to *un-3 a*. E86 was also crossed to *un-3 a* and generated 86-1. Crosses between E89 to *un-3 a* gave 89-6 and 89-9. Strains 196-2, 196-3 and 196-4 were generated from crosses of T196 with *un-3 a*.

G11		G12	
1st generation	2nd.generation	1st generation	2nd.generation
E47	57-1	T48	196-2
E57	57-3	T161	196-3
E86	57-4	T196	196-4
E89	57-5	T211	
	86-1		
	89-6		
	89-9		

Table 3-3 Progeny selected from *mt A-2* transformants for methylation and/or RFLP analyses. See text for parent of second generation progeny.

Figure 3-6 shows Southern blots of genomic DNA of G11 and its *A* progeny. DNA methylation analysis of the progeny was done by Southern blot of DNA digested with the isoschizomers *Sau3A*I and *Mbo*I (methylation sensitive and insensitive, respectively). When a *mt A*-2 probe was used, three bands (0.5, ~0.9 and ~1.6 kb), corresponding to the resident gene, were observed (see recipient strain, RLM 52-22). The initial transformant, G11, had these three resident bands but also had two extras bands corresponding to the *mt A*-2 ectopic copy (1.4, 1.8 kb). The presence of some bands larger than 3 kb when G11 was digested with *Sau3A* I suggests that methylation of *mt A*-2 and *qa-2* sequences occurred during vegetative growth of this transformant. Three classes of G11 *A* progeny can be seen in Figure 3-6: 1) One in which the ectopic copy was lost (E47, 57-3, 57-4); 2) A class in which the ectopic copy was maintained but little or no methylation was observed associated with *mt A*-2 sequences (E86 and E89) and 3) A class in which the ectopic *mt A*-2 shows methylation (57-1 and 57-5). Progeny 57-5 also shows RFLP associated with the resident *mt A*-2, suggesting mutation occurred due to RIP. Re-probing the blot with the *qa-2*$^+$ gene provided a control for efficiency of restriction site digestion and methylation associated with the introduced *qa-2*$^+$ sequences. DNA from classes where the ectopic sequences were maintained showed methylation of *qa-2*$^+$. Progeny E86 also showed restriction site alteration associated with *qa-2* (Panel B). The methylation that occurred during

vegetative growth of G11 seemed to be more associated with the *qa-2* gene than with *mt A*-2 sequences (see Figure 3-6 panel B).

Southern blots of transformant G12 and its *A* progeny are shown in Figure 3-7. G12 has only one extra band corresponding to integrated *mt A*-2 sequences. As with G11, methylation of *mt A*-2 sequences also occurred after transformation and prior to being taken through a cross. Unlike G11 however, this methylation was more apparent with *mt A*-2 sequences rather than with *qa-2* sequences (Figure 3-7). Progeny of G12 either do not contain ectopic copies (196-2, 196-3 and 196-4) or contain the ectopic copy with associated methylation of *mt A*-2 and *qa-2* sequences (T48, T161 and T211). T211 methylation resembles the pattern seen in G12 (including the simple hybridisation profile with *qa-2* sequences). T48 and T161 show a high degree of methylation of the *mt A*-2 sequences. T161 also exhibited a restriction site alteration associated with the ectopic *mt A*-2 and *qa-2* sequences.

Independently of the Southern blot analysis, a few progeny derived from G11 (E47, 57-1, 57-3, 57-4, 57-5, E86) and G12 crosses (T161, T211, T196, 196-2, 196-3, 196-4) were screened for mutations using PCR and restriction enzymes to detect RFLP changes in the resident *mt A*-2 gene. DNA from these progeny was used for PCR amplification. *mt A-2* was amplified using primers rI.1/rI.2 (see Appendix 1), that specifically amplify the resident copy. PCR products were then digested with *Hinf* I, *Mbo* II and *Hae* III, which recognise several sites in the *mt A-2* sequence. These sites mostly contain cytosines and guanines and changes in RFLP patterns would imply G:C to A:T mutations which are typical of RIP. Alterations in the restriction pattern of the resident *mt A-2* copy were not observed with any of these progeny (data not shown).

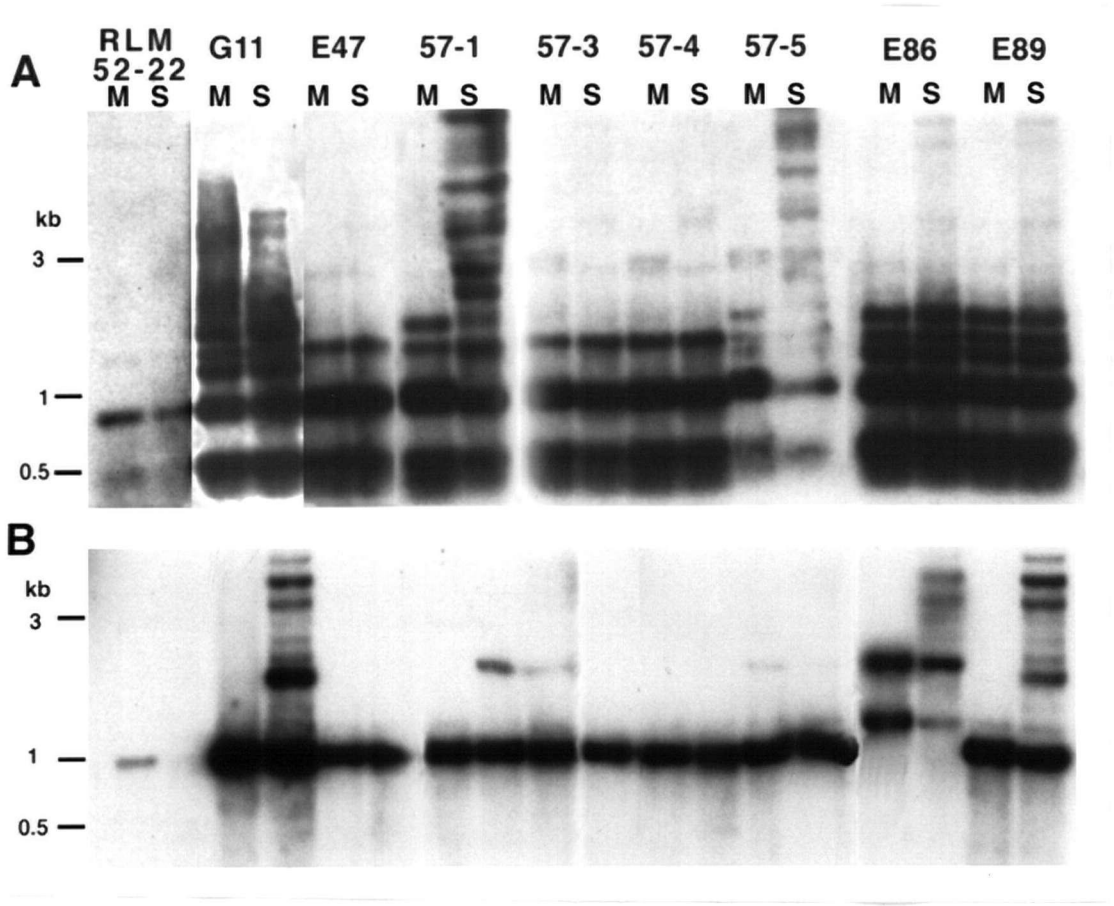


Figure 3-6 Southern blot of genomic DNA of G11 and *A* progeny. DNA was digested with methylation-insensitive and -sensitive enzymes, *Mbo*I and *Sau*3AI, respectively. Panel A shows hybridisation with a 1.1 kb *mt A-2* fragment. Panel B shows hybridisation to a 0.9 kb *qa-2* fragment. M=*Mbo*I; S=*Sau*3AI.

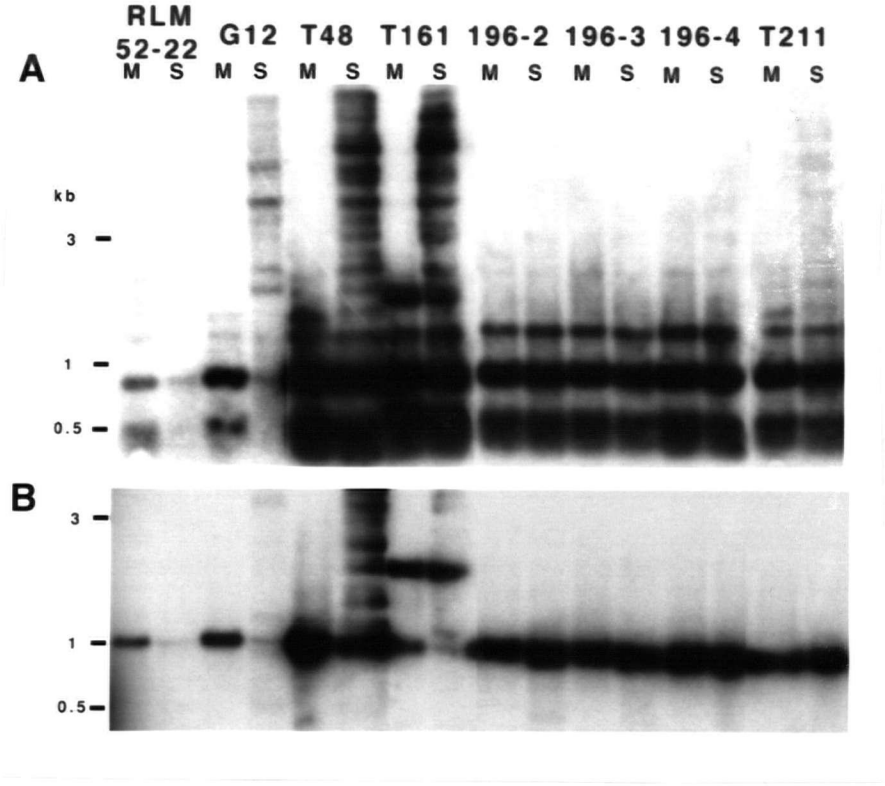


Figure 3-7 Southern blot of genomic DNA of G12 and *A* progeny. DNA was digested with methylation-insensitive and -sensitive enzymes, *Mbo*I and *Sau*3AI, respectively. Panel A shows hybridisation with a 1.1 kb *mt A-2* fragment. Panel B shows hybridisation to a 0.9 kb *qa-2* fragment. M=*Mbo* I; S=*Sau*3A I.

Ten progeny from crosses of E86 and E89 to *un-3 a* were analysed in Southern blots. The DNA was digested with *Mbo*I and *Sau*3AI and the blots were hybridised with an *mt A*-2 fragment. No RFLP or DNA methylation was observed in the 20 *A* progeny, suggesting RIP mutations did not occur in *mt A*-2 (data not shown).

3.3.3.4 *mt A-2* mutational analyses

Based on DNA methylation and RFLP analyses, a few progeny were selected for DNA sequence analysis of *mt A-2*. The resident copy of *mt A-2* in E47, 57-1, E86 (and progeny 86-1), E89 (and progeny 89-6 and 89-8), T48, T161 and T211 was amplified by PCR using primers rI.1/rI.2 and their DNA sequence determined. Sequencing was initially done with primer rI.1 (upstream of *mt A*-2 start codon). A number of mutations were found in strain 57-1 which was then renamed A-2^{m1}. No mutations were found in the first 500 bases sequenced from other progeny.

About 1kb of the *mt A-2* DNA sequence of the A-2^{m1} was determined. Twenty-seven base pair changes were found in the A-2^{m1} mutant (Figure 3-8) and these mutations lead to 8 amino acid substitutions and two stop codons in the *mt A-2* ORF. The first stop codon (a TGG to TAG) would result in a truncated MT A-2 polypeptide of 195 aa instead of 324 aa (see Figure 3-9). About two thirds of the mutations occurred in 5'CpA 3' dinucleotides as in other RIP events (Grayburn and Selker, 1989). All mutations were of the same polarity, resulting in G to A changes on the *mt A*-2 coding strand.

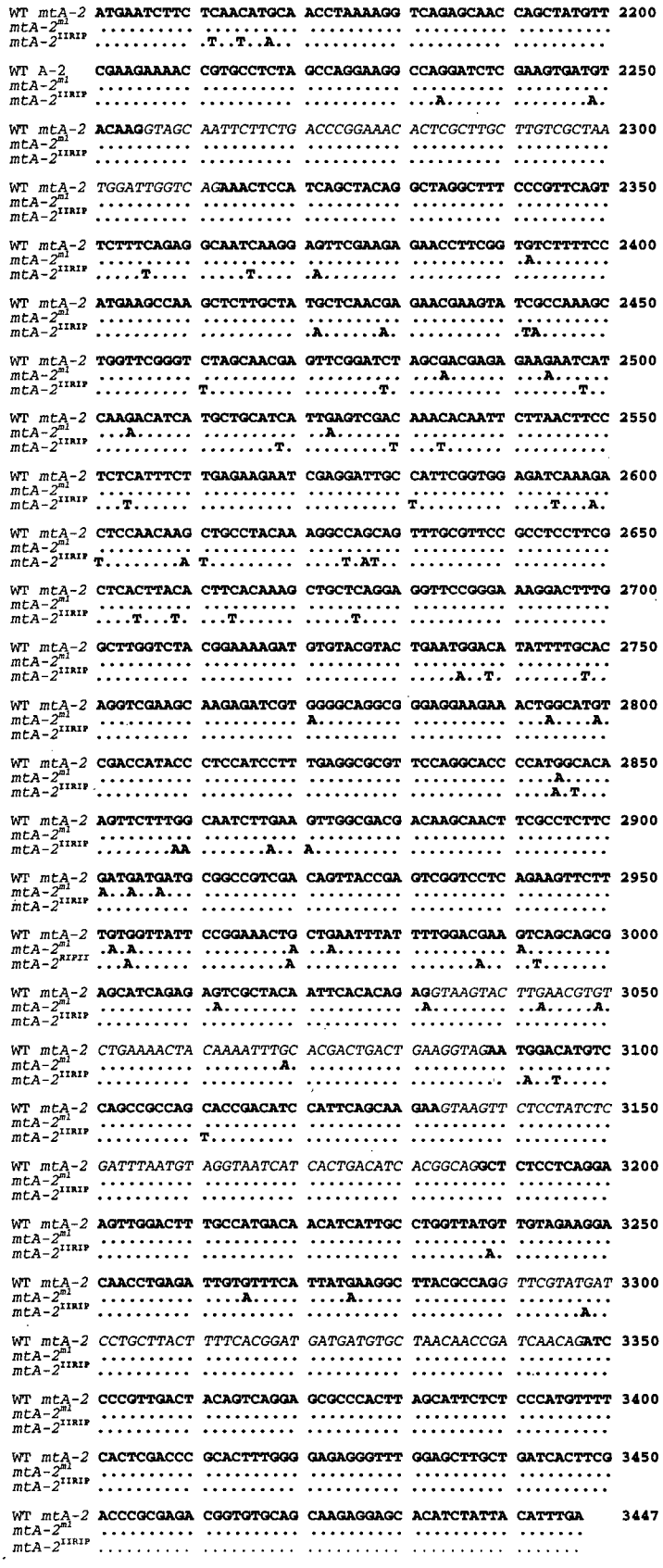


Figure 3-8 DNA sequence alignment of *mt A*-2 WT and the A-2^{m1} and *A*^{IIRIP} mutants. G:C to A:T transition are shown under the WT sequence and identity is represented by dots. Exons are bold and introns are italicised. Numbers on the right correspond to the idiomorph sequences as deposited in Gen Bank (accession no. M33876).

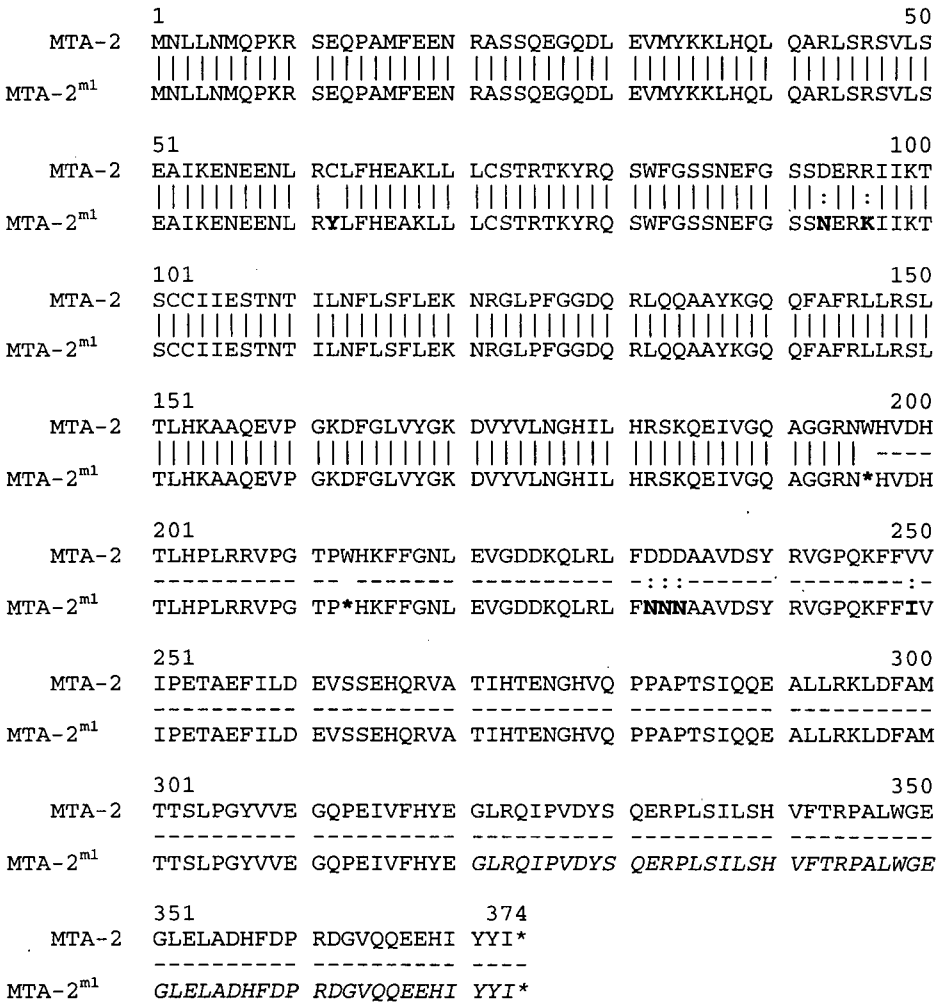


Figure 3-9 Protein sequence changes of MT A-2 in the A-2^{m1} mutant. Changes are shown in bold. Sequences presumably absent from the translated protein are indicated with horizontal bars. Asterisks indicate stop codons. Italics represent sequences not determined by DNA sequencing in the A-2^{m1} mutant.

3.3.4 Isolation of *mt A-3* through RIP

3.3.4.1 Isolation of *mt A-3* homokaryotic transformants

Transformants L22 and L25 were obtained after transformation of pAL2 into the C(2)2-9 strain. About 25 HygB^r transformants were randomly selected and grown with 250 U HygB/ml. The *mt A-3* transformants (Figure 3-10) were probed with 1.3 kb of the *hph* gene because *mt A-3* sequences weakly cross-hybridise with ribosomal DNA (Figure 3-11; S. Saupe pers. comm.) making it difficult to differentiate single and multiple copy transformants. Transformants L22 and L25 were selected for a RIP cross. Homokaryotic transformants were obtained after 4 subcultures of single conidial isolations on medium containing hygromycin.

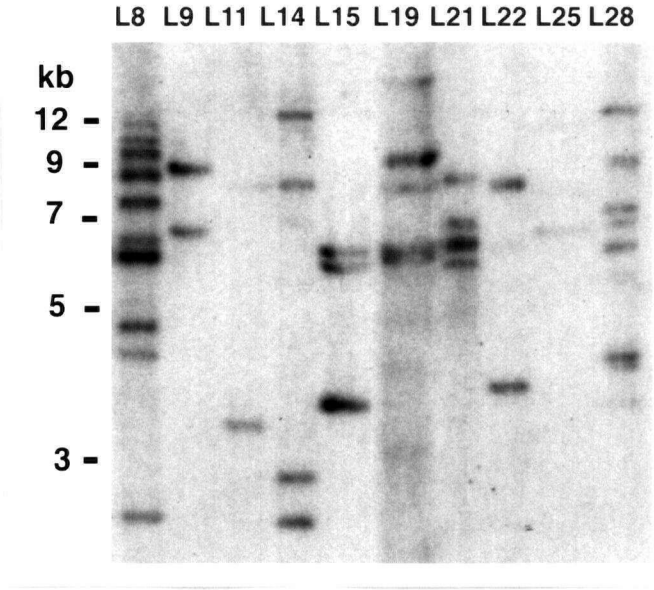


Figure 3-10 Southern Blot of genomic DNA of *mt A-3* transformants. DNA of transformants containing different number of integrated copies was digested with *Hin*dIII and hybridised with a 1.3 kb *Bam*HI *hph* probe.

3.3.4.2 Isolation of *mt A-3* RIP progeny

The L22 and L25 homokaryotic transformants were crossed to an *un-3 a* strain so that *a* progeny could be negatively selected. Because the *un-3* gene is very closely linked to *mt*, progeny carrying the *un-3* mutant allele are temperature sensitive and have the *a* mating-type. Growth of heat-shocked ascospores at 30°C selects against *a* progeny.

As for *mt A-2* transformants, the fertility of progeny from the *mt A-3* RIP crosses was tested on both *fl A* and *fl a* tester strains. The ratio *A/a* progeny were approximately 1:1 in the L22 and L25 crosses. Table 3-4 shows the number of progeny from L22 and L25 transformants tested for fertility. Ascospore formation was scored at 14 days after fertilisation by observation of ascospores on filter paper that was laid on top of the crosses. Analyses of progeny from *mt A-3* RIP crosses did not reveal any abnormal sexual phenotype. The failure to observe a phenotype in the RIP crosses could be due to similar reasons as that of the *mt A-2* transformants.

The L22 *mt A-3* transformant gave 35% of progeny that had an abnormal growth phenotype (excessive branching). This phenotype was not due to RIP of mating type sequences or to RIP of a gene closely linked to it, because a number of *a* progeny also exhibited the growth abnormality. As the initial transformant does not exhibit the abnormal phenotype it could not be explained by disruption of a gene upon insertion of the plasmid. Since RIP has been shown to extend beyond the duplicated sequences (Irelan *et al.*, 1994), this phenotype is probably due to RIP of a gene located close to the insertion point of the duplicated sequence. This indirectly suggested that RIP was triggered by duplication of the *mt A-3* sequences and thus *mt A-3* was also likely mutated.

3.3.4.3 DNA Methylation and RFLP Analyses of *mt A-3* RIP progeny

A few *mt A*-3 first and second generation progeny (Table 3-5) were selected for RFLP, DNA methylation and sequence analyses. The strain V24, which exhibited the abnormal growth phenotype, and three of its progeny, 24-14, 24-19 and 24-46 (from cross with OR *a*) were analysed. Additional second generation progeny were generated by crossing V20 and B5 to OR *a* (see Table 3-5) and subsequently analysed.

Homokaryotic transformant	**Total progeny isolated from cross to *un-3 a***	**Total *A* progeny tested for fertility on *fl a***
L22	100	100
L25	100	100

Table 3-4 Progeny of L22 and L25 homokaryotic transformants tested for fertility on *fl* tester plates.

In contrast to the *mt A*-2 transformants (G11 and G12) the two initial *mt A-3* homokaryotic transformants, L22 and L25, did not exhibit DNA methylation of *mt A-3* sequences prior to a cross (Figure 3-11 and Figure 3-12). *Mbo* I restriction sites are common in the *mt A-3* sequence and thus fragments observed in Southern blots are relatively small (< 0.5 kb) in the recipient strain C(2)2-9 (Figure 3-11 Panel A). In addition, a non-specific band of 0.8 kb is present as shown by hybridisation of the *mt A-3* probe to an *a* strain (Figure 3-11 Panel A).

Figure 3-11 shows Southern blots of transformant L22 and some *A* first and second generation progeny. L22 has a 0.7 kb band corresponding to the integrated plasmid copy. Because of the small sizes of fragments generated by *MboI/Sau3A*I digestion, DNA methylation was not easily recognised. The occurrence of high molecular weight bands may be due to partial digestion, which were confirmed in some cases (24-46 and V92) when the blot was probed with *qa-2* (Panel B) (pAL2 transformants do not contain duplicated copies of the *qa-2* gene - see

section 3.2.2). Methylation of *mt A-3* occurred in V20, 20-6, 24-19, 24-46 and V92. In these lanes, digestion with the methylation sensitive enzyme *Sau*3AI revealed a decrease of low molecular weight bands and appearence of higher molecular weight bands in some cases (20-6, V24, 24-46 and V92) The 0.7 kb band corresponding to the ectopic copy of *mt A-3* was lost in 24-46, V32 and V92 and in 24-46 and V32 a new 1 kb band appeared with both restriction enzymes which was probably the result of mutation in *mt A-3*.

L22		L25	
1st.generation	2nd.generation	1st.generation	2nd.generation
V20	24-14	B5	5-1
V24	24-19	B29	5-3
V32	24-46	B68	5-6
V92	20-6	B69	
		B76	
		B99	
		B100	

Table 3-5 Progeny selected from *mt A-3* transformants for methylation and/or RFLP analyses.

Southern blots of transformant L25 and its progeny are shown in Figure 3-12. In the L25 transformant, the ectopic integrated fragment that hybridised with *mt A-3* sequences was ~ 0.8 kb and only progeny B5, B99 and B100 retained it (Figure 3-12- Panel A). Digestion of DNA from progeny B99 and B100 with *Sau*3AI revealed appearence of high molecular weight bands, indicative of DNA methylation in *mt A-3* sequences. These bands cannot be attributed to partial digestion because the control blot with *qa-2* sequences showed restriction digestion was not partial (see Panel B).

Some progeny of *mt A-3* transformants were also screened for mutations through RFLP changes in the 1.1 kb resident *mt A-3* PCR product. DNA from V24, 24-14, 24-46, V32 and B99 were used for PCR amplification of the resident copy of *mt A-3* with primers rII.1/rII.2 (see Appendix 1). As in the *mt A-2* RFLP analyses, PCR products were digested with three restriction enzymes (*Hinf* I, *Mbo*II and *Hae*III) which have multiple sites in the *mt A-3* sequence. RFLP

changes were observed in strain 24-46 as compared to the WT control when cut with *Hinf* I (data not shown) suggesting a mutation had occurred in *mt A-3* in this strain.

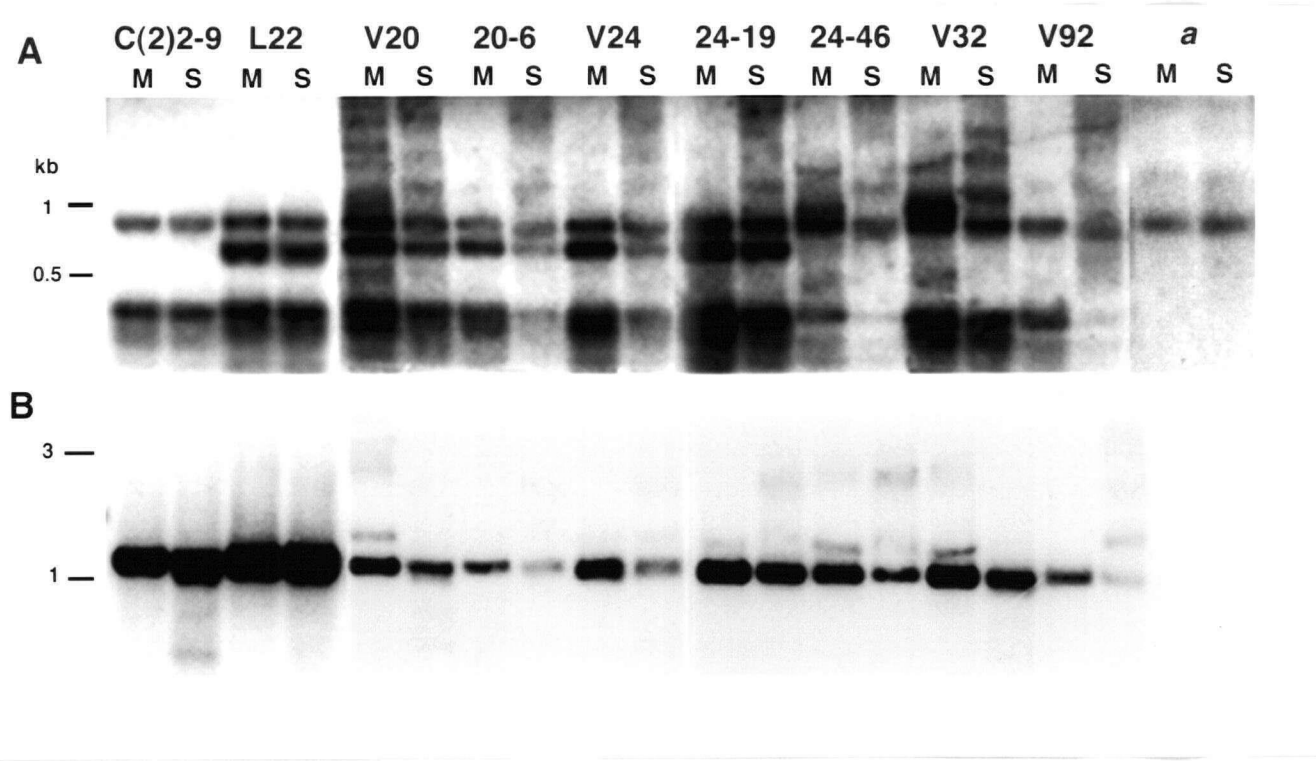


Figure 3-11 Southern blot of genomic DNA of L22 transformant and progeny. DNA was digested with methylation-insensitive and -sensitive enzymes, *Mbo*I and *Sau*3AI, respectively. An *a* strain was included as a control of non-specific cross-hybridisation to *mt A-3* sequences, presumably ribosomal DNA. Panel A shows hybridisation with a 1.1 kb *mt A-3* fragment. Panel B shows hybridisation to a 0.9 kb *qa-2* fragment. M=*Mbo*I; S=*Sau*3AI.

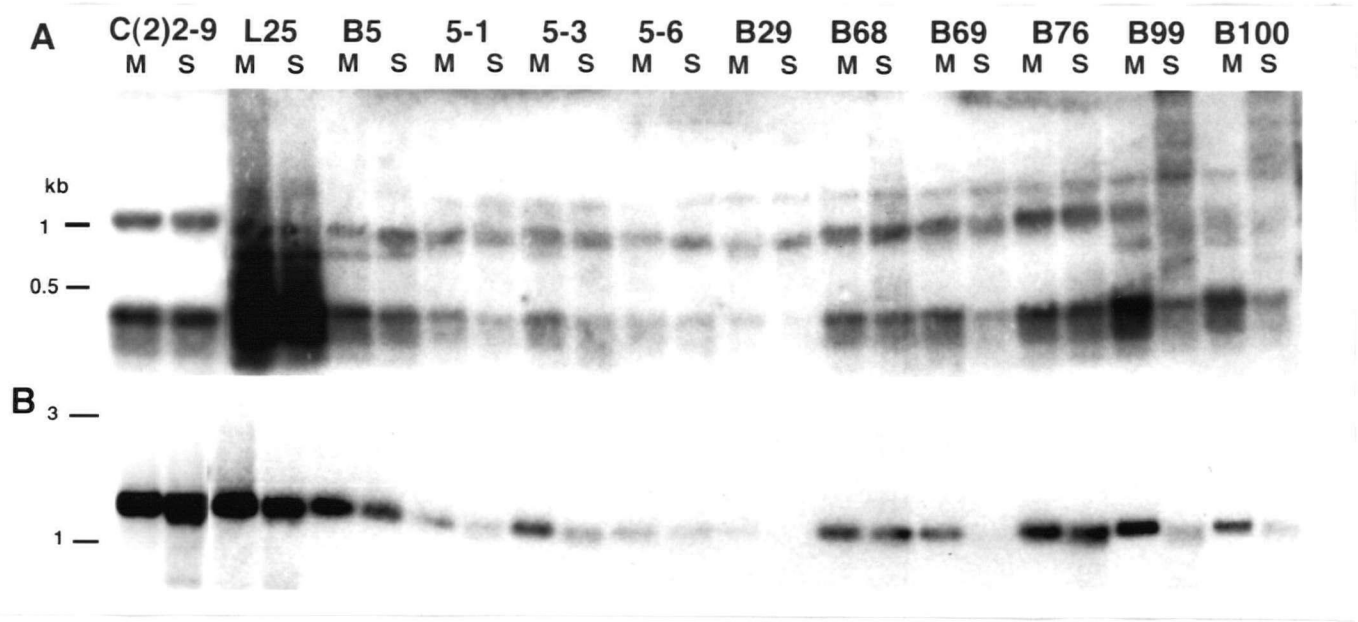


Figure 3-12 Southern blot of genomic DNA of L25 transformant and *A* progeny. DNA was digested with methylation-insensitive and -sensitive enzymes, *Mbo*I and *Sau*3AI, respectively. Panel A shows hybridisation with a 1.1 kb *mt A-3* fragment. Panel B shows hybridisation to a 0.9 kb *qa-2* fragment. M=*Mbo*I; S=*Sau*3AI.

3.3.4.4 *mt A-3* mutational analyses

A few progeny from the *mt A-3* transformants were chosen for DNA sequence analysis. V24 is one of the L22 progeny likely to be a result of RIP because it exhibited the abnormal growth phenotype (section 3.3.4.2) and was selected for DNA sequence analyses. Progeny 24-46 and V32, which showed RFLP changes in Southern analyses (Figure 3-12) and 24-14 were also sequenced. About 400 bp of *mt A-3* PCR fragments were initially sequenced with a primer located about 50 bp before the start codon. G:C to A:T mutations in *mt A-3* were observed in strain 24-46 (but not in the parental V24) and in progeny V32 (see Figure 3-13). Progeny 24-46 and V32 were renamed A-3^{m1} and A-3^{m2}, respectively.

There were 28 mutations in the first 600 bp of the *mt A-3* ORF in the A-3^{m1} mutant (Figure 3-13). They lead to only 3 aa substitutions and a nonsense mutation at position 84 of the protein, originally a tryptophan TGG codon in the *mt A-3* ORF (Figure 3-14). Fifty-seven percent of the changes occurred in 5'CpA 3' dinucleotides (Figure 3-13). The *mt A-3* ORF of the A-3^{m2} mutant suffered only 11 base changes in exons and another 6 mutations in the second intron (Figure 3-13). A nonsense mutation occurred at position 112 of the protein due to a change from a CAA glutamine to a TAA in nucleotides 661-659 (Figure 3-14). Both mutants have stop codons in the ORF before the region coding for the HMG domain.

3.3.5 Expression of *mt A-2* and *mt A-3* in mating type mutants

Because A-2^{m1} and A-3^{m1} mutants were able to produce abundant ascospores, the severe phenotype observed in the A^{IIRIP} mutant was suspected to be due to absence of both *mt A-2* and *mt A-3*. Sequencing of the promoter region of *mt A-2* and *mt A-3* of the A^{IIRIP} mutant showed mutations in possible regulatory regions. To determine if *mt A-2* and *mt A-3* transcripts were produced in the mating type mutants, a series of RT-PCR experiments was performed. Total RNA from WT *A*, A-2^{m1}, A-3^{m1} and A^{IIRIP} strains grown in crossing media was used for the reverse transcriptase reaction. Figure 3-15 shows the separation of PCR fragments after amplification with primers specific for the three *A* mating-type genes (see Appendix 1 for primers). Amplification of *mt A-1* transcripts was used as the control for efficiency of the reverse-transcriptase reaction. Transcripts corresponding to *mt A-2* were absent in cDNA preparations from the A-2^{m1} and the A^{IIRIP} mutants (Panel A). PCR with the cDNA preparation from A-3^{m1} mutant did not contain a band corresponding to the *mt A-3* transcript. With the A^{IIRIP} mutant no fragment corresponding to the *mt A-3* cDNA was observed in amplifications (Panel B). The *mt A-1* transcripts were present in all A-2^{m1}, A-3^{m1} and A^{IIRIP} mutants indicating that

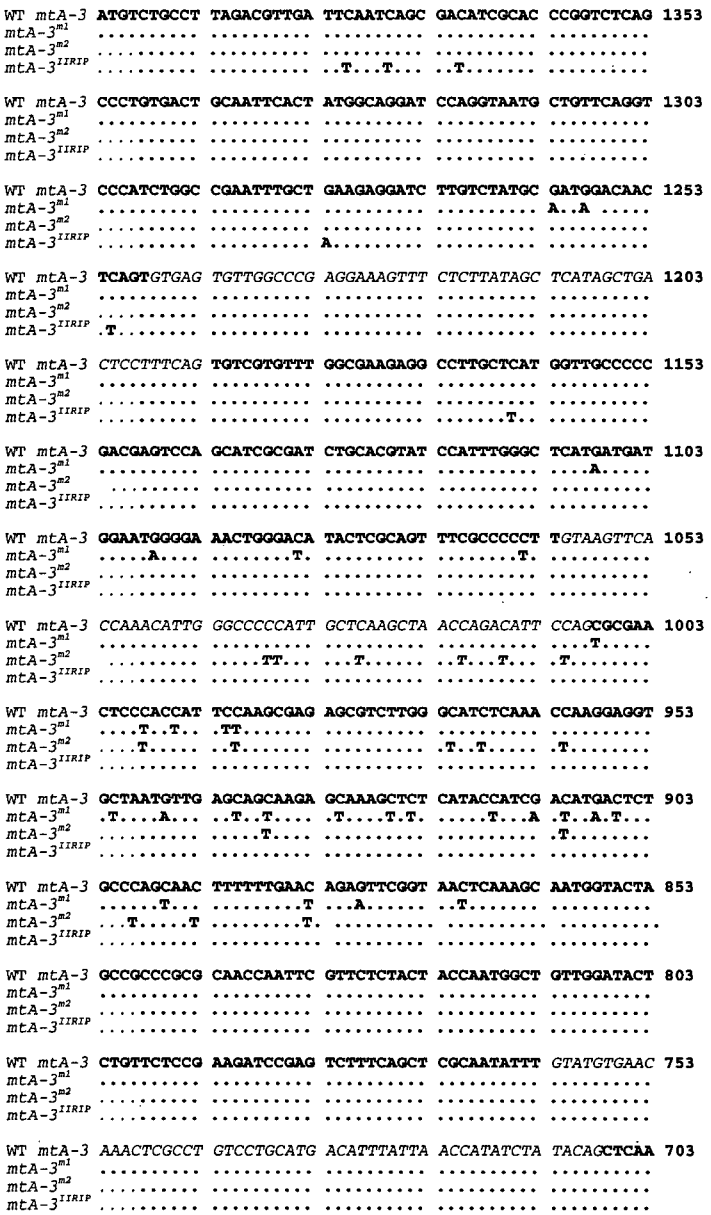


Figure 3-13 DNA sequence alignment of *mt A-3* mutants with the wild-type sequence. G:C to A:T transition changes are shown under the WT sequence and identity is represented by dots. Exons are bold and introns are italicised. Numbers on the right correspond to the idiomorph sequences as deposited in Gen Bank (accession no. M33876). Only sequenced segments are shown.

A

```
WT      1 MSALDVDSISDIAPGLSPVTAIHYGRIQVMLFRSHLAEFAEEDLVYAMDN 50
          |||||||||||||||||||||||||||||||||||||||||||||||.:||
A-3m1   1 MSALDVDSISDIAPGLSPVTAIHYGRIQVMLFRSHLAEFAEEDLVYTIDN 50

         51 SVVVFGEEALLMVAPDESSIAICTYPFGLMMMEWGNWDILAVSPPSRTPT 100
            ||||||||||||||||||||||||||||:||| ----------------
         51 SVVVFGEEALLMVAPDESSIAICTYPFGLIMME*GNWDILAVSPLLRTPT 100

        101 IPSESVLGISNQGGANVEQQEQSSHTIDMTLPSNFFEQSSVTQSNGTSRP 150
            --------------------------------------------------
        101 IPSESVLGISNQGGANVEQQEQSSHTIDMTLPSNFFEQSSVTQSNGTSRP 150

        151 RNQFVLYYQWLLDTLFSEDPSLSARNISQIVAGLWNSEHPAAKARFRELA 200
            --------------------------------------------------
        151 RNQFVLYYQWLLDTLFSEDPSLSARNISQIVAGLWNSEHPAAKARFRELA 200

        201 EMEVHRHRAENPHLYPDQPRFPTTDPVPPRMRYPCVISPEDRQRILRMLD 250
            --------------------------------------------------
        201 EMEVHRHRAENPHLYPDQPRFPTTDPVPPRMRYPCVISPEDRQRILRMLD 250

        251 FVWEESNGQLAAEEAALNDVVQPQQAEEVGPFPDFEWEEPNHIIDMSTDL 300
            --------------------------------------------------
        251 FVWEESNGQLAAEEAALNDVVQPQQAEEVGPFPDFEWEEPNHIIDMSTDL 300

        301 SVAQDPDFMMTEDDSMRFLLKQAS* 325
            -------------------------
        301 SVAQDPDFMMTEDDSMRFLLKQAS* 325
```

B

```
WT      1 MSALDVDSISDIAPGLSPVTAIHYGRIQVMLFRSHLAEFAEEDLVYAMDN 50
          ||||||||||||||||||||||||||||||||||||||||||||||||||||
A-3m2   1 MSALDVDSISDIAPGLSPVTAIHYGRIQVMLFRSHLAEFAEEDLVYAMDN 50

         51 SVVVFGEEALLMVAPDESSIAICTYPFGLMMMEWGNWDILAVSPPSRTPT 100
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
         51 SVVVFGEEALLMVAPDESSIAICTYPFGLMMMEWGNWDILAVSPPSRTPT 100

        101 IPSESVLGISNQGGANVEQQEQSSHTIDMTLPSNFFEQSSVTQSNGTSRP 150
            | ||||||||| ||||||| ------------------------------
        101 ILSESVLGISN*GGANVEQ*EQSSHTIDMTLPSNFFEQSSVTQSNGTSRP 150

        151 RNQFVLYYQWLLDTLFSEDPSLSARNISQIVAGLWNSEHPAAKARFRELA 200
            --------------------------------------------------
        151 RNQFVLYYQWLLDTLFSEDPSLSARNISQIVAGLWNSEHPAAKARFRELA 200

        201 EMEVHRHRAENPHLYPDQPRFPTTDPVPPRMRYPCVISPEDRQRILRMLD 250
            --------------------------------------------------
        201 EMEVHRHRAENPHLYPDQPRFPTTDPVPPRMRYPCVISPEDRQRILRMLD 250

        251 FVWEESNGQLAAEEAALNDVVQPQQAEEVGPFPDFEWEEPNHIIDMSTDL 300
            --------------------------------------------------
        251 FVWEESNGQLAAEEAALNDVVQPQQAEEVGPFPDFEWEEPNHIIDMSTDL 300

        301 SVAQDPDFMMTEDDSMRFLLKQAS* 325
            -------------------------
        301 SVAQDPDFMMTEDDSMRFLLKQAS* 325
```

Figure 3-14 Protein sequence changes of MT A-3 in the A-3^{m1} (Panel A) and in the A-3^{m2} mutants (Panel B). Amino acid changes are shown in bold. Sequences presumably absent in the translated protein are indicated with horizontal bars. Asterisks indicate stop codons.

reverse-transcriptase reaction was efficient and yielded sufficient quantities of cDNAs for PCR amplification (Panel C). This experiment suggested that A^{IIRIP} expresses *mt A-1* but not *mt A-2* or *mt A-3* and that A-2^{m1} and A-3^{m1} do not express *mt A-2* and *mt A-3*, respectively.

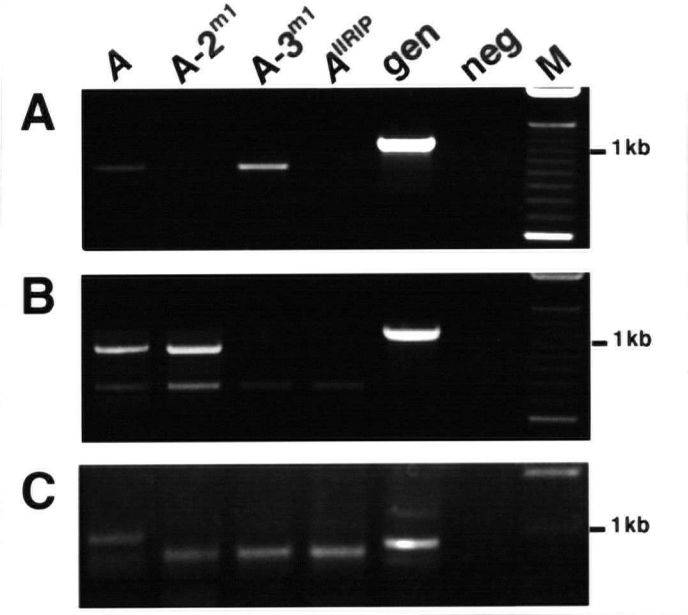


Figure 3-15 Amplification of cDNA of mating-type genes in the WT OR *A*, A-2^{m1}, A-3^{m1} and A^{IIRIP} mutants by RT-PCR. Genomic DNA (from WT OR *A*) was included in all experiments as a control for amplification reaction and for size comparison with cDNA. Panels A and B show amplification products (~0.9 kb cDNA, 1.1 kb genomic) using primers specific for *mt A-2* and *mt A-3*, respectively. Panel C shows amplification of *mt A-1* cDNA used as control for reverse-transcriptase reaction (~0.8 kb). Some cDNA preparations contained traces of genomic DNA that was also amplified in the PCR. A non-specific 0.6 kb band appears in *mt A-3* cDNA amplifications.

3.3.6 Phenotypic characterisation of *mt A-2* and *mt A-3* mutants

To determine if a sexual function was specifically associated with *mt A*-2 and *mt A-3*, crosses of the A-2^{m1}, A-3^{m1} and A-3^{m2} mutants to WT strains were analysed in detail. Crosses between *P. anserina* strains mutated individually at each mating-type gene to wild type strains produced perithecia of different sizes, a decrease in the number of ascospores and abnormal ascospores, haploid croziers and uniparental progeny (Zickler *et al.*, 1995). In order to establish if *N. crassa mt A*-2 and *mt A-3* mating-type genes function in a similar manner, the number of ascospores were scored and the possible production of uniparental progeny was tested in crosses with A-2^{m1}, A-3^{m1} and A-3^{m2} mutants.

3.3.6.1 Microscopic analyses of mutants

Crosses were made to different *a* strains,Oak Ridge (OR) and Mauriceville background, using the mutants as either the maternal or paternal partner. Crosses were initially made to OR strains because they are the most common laboratory strains. Figure 3-16 to Figure 3-19 show perithecial squashes of A-2^{m1}, A-3^{m1} and A-3^{m2} crosses to the *ad-3A a* strain (RLM 52-22) as compared to the untransformed and initial transformant controls at 8 and 11 days post-fertilisation.

In a wild-type cross, karyogamy occurs 4 days post-fertilisation and meiosis procedes immediately after nuclear fusion. At 8 days post-fertilisation, most ascospores are delimited and start to become mature (black). Ascospore discharge begins at 8 days post-fertilisation and can still be observed 12-15 days after a cross (for more details see Chapter 1 section 1.3.3.2). Crosses of G11 and A-2^{m1} to the OR strain exhibited a few asci with less than eight ascospores (Figure 3-16). A few asci in A-2^{m1} crosses also contained ascospores which were twice as large as wild-type size. These type of asci also appeared (though less frequently) in crosses of G11. Most of the crosses to the OR strain, including control crosses, exhibited "bubble asci " (Raju *et al.,* 1987) which can be observed as 8 small round structures inside the asci (Figure 3-16 to Figure 3-19). In crosses between inbred strains it has been observed that up to 75% of asci abort after completion of the nuclear divisions and the asci contain eight very small vacuolated inviable ascospores. Genetic analyses indicate that they are the result of a combination of weak deleterious recessive genes when inbred lines are crossed (Raju *et al.,* 1987). When *mt A-2* and *mt A-3* mutants (as well as the transformant controls) were crossed to a distantly related strain (Rockefeller-Lindegren), ascospore formation was normal (N. Raju's observations). Figure 3-20 and Figure 3-21 show perithecial squashes of the mutants crossed to a Mauriceville *a* strain (FGSC 2226),

which is also distantly related to the laboratory OR strains. Rosettes contained more asci with viable ascospores than in the OR crosses. Very few bubble asci were present. Qualitatively, the mutants and control crosses did not differ. Abnormalities previously observed in crosses of G11 and A-2^{m1} to OR *a* strain, such as large ascospores or asci containing less than eight ascospores, occurred very infrequently (not shown). This observation suggests the abnormalities are also recessive deleterious mutations due to inbred crosses.

3.3.6.2 Ascospore production

Crosses were made between A-2^{m1}, A-3^{m1} and A-3^{m2} mutants and an *a* strain (NLG R1-42) and the number of ascospores were counted at both 8 and 11 days post-fertilisation. At 7 days post-fertilisation ascospores start to become black. At 11 days post-fertilisation most ascospores are mature and start to be ejected from the perithecia (see Chapter 1 section 1.3.3.2). A total of 5 samples containing 10 perithecia each, randomly selected from one crossing plate, was collected. Table 3-6 and Table 3-8 show the number of ascospores per 10 perithecia, derived from hematocytometer counts. Statistical analyses to determine if ascospore reduction was due to the *mt A*-2 and *mt A-3* mutations were done using ANOVA (Appendix 3) and Student's *t*-test at confidence levels of 0.05. Probabilities of the *t*-test associated with each mutant are shown in Table 3-7 and Table 3-9 . All calculations were done in comparison to the original untransformed control, using two-tailed distribution and considering data as two-sample of unequal variance. The transformant control was also included in the analyses so a decrease in ascospore formation due to factors such as localisation of transforming DNA or other factors intrinsic to the original transformant could be evaluated.

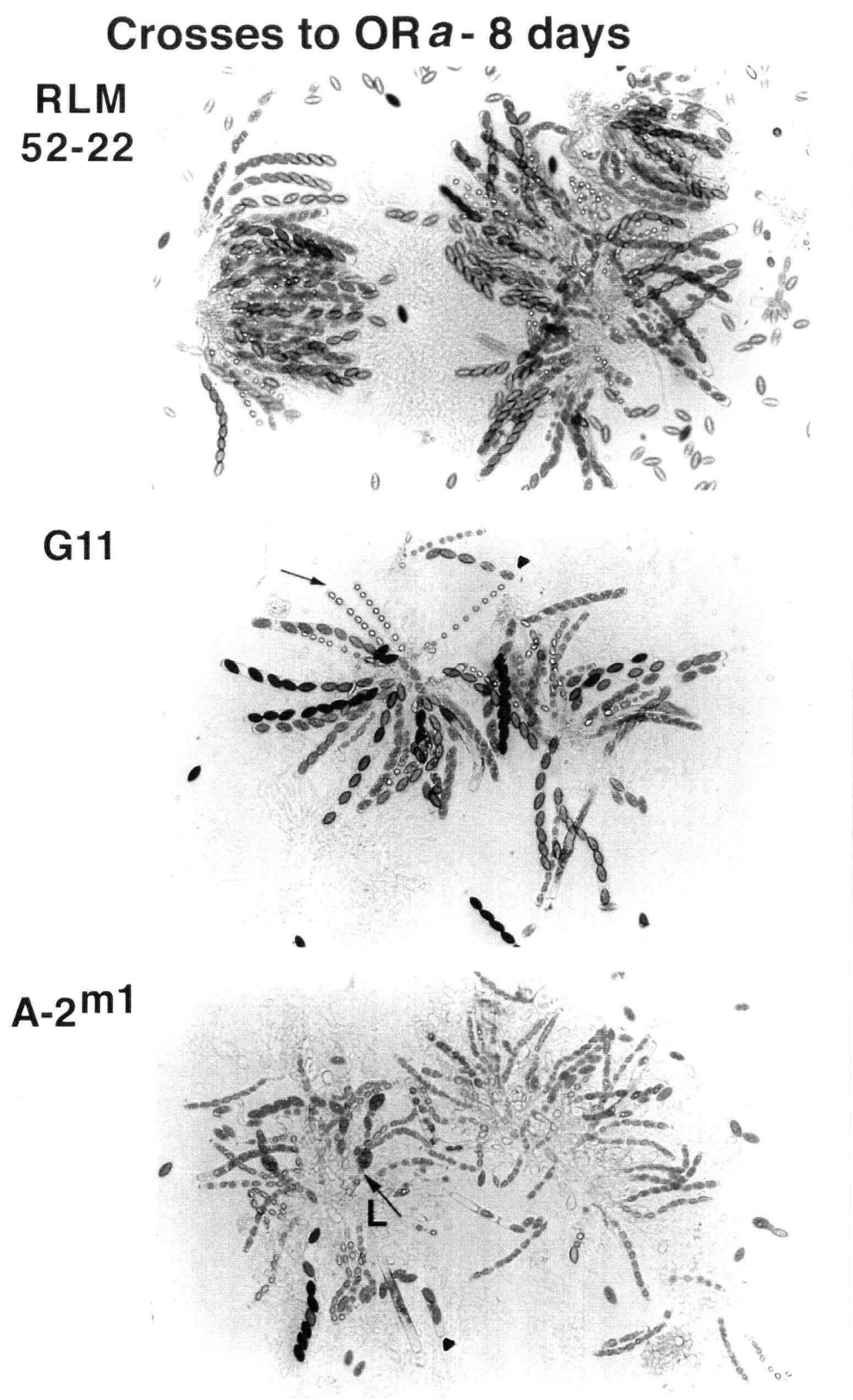


Figure 3-16 Perithecial squashes of crosses between the *mt A-2* mutant and controls (recipient RLM 52-22 and transformant G11) to the OR strain at 8 days post-fertilisation. Most asci in all three crosses have eight imature ascospores (white). Arrows indicate bubble asci with inviable ascospores. Bubble asci are more common in the transformant G11 and the A-2^{m1} mutant than the control RLM 52-22. Small arrow heads show asci containing less than eight ascospores. L shows one ascus containing abnormally large ascospores.

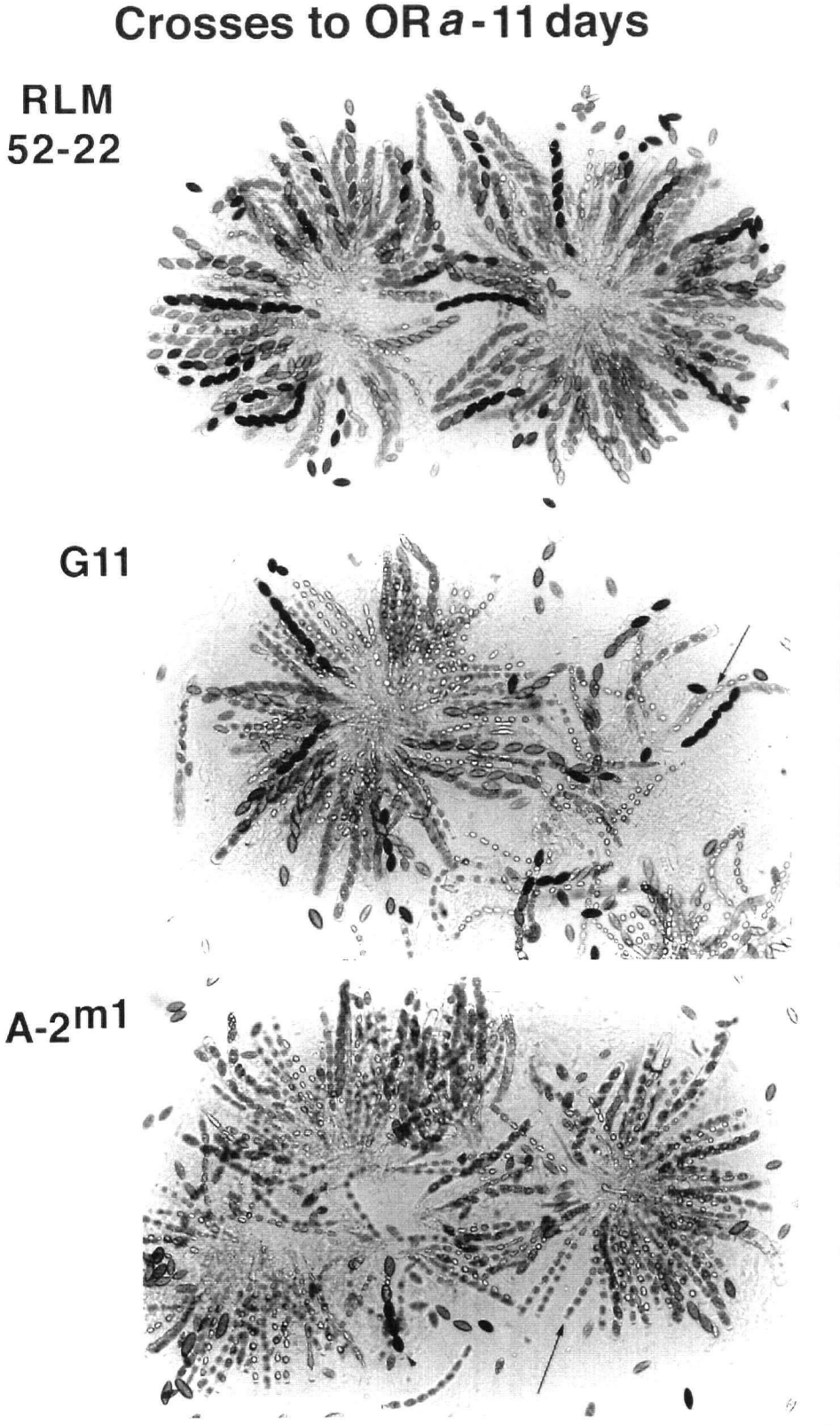


Figure 3-17 Perithecial squashes of crosses between the *mt A-2* mutant and controls to the OR *a* strain at 11 days post-fertilisation. A larger number of asci and mature ascospores (black) is observed in the control RLM 52-22. At this point discharge of ascospores have started. Bubble asci are more frequent in A-2^{m1} (arrows). Small arrowheads indicate ascospore containing less than eight spores.

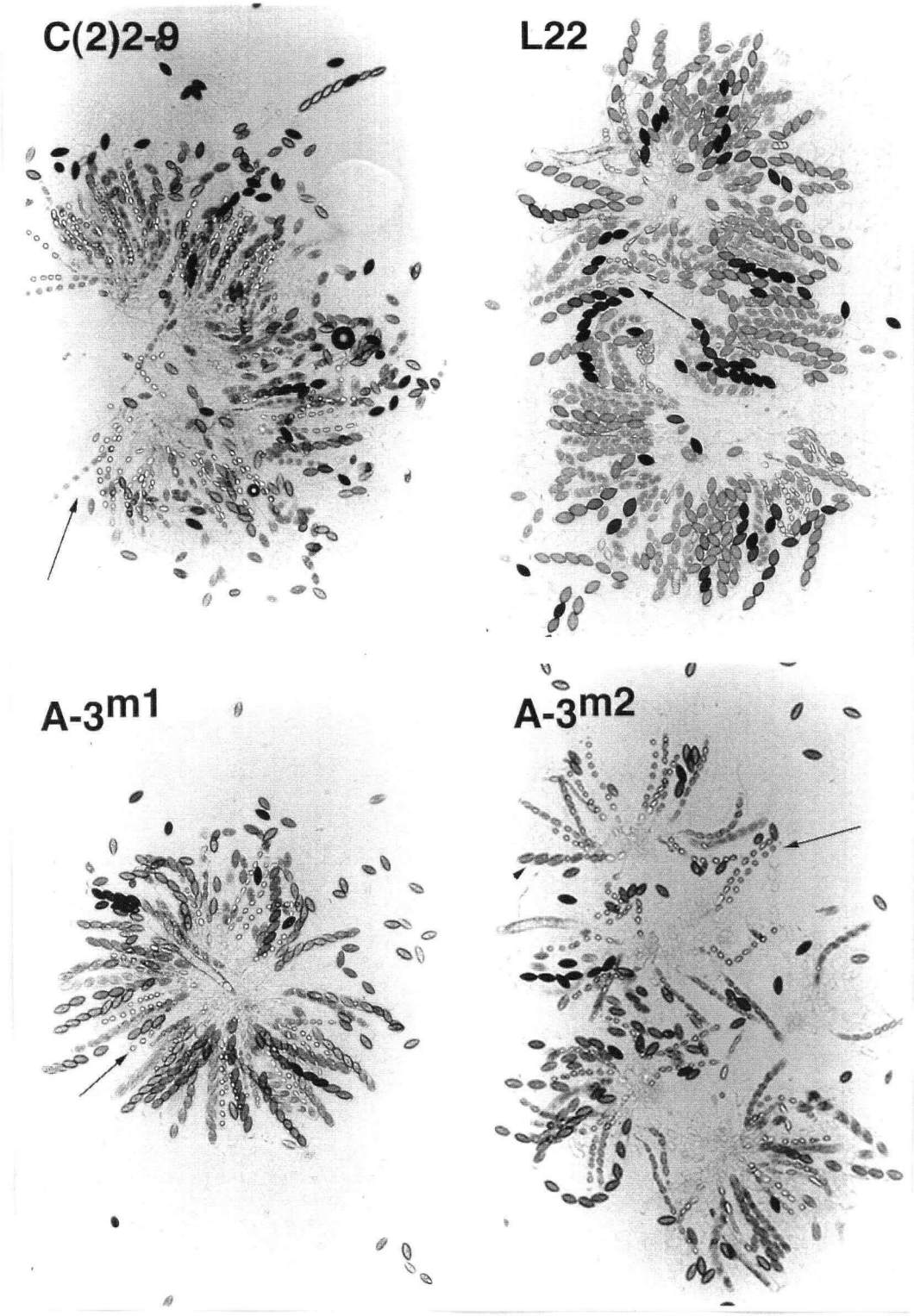


Figure 3-18 Perithecial squashes of crosses between the *mt A-3* mutants and controls (recipient NLG C(2)2-9 and initial transformant L22) to the OR *a* strain at 8 days post-fertilisation. Arrows as in Figure 3-16.

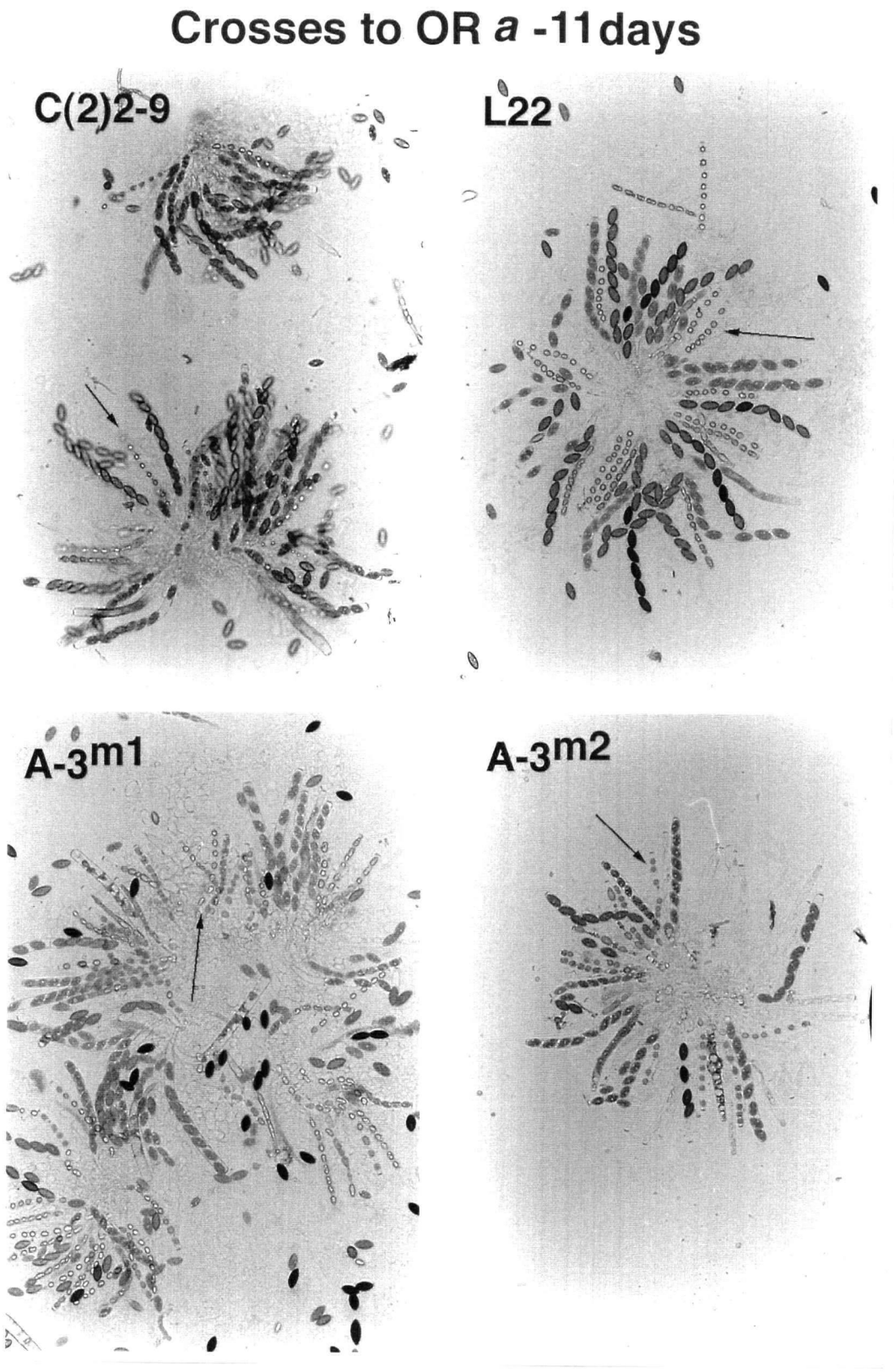


Figure 3-19 Perithecial squashes of crosses between the *mt A-3* mutants and controls to the OR *a* strain at 11 days post-fertilisation. Arrows indicate aborted asci.

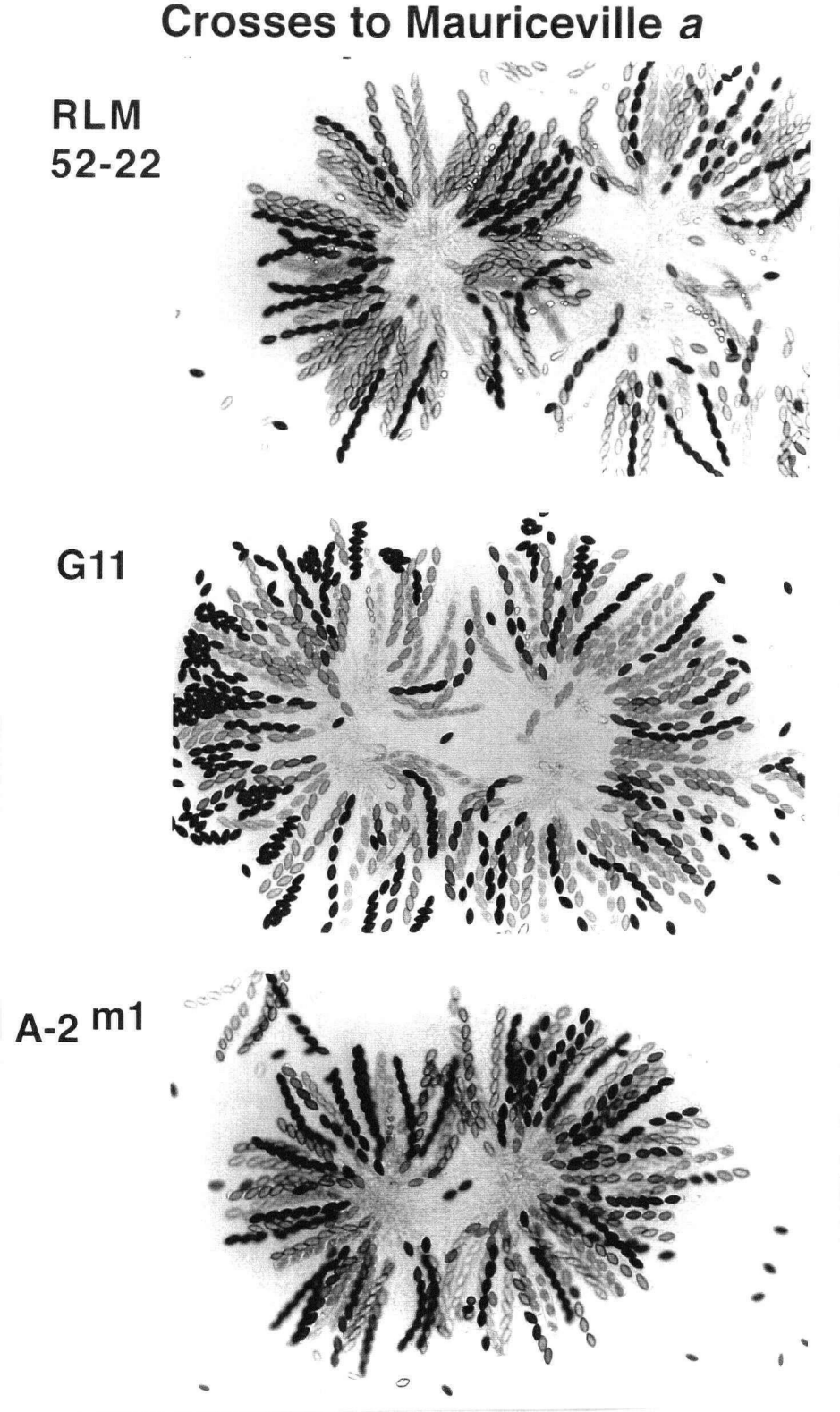


Figure 3-20 Perithecial squashes of the A-2^{m1} mutant and controls crossed to a Mauriceville *a* strain at 11 days post-fertilisation. More fertile asci are observed in these crosses than in crosses with the OR background.

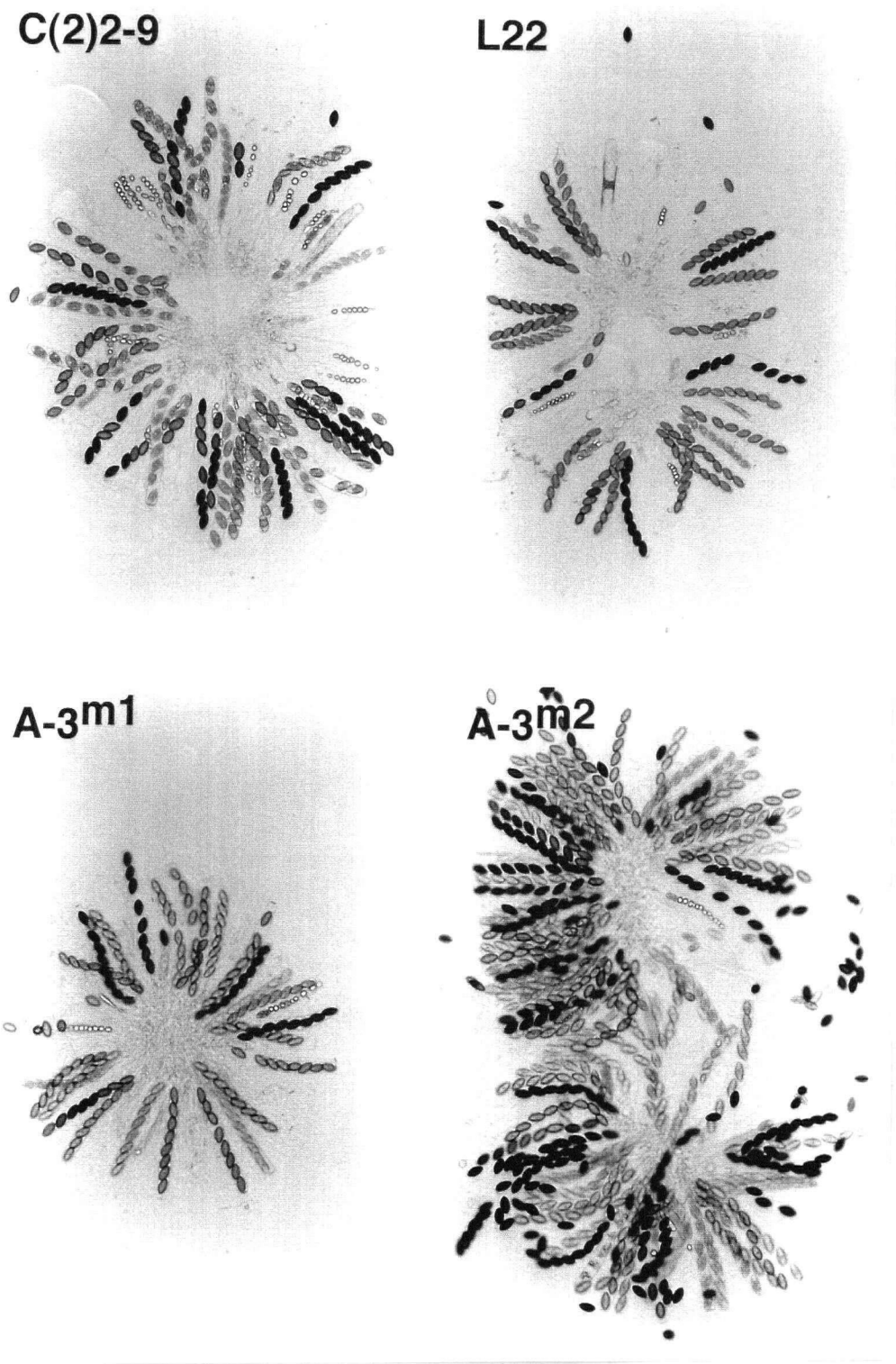


Figure 3-21 Perithecial squashes of A-3^{m1} and A-3^{m2} mutants and controls crossed to a Mauriceville *a* strain at 11 days. Legend as in Figure 3-20

ANOVA test between the A-2^{m1} mutant and the RLM 52-22 and G11 controls indicated variation at 8 days post-fertilisation (F=7.7) and not at 11 days (F=2.6). t-test indicated that the A-2^{m1} mutant showed decrease in ascospore number at 8 days after fertilisation ($p<0.005$) while a decrease was not observed in the parent transformant G11 ($p>0.5$). These results indicate a delay in the development of ascospores in the A-2^{m1} mutant as compared to the parental strain.

	RLM 52-22	G11	A-2^{m1}
8 days	6625	5062.5	1125
	6750	3437.5	1312.5
	4000	4250	1125
	4250	2812.5	1687.5
	5437.5	10062.5	1375
Average	**5412.5**	**5125**	**1325**
SD	1483.8	978.1	265.1
11 days	7375	5937.5	6250
	5875	6687.5	5000
	8875	5937.5	8062.5
	6125	6437.5	2375
	7000	5562.5	3625
Average	**7050**	**6112.5**	**5062.5**
SD	1375	375	2388.8

Table 3-6 Number of ascospores produced per perithecium by A-2^{m1} mutant and parental strains, 8 and 11 days post-fertilisation. SD=standard deviation.

	t-Test probability	
	8 days	**11 days**
A-2^{m1}	0.0017	0.1270
G11	0.8460	0.1591

Table 3-7 t-test probability values for A-2^{m1} mutant and initial transformant G11 at 8 and 11 days post-fertilisation. Probabilities were generated by comparisons done to number of ascospores of the recipient strain RLM52-22. Confidence levels at $p \leq 0.05$.

The two *mt A-3* mutants showed different results in this experiment. ANOVA test including A-3^{m1}, the controls NLG C(2)2-9 and L22 show significant decrease in ascospore

formation at 8 days but not 11 days (F=9.5 and 3.3, respectively). This decrease, however, occurred in the transformant L22 and not the A-3^{m1} mutant (see t-test Table 3-9). ANOVA tests for the A-3^{m2} mutant and controls had F values of 28.1 and 14.3 at 8 and 11 days post-fertilisation. t-tests indicated that a significant decrease in ascospore number ocurred for A-3^{m2} ($p< 0.005$ for both days).

	NLG C(2)2-9	L22	A-3^{m1}	A-3^{m2}
8 days	7187.5	1250	7375	687.5
	6312.5	3000	3125	1125
	5812.5	3250	4937.5	1937.5
	6000	3500	6437.5	750
	9562.5	4062.5	6812.5	2812.5
Average	**6975**	**3012**	**5737**	**1462**
SD	608.9	1020.6	1857.2	575.0
11 days	10562.5	8875	3562.5	2375
	9125	8625	8000	2125
	6750	4625	5062.5	2875
	6562.5	3562.5	2500	375
	6500	5437.5	2250	1750
Average	**7900**	**6225**	**4275**	**1900**
SD	1933.1	2724.9	2389.4	1087.3

Table 3-8 Number of ascospores produced per perithecium by A-3^{m1} and A-3^{m2} mutants and parentals , 8 and 11 days post-fertilisation. SD= standard deviation.

	t-Test probability	
	8 days	**11 days**
A-3^{m1}	0.2648	0.0282
A-3^{m2}	0.0003	0.0006
L22	0.0020	0.2536

Table 3-9 t-test probability value for A-3^{m1} and A-3^{m2} mutants at 8 and 11 days post-fertilisation and initial transformant L22. Probabilities were generated by comparisons done to number of ascospores of the recipient strain NLG C(2)2-9. Confidence levels at $p \leq 0.05$.

A number of factors can affect the number of ascospores produced in a WT cross. The results cannot be taken separately from the fact that these crosses were done to a strain of Oak Ridge background. A decrease in ascospore formation would be expected due to formation of bubble asci (see section 3.3.6.1 and Figure 3-16). A significant decrease was only observed with

the A-3^{m2} mutant. This mutant, however did not originate from the second generation thus the expected frequency of bubble asci was smaller than for the other mutants. The lower production of spores by the A-3^{m2} mutant may be due to the fact that this mutant produced smaller perithecia. Size of perithecia can be related to several causes including strain background and nutrition. If a significant decrease in ascospore formation had been observed for all mutants, these experiments would have to be repeated using an unrelated strain.

The number of ascospores from A^{IIRIP} crosses was also counted at 8 days post-fertilisation. The average number was 10 ascospores per 10 perithecia. A^{IIRIP} was not included in the statistical analyses. Figure 3-22 Panel B shows an example of a rosette of this mutant. A few perithecia contain a few, apparently normal, 8-spored asci.

3.3.6.3 Uniparental progeny analyses

The A-2^{m1}, A-3^{m1}, A-3^{m2} and A^{IIRIP} mutants were crossed to the *R al-1 a* (FGSC 2088) strain to test for the production of *A* uniparental progeny. The presence of spindle-shaped ascospores in crosses with *R a* means that no *a* nucleus entered the crozier prior to karyogamy because the *R* mutation is ascus dominant and thus all eight ascospores (4*A* and 4*a*) are normally round. Over 50 perithecia from each cross were squashed at 8 days after fertilisation. Considering about 200 asci per perithecia (except for A^{IIRIP}) more than 10000 asci were observed for each cross. In all of the crosses, only round ascospores were observed. Figure 3-22 shows two examples: Panel A is a cross between A-3^{m2} to *R al-1 a* and Panel B is a cross of A^{IIRIP} to *R al-1 a* showing one of the very few perithecia that contained ascospores. These results indicate that the *N. crassa* mating type genes, unlike the *P. anserina* homologs, do not seem to be required for cellularisation of opposite nuclei in the crozier. The only report of uniparental progeny production in *N. crassa* was from a cross between *chol-2, ylo-1, trp-2, a* and a translocation

strain *T(IIR;VIR)R2459 arg-12 fl A* (Barry, 1995). Only a single uniparental ascus was observed and seemed to be the result of a failure to distinguish self from non-self due to unknown mechanisms.

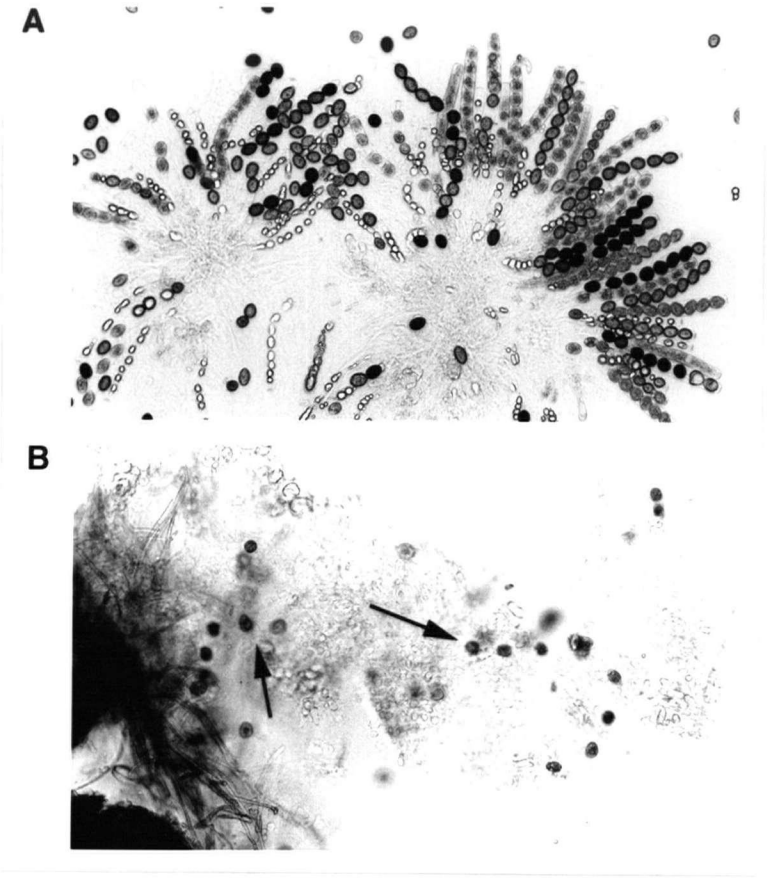


Figure 3-22 Perithecial squashes of crosses of A-3^{m2} and *A^{IIRIP}* mutants to *R a.* Panel A shows A-3^{m2} rosettes 8 days after fertilisation showing production of round spores. Panel B show cross between *A^{IIRIP}* and *R*. Most perithecia from *A^{IIRIP}* crosses do not contain rosettes; only a small number of perithecia produce a few asci bearing ascospores. Arrows indicates asci containing 8 ascospores.

3.3.6.4 Heterokaryon incompatibility tests

MT A-1 is responsible for mating identity and for mating-type associated heterokaryon incompatibility during vegetative growth. All of the *mt A-1* mutants (with one exception) lose their mating and heterokaryon incompatibility functions simultaneously (Griffiths, 1982, Saupe *et al.,* 1996). However, it is not known whether *mt A-2* and *mt A-3* are intact in these mutants. Although compatible heterokaryons between A^{IIRIP} and *un-3 a* were never observed (Glass and Lee, 1992) it is not known if the *mt A-2* and *mt A-3* genes play separate roles in heterokaryon incompatibility.

The A-2^{m1} and A-3^{m1} mutants were tested for loss of heterokaryon incompatibility function. *A* progeny with appropriate auxotrophic markers were isolated from crosses between A-2^{m1} and A-3^{m1} mutants with *a* strains carrying different markers (Table 3-1). The A-3^{m1} *pyr-4* strain was obtained after cross to RLM 57-26. A-2^{m1} *ad-3B* was obtained from a cross to NLG R1-42. Figure 3-23 and Figure 3-24 show graphs comparing the growth of heterokaryons formed between mating-type mutants and *a* strains with incompatible or compatible controls. All tests were done in triplicate. One replicate value (from A-3^{m1}/a) was withdrawn from the analysis from day 3 because it had "escaped" and thus exhibited wild-type growth (DeLange and Griffiths, 1975). The growth of A-2^{m1}/*a* and A-3^{m1}/*a* heterokaryons was similar to the growth of an incompatible *A/a* heterokaryon. Hence *mt A-2* and *mt A-3* do not appreciably affect heterokaryon incompatibility mediated by mating-type.

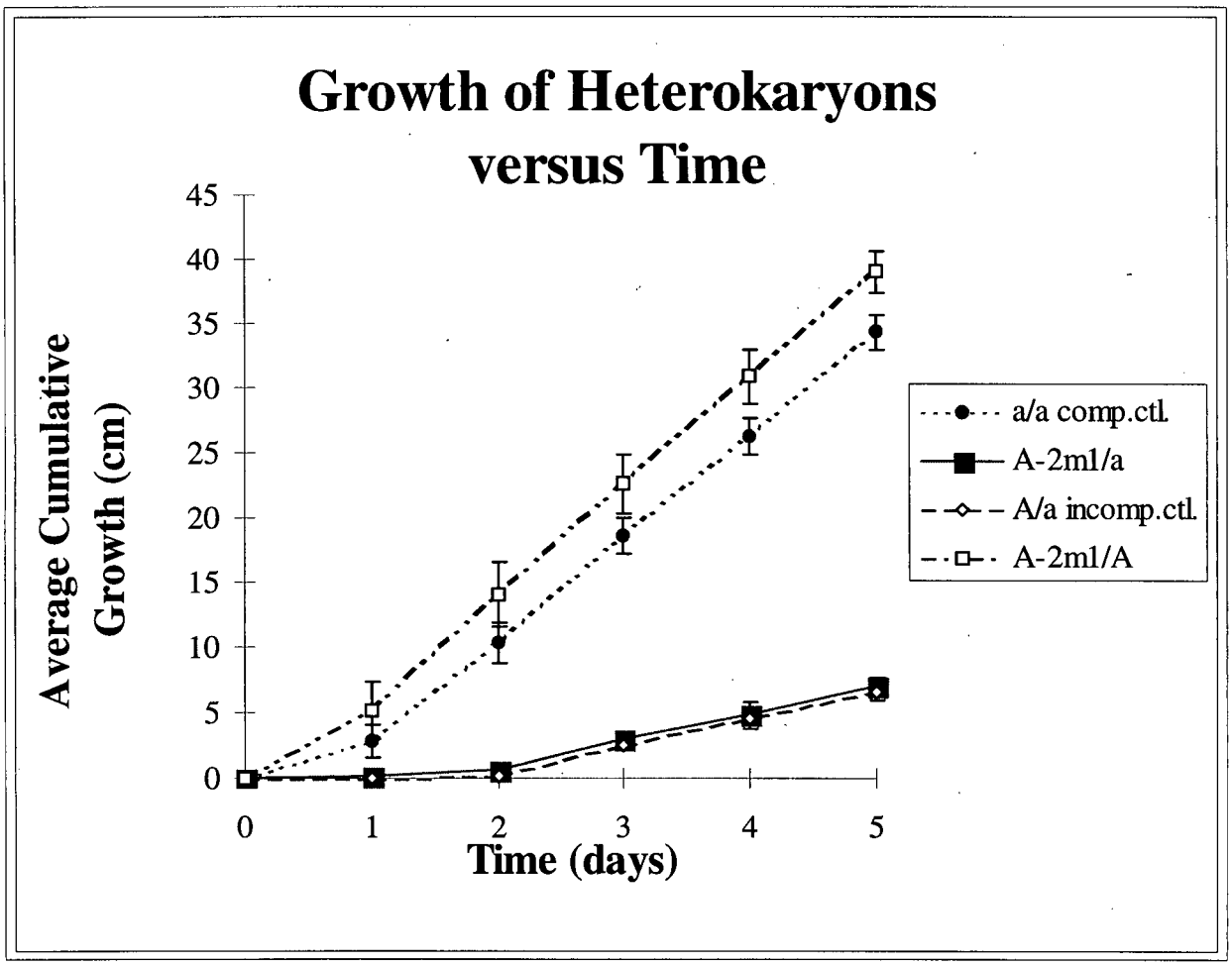


Figure 3-23 Linear growth of A-2^{ml} mutant heterokaryons and heterokaryon compatible and incompatible controls. Compatible control (*a/a*) was a heterokaryon formed between strains carrying *ad-3B a* (NLG R1-42) and *qa-2 aro-9 a* (NLG R2-54); *A/a* incompatible control was a *ad-3B arg-1 a* (I-20-26)/*qa-2 aro-9 A* (RLM 52-22). Heterokaryons were forced between the A-2^{ml} mutant and *A* and *a* strains bearing *qa-2 aro-9* markers.

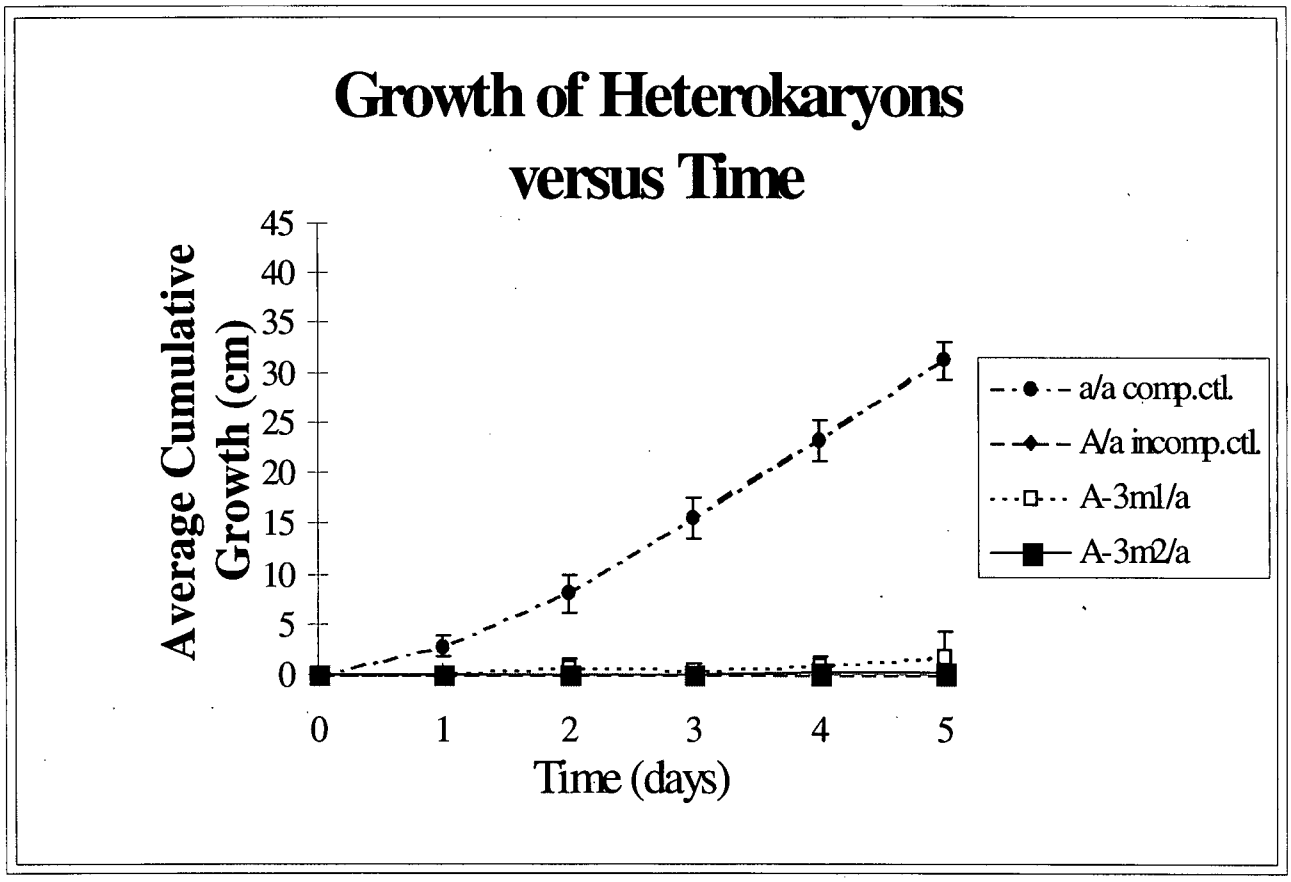


Figure 3-24 Linear growth of A-3^{m1}/*a* and A-3^{m2}/*a* heterokaryons and heterokaryon compatible and incompatible controls. Compatible control (*a/a*) was a heterokaryon formed between strains carrying *pyr-4 arg-5 inl pan-2 a* (RLM 57-26) and *qa-2 aro-9 a* (NLG R2-54); *A/a* incompatible control was a *pyr-4 cyh-1 a* (RLM 57-30)/*qa-2 aro-9 A* (RLM 52-22). Heterokaryons were forced between the *qa-2 aro-9 a* and the A-3^{m1} and A-3^{m2} mutants.

3.4 DISCUSSION

3.4.1 Gene replacement and RIP of mating-type sequences

Functional studies of mating-type genes have to be done at the *mt* locus because full sexual development cannot be restored in mating-type mutants by transformation with ectopic copies. Gene replacement would be the first choice for mutational analyses because of its specificity: unlike methods of random mutagenesis, gene replacement targets mutation specifically to the gene and the analyses are not complicated by mutations at other genes.

Various methods were used in this study and by others in our laboratory to obtain gene replacement at the *mt* locus by homologous recombination. Nevertheless, gene replacement strains were not obtained. The recombination frequency at the *mt* locus was estimated to be not greater than 0.25% (Chang and Staben, 1994). This estimate was based on a gene replacement of *mt A* with more than 3 kb of non-homologous sequences (*mt a* and the *hph* gene) and mating-type flanking sequences. The size of the fragment used for *mt A-3* disruption was considerably smaller (~1.6 kb) than that used in *a* replacement. The number of transformants screened in the experiments using pHA-3 was high enough to uncover at least one gene replacement strain, based on previous recombination frequency estimates at the *mt* locus. If the extent of homology of *mt A-3* sequences to the recipient strain is taken into account, more gene replacement events should have been observed.

Length and/or percentage of homology is known to play a very important role in homologous recombination in other organisms and may also play a role at the mating-type locus. Apparently, the *mt* locus requires higher homology in order for homologous recombination to occur. Gene replacement at the *mt* locus was obtained with a cosmid containing the whole *mt A* locus with more than 13 kb of flanking sequences on each side (T. Vellani, pers. comm.) This

cosmid was transformed into an *a* strain mutated at *tol* (Newmeyer, 1970) which does not exhibit heterokaryon incompatibility associated with mating type. Among the 700 $HygB^R$ transformants selected, six were able to complete the sexual cycle and produced abundant ascospores. Genetic and molecular analyses indicate that the strains are the result of two-step gene replacements.

Other experiments designed to obtain gene replacements using clones containing intact or partially deleted *mt A* sequences plus approximately 1 kb of flanking sequences at each side have been performed (S. Saupe, unpubl. results). These constructs were introduced into *a* strains and the selection used was similar to that of Chang and Staben (1994). As with the *mt A-3* replacement experiments, the number of transformants screened was high enough to uncover possible gene replacement strains, although they were not recovered. It is difficult to explain why the replacement of the *A* resident by *a* sequences (Chang and Staben, 1994) was more frequent as compared to the replacement of *a* resident with *A* sequences. It has been proposed that a selection against certain types of replacement events at the *mt* locus may lower the recombination frequency (Chang and Staben, 1994). Since the level and length of homology in the flank sequences was the same in the two types of experiments, it could be that it is easier to replace a large sequence (*A*) by introducing a small fragment (*a*) than it is to displace a smaller genomic segment to include a larger one. Alternatively, because there are *A* mating-type specific genes in the centromere-proximal flanking region of the *A* idiomorph, it may be that recombinants which have *A* idiomorphic sequences with *a* flanking sequences would not be viable. In Chang and Staben's work (1994), only the centromere distal flank of the gene replacement strain was shown to be derived from the *A* recipient. The development of a gene-replacement method for the *mt A* is of special interest when fine analyses of different parts of the genes are to be assayed for

function (using site directed mutagenesis for example). For initial analyses of *mt A-2* and *mt A-3* function, RIP proved to be the best strategy.

Screening of RIP mutants was based on reduction in the number of ascospores based on the known phenotype of the A^{IIRIP} mutant. However, the *mt A-2* and *mt A-3* mutants did not exhibit a drastic reduction in ascospore number. RIP frequency values could not be accurately estimated since not every progeny was analysed for methylation and/or mutation. However, the failure to observe a high frequency of progeny that exhibited RFLP or methylation in Southern blots suggested that low frequencies of RIP occurred. Additional evidence for low frequency of RIP of *mt A-2* and *mt A-3* sequences was the failure to observe RIP in second generation progeny from individuals that escaped RIP in the first cross (E86 and E89). Although E86 and E89 still carried the *mt A-2* duplication, none of the 20 progeny derived from the E86 and E89 crosses analysed in Southern blots revealed RFLP or methylation of the *mt A-2* gene. Sequencing of 500 bp of *mt A-2* in three of these progeny also failed to reveal mutations. RIP is thought to involve DNA:DNA interactions and the length and extent of homology of the duplicated fragment are important for RIP efficiency (reviewed in Singer and Selker, 1995). The sizes of the duplicated mating-type sequences in the $A\text{-}2^{m1}$ and $A\text{-}3^{m1}$ RIP crosses were 1.3 and 2.5 kb respectively, and are thus in the range of sizes that can be easily mutated by RIP. The *SstII* fragment, used for RIP of the A^{IIRIP} mutant, was 3 kb and yet it only yielded one single mutant out of 157 (Glass and Lee, 1992). In contrast, the number of *mt A-1* RIP mutants was more frequent than for *mt A-2* and *mt A-3*; fourteen *mt A-1* RIP mutants out of 72 progeny were isolated (Glass and Lee, 1992). The relatively low RIP frequency of *mt A-2* and *mt A-3* in this study and that of Glass and Lee (1992) suggests that *mt A-2* and *mt A-3* are protected against RIP. Repeated attempts to isolate an *a* mutant by RIP have failed (J. Grotelueschen and R. Metzenberg, pers. comm.). A protection

against RIP associated with the mating-type sequences may be evolutionarily important since the sexual machinery would be compromised if mating-type genes were rendered non-functional. However, a more rigorous series of comparative RIP experiments using both *mt A* and *mt a* would be necessary to answer these questions.

Homologous recombination was shown to be related to transfer of DNA methylation (MIP) in *Ascobolus immersus* (Colot *et al.,* 1996). Even though it has not be shown that frequencies of meiotic recombination are associated with RIP methylation and mutation in *N. crassa* (Irelan *et al.,* 1994), DNA:DNA pairing seems to be important for RIP because RIP operates in a pair-wise manner (Fincham *et al.,* 1989). The lack of complementation by ectopic copies of mating-type sequences, low recombination rates and low RIP efficiency at the mating-type locus may explain several difficulties encountered in attempts to obtain a gene replacement and to induce RIP in the mating-type sequences. The same phenomenon may be responsible for problems with both gene replacement and RIP of *mt A-2* and *mt A-3*. One possibility is the presence of cis-acting elements that may influence DNA pairing and thus interfere with recombination and RIP. These sequences could be responsible for the existence of a different state of the chromatin at the *mt* locus during vegetative and sexual stages that may regulate silencing and recombination of mating-type sequences.

3.4.2 Functional analyses of *mt A-2* and *mt A-3* mutants

RT-PCR indicated that the A-2^{m1}, A-3^{m1} and A^{IIRIP} were null mutants. Perhaps *mt A-2* and *mt A-3* transcripts could not be detected in the A-2^{m1}, A-3^{m1} and A^{IIRIP} mutants due to mRNA instability. A phenomenon termed nonsense codon-mediated mRNA degradation has been shown in prokaryotes and eukaryotes (reviewed in Peltz and Jacobson, 1993). In the *S. cerevisiae* phosphoglycerol kinase, a nonsense codon within the first two thirds of the coding region led to

mRNA instability and degradation (Peltz *et al.,* 1994). Except for the *mt A-3* ORF in the A^{IIRIP} mutant, all of the other mutants have stop codons in the first half of the ORFs.

Mutations in either *mt A-2* or *mt A-3* alone do not lead to any drastic sexual development phenotype. Conversely, if both *mt A-2* and *mt A-3* are mutated, as in the A^{IIRIP} mutant, sexual development is strongly affected. The *mt A-2* and *mt A-3* mutants (including the A^{IIRIP} mutant) do not have an altered vegetative phenotype. They produce large number of conidia, form normal protoperithecia when grown in crossing media and do not interfere with heterokaryon incompatibility. Although *mt A-2* and *mt A-3* transcripts were observed in the vegetative phase, it is possible that they are post transcriptionally regulated as suggested by structural analyses of transcripts (see Chapter 2).

The altered sexual phenotype of the A^{IIRIP} mutant is very obvious even at the macroscopic level (perithecia are small and do not form pronounced beaks). The A-2^{m1} and A-3^{m} mutants may have a very subtle effect on the sexual cycle that was not recognized by microscopy. If A-2^{m1} and the two A-3^{m} mutants had been isolated before the A^{IIRIP} mutant, we may not have assigned *mt A-2* and *mt A-3* to any sexual function in *N. crassa* or may have concluded that they are redundant in function. These conclusions would have had some support based on data from other *Neurospora* species and from *C. heterostrophus. N. terricola* is a homothallic species in which both *A* and *a* idiomorphs have been cloned and sequenced (Beatty *et al.,* 1994; T. Vellani, unpubl. results). *N. terricola mt a-1* and *mt A-1* genes are very similar to the *N. crassa* homologs and can confer mating identity and heterokaryon incompatibility when introduced into *N. crassa* (Beatty, 1993; T.Vellani, unpubl. results). However, in *N. terricola,* sequences corresponding to *mt A-3* are absent and the *mt A*-2 ORF is interrupted by a nonsense codon. Moreover, the *N. terricola mt A-2* cDNA was not detected in RT-PCR experiments (T.Vellani, unpubl. results).

Although *N. terricola* is self-fertile and therefore does not mate *per se* the initial steps of the fertilisation process, karyogamy and some aspects of ascospore formation are similar to those of the heterothallic species (Raju,1978). *C. heterostrophus* MAT-1 and MAT-2 idiomorphs are very small and each contains only one gene functionally similar to either *mt A-1* and *mt a-1,* respectively (Turgeon *et al.*; 1993). There is no evidence for the presence of *mt A-2* or *mt A-3* homologs in *C. heterostrophus* and the sexual cycle completes in a fashion similar to that of *N. crassa,* with production of apothecia and ascospores. Although it is possible that homologs or analogs of *mt A-2* and *mt A-3* exist elsewhere in the genome of *N. terricola* or *C. heterostrophus,* this hypothesis has not been examined.

It is remarkable that the A^{IIRIP} mutant still manages to produce a few progeny which show normal segregation of the mating-type, since steps preceding karyogamy are greatly compromised (Glass and Lee, 1992). These few progeny come from normal asci, indicating that it is not an occasional spore that can survive from one ascus, but rather it is an occasional successful karyogamy that can proceed in spite of gene malfunction. Multiple rounds of mitosis occur in the ascogonium, prior to the migration of opposite mating-type nuclei into the crozier. After formation of the first crozier, additional croziers normally arise from adjacent ascogenous cells (Raju, 1992). Mutations of *mt A*-2 and *mt A-3* could interfere with the multiple mitotic divisions that give rise to the numerous nuclei in the ascogenous hyphae; in the A^{IIRIP} mutant this process, and not karyogamy, may be impeded. The data presented here suggests that the *mt A-1* and *mt a-1* genes are critical for sexual reproduction and *mt A*-2 and *mt A-3* are accessory and promote the production of numerous progeny.

3.4.3 *N. crassa* and *P. anserina* mating-types: functional conservation

The similarity in the structure of the mating-type loci of *P. anserina* and *N. crassa* strongly suggests that they have evolved from a common ancestor. However the levels of identity of mating-type genes also suggest that these genes may have diverged enough to function differently. The lack of vegetative incompatibility function in the *P. anserina FMR1* and *FPR1* genes is a good example of functional divergence. Even when introduced into *N. crassa, FMR1* and *FPR1* do not trigger vegetative incompatibility (Arnaise *et al.,* 1993). The similarity between MT A-2/SMR1 and MT A-3/SMR2 is mostly restricted to a stretch of 17 aa and the HMG domain, respectively, indicating that evolution of function could be greater than with the MT A-1/FMR1 and MT a-1/FPR1 proteins. The failure to complement post-fertilisation functions with heterologous mating-type genes between *N. crassa* and *P. anserina* suggested that either those genes are not functionally conserved or that *N. crassa* mating-type genes cannot be expressed ectopically in *P. anserina* (Arnaise *et al.,* 1993).

The present study shows that the *N. crassa* mating-type genes are not functionally equivalent to the *P. anserina* mating-type genes. The A-2^{m1} and A-3^{m} mutants did not exhibit a drastic reduction in ascospore number and uniparental progeny were not evident in crosses with these mutants. Uniparental progeny were not observed even in the few asci formed in the A^{IIRIP} double mutant . When *P. anserina* mating-type mutants were crossed to the opposite mating type wild-type, a decrease in ascospore production and a high incidence of uniparental asci and uninucleate croziers was observed (Zickler *et al.*, 1995). From the *P. anserina* study, it was suggested that the mating-type genes play an essential role for distinguishing self-non-self during crosses, allowing cellularisation of two opposite mating-type nuclei in the crozier (Zickler *et al.*, 1995). We cannot rule out the possibility that *mt A-2* and *mt A-3* are involved in the recognition

of opposite mating-type nuclei, but factors, in addition to MT A-2 and MT A-3, must be required for this process. *N. crassa* mating-type genes are transcribed during vegetative and sexual phases (C. Staben and T. Randall, pers. comm.; Saupe *et al.,* 1996; Chapter 2) but trancripts from *P. anserina* mating-type genes could only be detected by RT-PCR in perithecial tissues (R. Debuchy, pers. comm). This fact may indicate a difference in stability or regulation of mating-type products in *P. anserina* versus that of *N. crassa*. *P. anserina*, being a pseudohomothallic fungus, may not require additional mechanisms of recognition of opposite mating-type nuclei that are present in the heterothallic *N. crassa*.

3.4.4 Mechanisms of regulation by the mating-type gene products

The lack of a change in the sexual phenotype for A-2^{m1}, A-3^{m1} and A-3^{m2} mutants suggests that *mt A-2* and *mt A-3* may be involved in two different pathways that would independently influence sexual reproduction. If that is the case, the two pathways would have to be redundant at least partially, and may converge at a certain point of sexual development. If, on the other hand, *mt A-2* and *mt A-3* are in the same pathway, we have to evoke interaction between them and another product to form or stabilise a transcription complex. Evidence for the function of the mating-type genes as transcription factors is based on similarity to other proteins and the fact that MT a-1 and MT A-3 polypeptides bind the same specific DNA sequences *in vitro* (Philley and Staben , 1994; Philley *et al.,* 1995).

Because vegetative and sexual functions are so distinct different sets of genes are expressed in the two phases of *N. crassa* life. Transcriptional regulation involves numerous general transcription factors and also specific activators and repressors to guarantee exact levels of expression. Nitrogen regulatory elements are involved in the control of the sexual cycle in *N.*

crassa, as are the mating-type genes. It will be interesting to know to what extent mating-type genes are involved in the switch between vegetative and reproductive phases.

N. crassa mating-type genes may work in a manner similar to that of *S. cerevisiae* (see Johnson, 1995 for a review and Chapter 1). By combining with each other and with other proteins, the mating-type products could regulate different sets of genes during sexual development as well as heterokaryon incompatibility during vegetative growth. However, interaction between the mating-type and other gene products can only be investigated by genetic analyses of target genes in the different mutants or by biochemical analyses, for example with the yeast two-hybrid system. The participation of the mating-type genes as regulators of sexual development is studied in Chapter 4.

4. Transcriptional Analyses of Sexual Development Genes

4.1 INTRODUCTION

Fungal development is affected by environmental factors, such as nitrogen and carbon starvation, and light (see Chapter 1 section 1.3.3.2). In *N. crassa,* the decision to grow by hyphal extension, to produce microconidia or macroconidia or to develop protoperithecia is triggered by the environment. The signal is transmitted into the cell by signalling systems and a response is achieved by different pathways.

Over 200 sexual development mutants have been identified in *N. crassa* and many of them have pleiotropic effects in the vegetative phase. Because there are so many steps in which sexual development can be impaired and because mutations can be pleiotropic, classification of sexual mutations in *N. crassa* can be difficult. However, the mutants can be divided into two main classes. The first class of mutations affect fertility of the mutants and the second class alters the morphology of perithecia, asci and ascospores. Mutants that are affected in male and female fertility are easily recognised because of their failure to cross with an opposite mating-type strain. In contrast, recessive mutations that affect post fertilisation events can be masked by the wild type partner in heterozygous crosses.

4.1.1 Mutations affecting fertility

4.1.1.1 Female-sterile mutants

Many female fertility mutants have been isolated. These mutants are either impaired in the production of protoperithecia or do not respond to fertilisation by the opposite mating-type male cell (Mylyk and Threlkeld, 1973; Tan and Ho,1970; Ho, 1972). However, these mutants

mate normally as male partners. Only twelve different mutants (called *fs-* or *ff*, for female-sterility or female-fertility) have been analysed genetically (reviewed by Perkins *et al.,* 1982). Another 32 mutants affecting only female fertility were mapped to 28 complementation groups (Johnson, 1978). Many of these mutants also showed defects such as slow growth during the vegetative phase. DeLange and Griffiths (1980a) isolated several mutants defective in sexual development, in which eight were impaired either in protoperithecia formation or produced abnormal perithecia. Some mutants defective in female fertility were initially isolated in screening for other traits. Examples of mutants that exhibit lower fertility as a secondary phenotype are: *Banana ascus, Round-spore, Spore-killer, colonial-2* (see Raju, 1992 for a review or refer to Perkins *et al.,* 1982 for description of other loci).

Many genes that affect female fertility are recessive and in several cases may be involved in general processes since they have defects in the vegetative phase. A few genes that affect female fertility have been recently analysed at the molecular level. The *wc-1* and *wc-2* genes (white collar) are involved in several processes that are controlled by blue light including sexual responses such as protoperithecia formation and phototropism of perithecial beaks (Degli-Innocenti *et al.,* 1983; Harding and Melles, 1983). The *wc-1* and *wc-2* genes have both been cloned and code for zinc-finger putative transcription factors (Ballario *et al.,* 1996, Linden and Macino, 1997). These two genes are involved in a signal transduction pathway involving several events induced by blue-light. Transcription of *wc-1* is under the control of the *wc-2* gene but it has been suggested that the proteins encoded by both genes may interact to regulate blue-light inducible genes (Linden and Macino, 1997). ?The *Asm-1* gene was cloned (Aramayo *et al.,* 1996) based on its homology to *stuA*, an *A. nidulans* gene involved in conidiophore and sexual development (Miller *et al.,* 1992). The ASM-1 protein has a potential DNA-binding motif

(proposed as the APSES domain, Aramayo *et al.*, 1996) present in transcription factors from *S. cerevisiae* that control the cell cycle. As in the *A. nidulans stuA* mutant, the *N. crassa Asm-1*⁻ mutant alters the vegetative phenotype and also cannot produce the female sexual organ. Mutations in the *rco-1* gene leads to female-sterility, block of conidiation at different stages and reduction of growth rates (Yamashiro *et al.*, 1996). The *rco-1* gene was cloned and has homology to the *S. cerevisiae TUP1* gene, which is involved in the regulation of several developmental processes (Tzamarias and Struhl, 1994, see Chapter 1 section 1.3.1). It is not known if these genes correspond to any of the genes mutated previously in classical genetics studies.

4.1.1.2 Male-sterile mutants

Thirty male-barren mutants (*mb*-) were isolated that were defective as a male at different stages of the sexual cycle (Weijer and Vigfuson, 1972). Complementation tests showed that at least four loci are responsible for early sexual development and the remaining genes control later stages of the cycle (Vigfuson and Weijer, 1972). Some of the functions possibly disrupted in these mutants may be: attachment and fusion of the trichogyne-conidium, migration of the male nucleus to the ascogonium, association of opposite mating-type nuclei in the ascogonium or karyogamy.

4.1.1.3 Female and male sterile mutants

The *fmf*-1 mutant (female and male sterile) has a block in perithecial development so that perithecia remain about one-third of the normal size (Johnson, 1979). The *fmf-1* mutation is recessive and can be complemented in heterokaryons. The *fmf*-1 product was shown to be needed for expression of several sexual development genes and appears to have regulatory functions (Nelson and Metzenberg, 1992).

All of the *mt A-1*, *mt a-1* and the A^{IIRIP} mutants (See Chapter 1) were also sterile as male and female partners. While *mt A-1* and *mt a-1* mutations affect initial steps in fertilisation, the A^{IIRIP} mutant is affected in later phases of the sexual development.

Female- and male-sterility also can be observed in crosses of aneuploid or partial duplication strains. Different steps of perithecial or ascus development can be impaired depending on which segment of the genome is duplicated (Raju and Perkins, 1978). RIP is the most probable cause of gene inactivation in crosses of duplication strains (Cambareri *et al.*, 1989).

4.1.2 Mutations affecting morphology of perithecia, asci and ascospores

Many of the sexual development mutations have pleiotropic effects on perithecia, asci and ascospore development. The protoperithecium starts to enlarge and develop into the perithecium within 24 hours of fertilisation (Bistis, 1981). The development of the perithecium can be arrested if a block occurs in early steps of cell fusion or karyogamy, indicating that development of asci and ascospores is closely connected to perithecial development.

4.1.2.1 Mutations affecting perithecial development

Because the perithecium tissues originate mostly from the maternal parent, a few perithecial mutations only have a phenotype if the mutant is used as the female partner. The *per-1* gene affects perithecial colour when the female partner carries the mutation (Johnson, 1976). The *per*-1 mutants have been used for the study of perithecial morphogenesis. The *pen-1* (*pe*rithecial *n*eck) mutation was observed as a failure in the development of perithecial beaks through which ascospores are discharged (DeLange and Griffiths, 1980a). Only 10 to 20 asci per perithecia were formed in female *pen-1* x *pen-1*$^{+}$ crosses (compared with about 200 asci in wild-type crosses).

Raju and Perkins (1978) studied several crosses that gave rise to barren perithecia, i.e. produced few or no ascospores; the mutants were impaired at different stages of sexual development. Three main classes of genotypes may give rise to the barren phenotype: chromosome rearrangements (translocation and duplications), recessive and dominant mutations. Duplication crosses are barren and it is likely that sterility is due to effect of RIP on the genes involved in the duplication (Cambareri *et al.*, 1989). Several mutants affected in meiotic genes show decreased ascospore production or aberrations. Most of them are either *uvs* (uv-sensitive), *mus* (mutagen sensitive) or *mei* (meiotic abnormality) (see Raju, 1992 for details and description of other mutants). Some barren mutants observed in the study of Raju and Perkins (1978) were defective in DNA repair (e.g. *uvs-3* , *uvs-5, uvs-6, mei-3*). Leslie and Raju (1985) found 32 severely-barren mutants when they screened 99 strains collected from nature for recessive genes that affected fertility. Some of the mutants were blocked at early stages of sexual development and croziers were absent or malformed. Other mutants had either abnormal chromosome pairing, blocks in several meiotic phases or perithecial beak abnormalities. The fact that an array of mutations can produce the same type of phenotypic defects during karyogamy and meiosis suggested that regulation of development and morphogenesis must involve interactions more complex than a one-dimensional pathway (Raju and Perkins, 1978).

4.1.2.2 Mutations affecting asci formation

Ascus-development mutants fall into several classes and can be affected in chromosome pairing, recombination and meiosis; asci and ascospore morphology; number, size, pigmentation, dormancy and viability of ascospores (Raju, 1992). As with the fertility mutants, many ascus-development mutants show pleiotropic effects during vegetative growth, indicating the basic nature of the mutations.

Formation of bubble asci (Chapter 3 section 3.3.6.1) is an example of failure in the development of asci observed in crosses between inbred strains that are due to several weak deleterious mutations (Raju *et al.,* 1987). Several mutations affecting ascus morphology have also been described. The *peak (pk)* mutation gives rise to non-linear balloon-shaped asci that lack apical pores through which ascospores are discharged and the mutants also have an abnormal colonial morphology during vegetative growth (Murray and Srb, 1962). *Scruffy* and *scumbo* produce nonlinear asci but have normal apical pores (Pincheira and Srb, 1969). Another mutant, *Perforated (Prf)* has multiple small pores instead of the normal single apical pore (Raju, 1987).

4.1.2.3 Mutations affecting ascospore morphology

An example of ascospore morphology mutation is *Round-spore (R)* (Mitchell, 1966; Chapter 3 section 3.3.6.1) which is an ascus-dominant mutation. *Round* ascospores have only one germination pore instead of the normal two in ellipsoidal ascospores. Three other different mutations lead to the production of one giant ascospore: *giant spore* (Srb *et al.,* 1973), *Banana* (Raju and Newmeyer, 1977) and *Perforated* (Raju, 1987). These three mutants have distinct phenotypes but all contain the four products of meiosis within a single spore.

Mutation in five different *Four-spore* loci *(Fsp)* reduce the number of ascospores to four instead of eight (reviewed in Raju, 1992). Some of the *Fsp* mutants are temperature-dependent and the defects seem to be in the post-meiotic mitosis.

4.1.3 Regulators of the sexual development in *N. crassa*

Although the pathways for nitrogen metabolism have been well studied in *N. crassa* (reviewed in Marzluf, 1993) little is known about the control of the sexual cycle by nitrogen. Starvation for ammonium ions and other primary sources of nitrogen (e.g. glutamine, arginine) is crucial for protoperithecia formation. A zinc-finger DNA-binding protein (NIT2) is required for

expression of genes involved in the utilisation of secondary nitrogen sources such as nitrates; nit-2$^-$ mutants have reduced levels of expression of some nitrogen metabolic genes (Fu and Marzluf, 1990). A negative regulatory protein (NMR) represses the synthesis of enzymes required for nitrate reduction by binding to NIT2 (Xiao *et al.,* 1995). In nmr$^+$ strains, high levels of primary nitrogen sources (as in Vogel's medium) repress expression of genes for utilisation of inorganic nitrogen sources. In *nmr* mutants, the nitrogen catabolic genes are constitutively expressed (reviewed in Marzluf, 1993). However, *nit-2, nmr* and other genes of the nitrogen circuit do not seem to be directly involved in protoperithecia development, since the female fertility of the mutants is not affected (G. Marzluf, pers. comm.). It seems that distinct pathways control nitrogen metabolism and entry into the sexual cycle. Alternatively, a common nitrogen sensing system, still to be discovered, might be utilised for expression of genes for both nitrogen metabolism and sexual development.

The production of protoperithecia seems to be independent of mating-type since several mating-type mutants, including a mating-type deletion strain, produce normal numbers of protoperithecia (Griffiths and DeLange, 1978; Griffiths, 1982; Glass and Lee, 1992; R. Metzenberg, pers. comm.). Fertilisation, however, is absolutely dependent on a functioning mating-type, namely the *mt A-1* and *mt a-1* products. All four mating-type genes are important for functions following fertilisation (Glass and Lee, 1992; Chang and Staben, 1994; Saupe *et al.,* 1996). A way to understand mating-type regulation of sexual development would be to find target genes directly controlled by the products of the *A* and *a* idiomorphs. In *S. cerevisiae,* the mating-type products associate with each other and other transcription factors to regulate different developmental pathways (see Chapter 1 section 1.3.1). Several target genes of the mating-type have been isolated in *S. cerevisiae* and a network of genes is known to control

sexual development. In *N. crassa* it is also likely that the regulation of sexual development involves the formation of complexes of the mating-type products that activate or repress genes in different pathways.

The key regulatory elements in *N. crassa* sexual development are likely to be *mt A-1* and *mt a-1*. Both genes are necessary for mating identity and also for post-fertilisation events (Chang and Staben, 1994; Saupe *et al.,* 1996). Target genes of *mt A-1* and *mt a-1* are likely to be expressed anytime from protoperithecia formation to karyogamy. Genes required for development during this period are likely to be those involved in making the protoperithecia receptive for fertilisation, development of specialised structures and nutrition of the protoperithecia. Target genes of *mt A*-2 and *mt A-3* are most likely to be genes expressed after fertilisation and before karyogamy, as suggested by the phenotype of the A^{IIRIP} mutant (Glass and Lee, 1992). If formation of a transcription activation complex is required for efficient transcription of sexual development genes, the presence of MT A-2 and MT A-3 proteins may be important, even in the early phase of ascus and perithecia development.

As very few of the genes identified in sexual development mutants have been cloned, little is known about regulation of target genes by mating-type. However, many genes preferentially expressed in the sexual cycle were isolated by subtractive hybridisation (Nelson and Metzenberg, 1992). Initial studies showed that a functional *mt A-1* is important for expression of some of these genes, suggesting that they may be direct targets of the mating-type products.

4.1.4 Sexual development genes (*sdv*)

By using a subtractive hybridisation technique, Nelson and Metzenberg (1992) isolated several clones that corresponded to genes expressed preferentially in the sexual phase. Some of

these genes are thought to be targets of regulators of sexual development, as their expression was different in the A^{m44} (mutated at *mt A-1*), *fmf-1* and *wc-1* mutants compared to the wild-type *A* strain (Nelson and Metzenberg, 1992; Merino and Nelson, 1995, Nelson *et al.,* 1995).

4.1.4.1 Isolation of the *sdv* genes

For subtractive hybridisations, RNA was isolated from a *A tol/a tol* heterokaryon grown 2-3 days in crossing media at 25°C (Nelson and Metzenberg, 1992). The RNA was used to synthesise ^{32}P-labelled cDNAs using random primers. RNA from an *a tol* strain grown under vegetative conditions was used for cDNA/RNA hybridisation. Single stranded cDNAs that did not hybridise to RNA from *a tol* were separated by column chromatography. The isolated cDNA pool was hybridised to a *N. crassa A* genomic cosmid library. About 7% of cosmids yielded a signal, but approximately 50% of these contained rDNA. Cosmids not bearing rDNA were further screened by probing RNA from an *a tol* strain (grown in vegetative medium) and *a tol/A tol* heterokaryon (grown in crossing media). Thirty-five cosmids containing putative sexual development transcripts were isolated corresponding to approximately 30 *sdv* genes.

4.1.4.2 Functional characterisation of the *sdv* genes

Initially, 14 *sdv* clones were transformed into an *A* strain in an attempt to disrupt sexual development function by RIP (Nelson and Metzenberg, 1992). Disruption of two genes (*sdv*-5 and *sdv-10*) generated recessive mutants. When both parents harbour a mutation in the *sdv-10* gene (renamed *asd-1*) aberrant asci are formed and ascospores are not delineated. The *asd-1*$^{+}$ gene codes for a rhamnogalacturonase which is abundant in young but not mature asci (Nelson *et al.,* 1995). Mutation in *sdv*-5 (*asd-2*) also lead to block in ascospore development.The *sdv*-15 gene was also disrupted and, in homozygous crosses, the phenotype of this mutant was shown by non-delimitation of ascospores (Merino and Nelson, 1995). The *sdv*-15 gene (renamed *asd-3*) is

thought to code for a sugar-transporter. Failure to identify a phenotype of other disrupted *sdv* genes suggested a few possibilities: 1) these genes are important but not essential for sexual development; 2) they are functionally redundant or 3) either parent can contribute the products (Nelson and Metzenberg, 1992).

4.1.4.3 The *sdv* genes as targets of the mating-type gene products

In the initial study, 14 *sdv* genes were used as probes in Northern analyses with the A^{m44} mutant. A functional *mt A-1* was required for expression of at least 7 of the *sdv* genes (Nelson and Metzenberg, 1992). Because the products of the majority of these genes are not known, we cannot predict when in sexual development they are needed. Even though *sdv* genes were expressed in early sexual development (protoperithecia formation) it is possible that mating-type genes other than *mt A-1* are also required for their expression.

In order to identify target genes of the mating-type products, expression of some *sdv* genes was analysed as described in this chapter. In addition to the *mt A-1* mutants, other mating-type mutants such as a^{m1}, A-2^{m1}, A-3^{m1}, A^{IIRIP} and a *mt A* deletion strain were assayed for expression of selected *sdv* genes. Since other genes expressed during different phases of fertilisation, such as cell fusion, pre-meiotic mitosis and karyogamy, are not available, RNA analyses with early *sdv* genes may provide an understanding of the intricate regulation of the sexual cycle by mating-type and elucidation of the mode of action of mating-type genes.

4.2 MATERIALS AND METHODS

4.2.1 Strains and growth conditions

Table 4-1 contains a list of the strains used in the transcript analyses of the *sdv* genes. For RNA isolation all strains were first grown in Vogel's minimal media (Vg), plus required supplements (adenine: 0.2 mg/ml; arginine and lysine: 0.6 mg/ml; thiamine: 10 µg/ml). Conidia were harvested at four days and used to inoculate liquid Westergaard's (WG) crossing media (described in Davis and De Serres, 1970) in flasks of one and four litres containing 100 or 300 mls, respectively to assure a large air-liquid interface. When required, supplements were added to WG medium at 1/20 of the recommended concentration for vegetative growth. Growth was for 2 to 5 days depending on the ability of the strain to form a thick mat in liquid media. Strain RLM 41DA was also grown on Vogel's vegetative media for 4 days. Perithecial RNA was obtained from a cross between *OR A* X *OR a,* six days after fertilisation on solid crossing media overlaid with Miracloth (Calbiochem).

Strain	Genotype	Reference
A	*qa-2 aro-9 A*	RLM 52-22
A^{IRIP}	*arg-2 mt A-1*^{m}	NLG R4-50
A-2^{m1}	*mt A-2* ^{m}	This study
A-3^{m1}	*mt A-3*^{m}	This study
A^{IIRIP}	*mt A-2*^{m} *mt A-3*^{m}	NLG R4-16
OR *a*	WT*a*	FGSC 532
a^{m1}	*mt a-1*^{m} *ad-3B cyh-1*	FGSC 4565
mt deletion*	*thi-4 lys-1 mt A del*	RLM 41-10DelA
mt partial duplication**	*trp-4 A/ a tol*	Gift of P. Shiu
OR *A*	WT *A*	FGSC 2489

Table 4-1 Strains used in the analyses of *sdv* genes. FGSC=Fungal Genetics Stock Center; NLG= L. Glass' Laboratory; RLM= R. Metzenberg's Laboratory. (*) see Figure 4-1 for construction of this strain. (**) contains a partial duplication of linkage group 1.

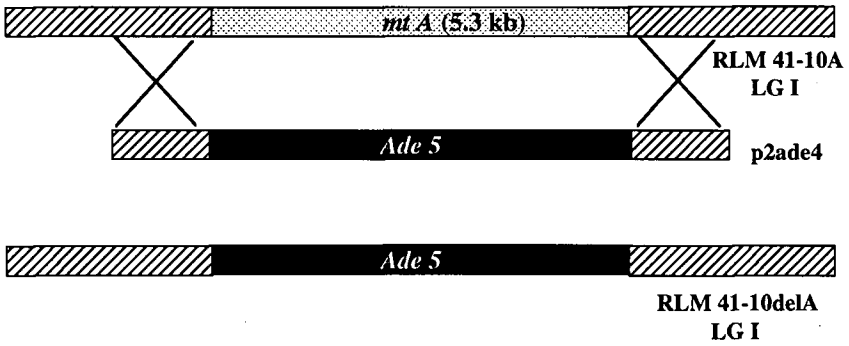


Figure 4-1 Construction of the *mt A* deletion strain. RLM 41-10delA was obtained by replacement of the *mt A* idiomorph sequences by the *Schizophyllum commune Ade5* gene (in plasmid p2ade4) into RLM 41-10A. The flanking sequences of the two strains are the same (R.L. Metzenberg, pers. comm.).

4.2.2 Clones

The *sdv* gene clones were kindly provided by Dr. M. A. Nelson (University of New Mexico).

Table 4-2 shows sizes of fragments used as probes and transcripts of the *sdv* genes (according to Nelson and Metzenberg, 1992):

sdv clone	Size of probe (kbp)	Size of transcript (kb)
sdv-1	3.0	1.4
sdv-3	5.5	1.4
sdv-4	12.0	2.0
sdv-7	7.0	1.5
sdv-10 (asd-1)	1.1 (cDNA)	2.0
sdv-14	3.0	2.4

Table 4-2- *sdv* genes selected for analyses. Clones and sizes according to Nelson and Metzenberg, 1992.

4.2.3 Northern Blot Analyses

Mycelial mats were isolated by filtration and stored frozen at -70°C . RNA isolation and Northern blots were done as described in Chapter 2 (see Materials and Methods, section 2.2.2.1) except that 3 µg of polyA$^+$ RNA was loaded per lane and hybridisation of membranes (Zeta-Probe -BioRad) was carried out overnight in a 0.5 M phosphate buffer pH 7.0/ 7% SDS solution

at 65°C. Washes were for 20 min. each in 2X SSC, 0.1% SDS at room temperature and in 0.1X SSC, 0.1% SDS at 65 °C. After a 24 h. exposure to X-ray films, blots were exposed to a phosphorscreen (Molecular Dynamics). The screen was scanned in a Phosphorimager scanner and amount of radiation was analysed using the Imagequant software (Molecular Dynamics). Amount of radiation emitted from blots was analysed for specific transcripts and for the constitutive gene controls. The method for background subtraction was object average (same size object was analysed where there was no bands). Controls included *N. crassa crp-1* gene for the small ribosomal subunit protein, (two transcripts of about 1 kb) (Kreader and Heckman, 1987) and/or the β-tubulin gene (tub-1 probe, 2.4 kb) (Orbach, 1986). Blots were allowed to decay or were stripped by boiling for 20 min. in 0.1X SSC, 0.5% SDS for subsequent analyses with other *sdv* genes.

Results are presented as the ratio of volume of radiation from the *sdv* genes and the constitutive gene control. All the comparative analyses were done relative to the *A* control (=1). Example of Northen hybridisations (usually after 24 h. exposure) are shown under graphs of the Phosphorimager analyses (even though most analyses were derived from several blots). Standard deviation bars are shown for those analyses in which repetitions were done (average is plotted in these cases). Data from different blots and calculated standard deviations are shown in Appendix 3.

4.3 RESULTS

4.3.1 Northern blots and Phosphorimager Analyses

The *sdv* genes chosen for this study were biased for genes positively controlled by *mt A-1*, except for *sdv-6* and *sdv-14* which had a slight increase in expression in the A^{m44} mutant (Nelson and Metzenberg, 1992). The A^{IRIP} mutant (Glass and Lee, 1992) was used as the control in this study. This study confirmed the results of Nelson and Metzenberg (1992) which showed *mt A-1* is required for expression of *sdv*-1, *sdv-4*, *sdv-7* and *asd-1*. Because these genes were not expressed in the WT *A* strain in vegetative media (Nelson and Metzenberg, 1992), another regulatory factor (or factors) seems to be involved in regulation of the *sdv* genes in limiting nitrogen conditions.

4.3.1.1 Effect of supplements on expression of *sdv* genes

The expression of the *sdv* genes is dependent on the concentration of nitrogen sources in the media. Growth in Vogel's media (rich in ammonium ions) inhibits the expression of most *sdv* genes in WT *A* (Nelson and Metzenberg, 1992). Some of the mating-type mutants required supplements containing nitrogen for their growth (see Material and Methods

Table 4-1). The supplements were added to a final concentration 20 times lower than in Vogel's medium, to guarantee growth of the mutants without saturating the media with primary nitrogen sources. A control experiment was done to measure the effect of supplements on expression of *sdv* genes. Figure 4-2 shows the expression of *sdv-1* in OR *A* in Westergaard's medium plus arginine, adenine and adenine + lysine + thiamine. The results show that, at least for *sdv*-1, no significant difference was observed (less than 1-fold).

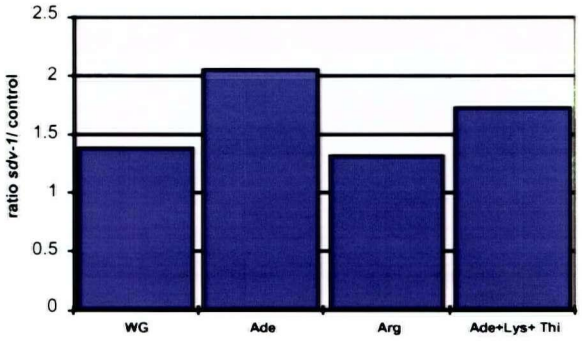


Figure 4-2 Effect of supplements on the expression of *sdv-1*. WT *A* was grown for 3 days in crossing media (WG) and crossing media containing adenine, arginine and adenine, lysine and thiamine. Variation was in the range of about 0.6.

4.3.1.2 Effect of time on expression of *sdv* genes

The expression of the *sdv* genes were first analysed in strains grown from 2 to 3 days depending on their growth rate (Nelson and Metzenberg, 1992). The effect of duration of growth on the expression of the *sdv* genes was analysed by probing RNA isolated from WT OR *A* with *sdv-1* and *sdv-4* (Figure 4-3). For both *sdv-1* and *sdv-4,* little difference in expression levels was observed between 3 and 5 days. The same analyses were performed using *asd-1*(not shown). Expression of *asd-1* was only observed at 3 days. Thus, expression of some *sdv* genes may be affected more than others by growth, or their expression may be transient.

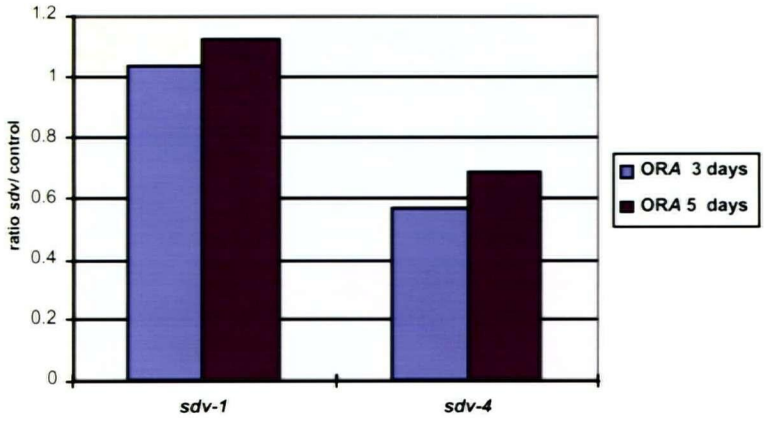


Figure 4-3 Effect of duration of growth on the expression of *sdv-1* and *sdv*-4.

4.3.1.3 Expression of *sdv-4*

The analysis of *sdv*-4 expression is shown in Figure 4-4. Of the five *A* mutants, only A^{IRIP} had undetectable levels of expression of *sdv-4* in all RNA preparations examined (see Appendix 3 for reference). In the *mt A-2* and *mt A-3* mutants *sdv-4* transcript levels were similar to those of WT *A*. In the *mt A* deletion strain expression of *sdv*-4 increased on average 8-fold as compared to the *A*. Growth of the *mt A* deletion strain in vegetative medium brought the expression of *sdv*-4 to levels similar to those of the WT *A* grown in crossing medium. *sdv-4* transcripts was not detected in most RNA preparations from the *mt* duplication strain or from RNA isolated from a cross. Expression of *sdv-4* was highly increased (7-fold) in the a^{m1} mutant.

The most dramatic differences in *sdv-4* expression as compared to WT *A* were the decrease in A^{IRIP} and high transcript level of *sdv-4* in the *A* deletion strain. *mt A-1* is required for

normal transcription levels of *sdv-4* in WT A. However its absence in the *mt A* deletion strain (in which *mt A-2* and *mt A-3* are also absent) did not cause a decrease in expression levels. As the main difference between WT *A* and the deletion strain is the absence of the mating-type sequences, the high level of *sdv-4* transcripts in the deletion strain may be explained by the loss of repression. As MT A-1 is important for activation of *sdv-4* in the WT *A*, repression has to come from MT A-2 and MT A-3 (or genes regulated by them). In the a^{m1} mutant, expression of *sdv-4* is also highly increased. Because the a^{m1} mutant is null for mating-type (Staben and Yanofsky, 1990) the expression levels observed must be due to absence of the *mt a* product. Thus, in *a* strains repression may occur by the action of MT a-1.

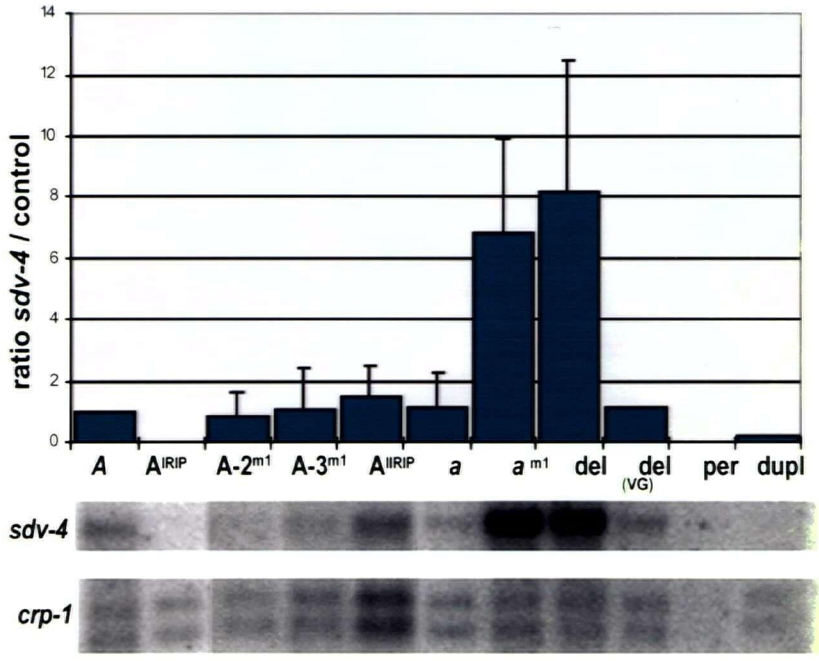


Figure 4-4 Expression of *sdv-4*. All counts were normalised based on the *A* strain (relative volume=1). Data were derived from at least three separate experiments, except for the deletion strain grown in vegetative medium and perithecial RNA (see Appendix 3). del= *mt* deletion; VG=Vogel's medium; per=perithecial RNA; dupl=mt duplication strain. *crp-1* has two transcripts. Standard deviation bars show variability observed.

4.3.1.4 Expression of *sdv-1*

Expression pattern of *sdv-1* is shown in Figure 4-5. The level of transcription of the *sdv-1* gene in the WT *a* was increased relative to WT *A* (at least 3-fold). The absence of *mt A-1* and *mt a-1* genes caused a decrease in the expression of *sdv-1* as compared to the respective WT strain (see A^{IRIP} and a^{m1} mutants). However, a^{m1} still had high levels of *sdv-1* transcripts. This result suggests that a functional *mt a-1* may not be very important for expression of *sdv-1* and an additional transcription factor may activate expression of the *sdv-1* gene in the *a* background. The A^{IRIP} mutant had almost undetectable levels of expression of the *sdv-1* suggesting *mt A-1* is required for its expression. However, in the deletion strain, *sdv-1* expression was slightly higher than that of WT *A* suggesting interaction with other regulatory products. Expression of *sdv-1* in the A-2^{m1} and A-3^{m1} mutants was higher than in *A* (about 2-fold for both mutants). In the A^{IIRIP} double mutant, expression was more than 3-fold higher than *A,* indicating an additive effect due to absence of both *mt A-2* and *mt A-3*. Together, these results suggest that, in the absence of MT A-1, MT A-2 and MT A-3 (or genes that they regulate) may act as repressors.

Transcripts corresponding to *sdv-1* were not detected in RNA from the deletion strain grown on vegetative medium (not shown). RNA from perithecia and from the *mt* duplication strain had slightly different levels of *sdv-1* transcripts.The partial duplication strain showed levels of *sdv-1* transcripts comparable to the WT *A,* and perithecial RNA contain higher levels of transcripts. Even though both mating-types are present in the *mt* duplication strain and in crossing cells, regulation of the *sdv-1* may be different during different developmental stages.

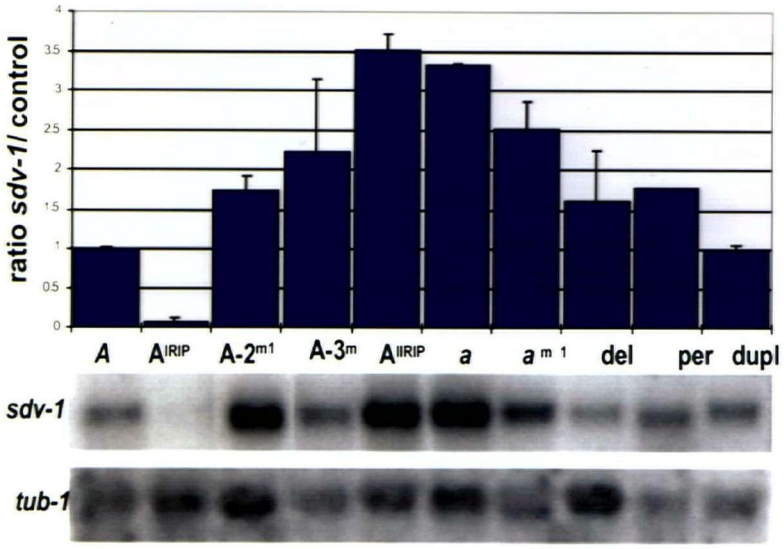


Figure 4-5 Expression of *sdv-1*. All counts were normalised based on the *A* strain (relative volume=1). Data are shown as average expression from at least three experiments, except for perithecial RNA and *mt A* partial duplication (see Appendix 3). Variability is shown by standard deviation bars.

4.3.1.5 Expression of *sdv-7*

In most preparations of *A* strain RNA (grown for 2-3 days in crossing medium), *sdv-7* transcripts could be detected (Figure 4-6). However, during a time course experiment, with RNA from WT *A* strain grown for 3 and 5 days, expression of *sdv-7* was not observed (not shown). In most RNA preparations of *A* (3 out of 5) *sdv-7* transcripts were detected after 24 hours of exposure of blots to x-ray films. In a^{m1} RNA, a very strong signal corresponding to *sdv-7* (more than 10-fold) was observed in all blots (with one exception). The A-2^{m1} mutant also showed very high levels of expression of *sdv-7* in one RNA preparation (more than 10-fold higher as compared to the WT *A* strain). The other strains showed the same expression as WT or lower in the majority of RNA preparations (Figure 4-6). Even though *sdv-7* expression seemed to be transient, MT a-1 and MT A-2 may be important for repression.

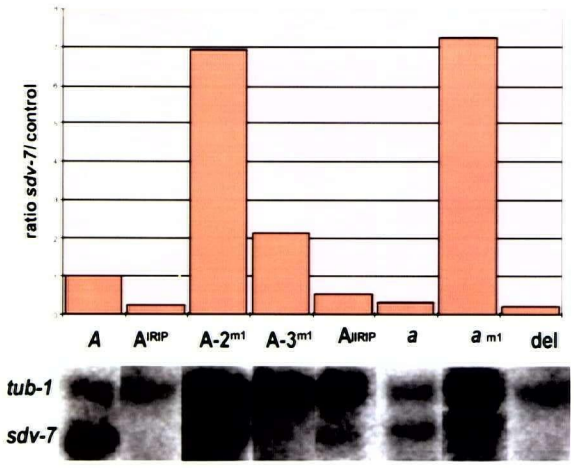


Figure 4-6 Expression of *sdv-7* . All counts were normalised based on the *A* strain (ratio=1). Data is shown as average from two different experiments. Blot shown was exposed for only 24 h.

4.3.1.6 Expression of *asd-1*

As with *sdv-7,* in some RNA preparations the level of *asd-1* expression in the a^{m1} and A-2^{m1} mutant was much higher than in the wild-type (more than 10-fold). The expression of *asd-1* seemed to be influenced by duration of growth (see section 4.3.1.2). Expression of *asd-1* was analysed in one experiment in which all strains were grown for 4 days (Figure 4-7). The expression of *asd-1* varied little between strains (ratio *asd-1*/control ranged from 1 to 1.6, Figure 4-7). A signal was not detected in A^{IRIP} RNA, and the a^{m1} mutant showed a two-fold increase in *asd-1* expression as compared to WT *a*. The *A* deletion strain showed reduced expression of *asd-1* as compared to WT *A*. These results suggest that regulation of *asd-1* may be different depending on the mating-type background, as with other *sdv* genes (see above). When mutants of

the three *A* mating-type genes were analysed, expression of *asd-1* was affected but *mt A-1* may be the most important activator, since its absence causes transcription to fall to undetectable levels.

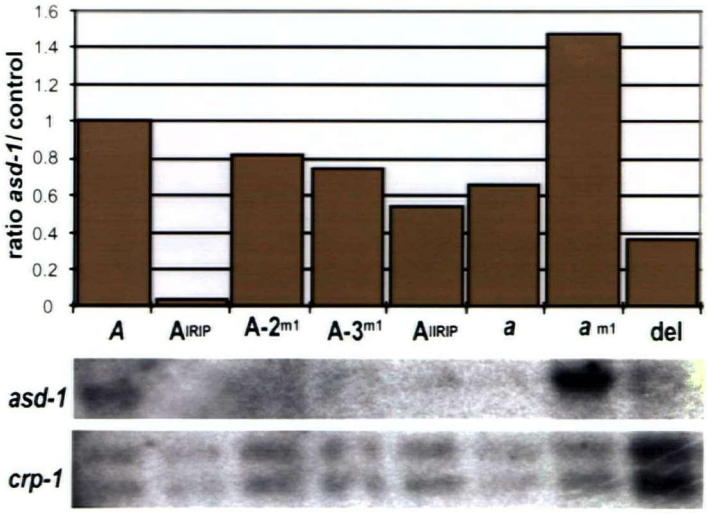


Figure 4-7 *asd-1* expression. All counts were normalised based on the *A* strain (ratio=1). Data shown is from one experiment in which all strains were grown for four days (24 h. exposure).

4.3.1.7 Analysis of other *sdv* genes

The A^{m44} mutant showed a decrease in the transcript level of *sdv-3* (Nelson and Metzenberg, 1992). The expression of the *sdv-3* gene was analysed in this study using $A\text{-}2^{m1}$, A^{IIRIP}, a^{m1} and the *mt* deletion strain, but not A^{IRIP} and $A\text{-}3^{m1}$. Two other *sdv* genes, *sdv*-6 and *sdv*-14, that showed higher expression in the A^{m44} mutant (Nelson and Metzenberg, 1992) were also analysed. These analyses are shown in Appendix 3, since it was impossible to perform a complete quantitative expression analyses with them.

4.4 DISCUSSION

In this chapter, the function of mating type genes as regulators of sexual development genes was analysed. Previously, only the *mt A-1* gene had been shown to regulate *sdv* genes (Nelson and Metzenberg, 1992). The availabity of *mt A-2* and *mt A-3* mutants and an *A* mating-type deletion strain made it possible to infer, through transcriptional analyses, how mating type regulates *sdv* genes. This is the first report demonstrating that, in addition to *mt A-1*, other mating-type genes are involved in the transcriptional regulation of sexual development genes.

4.4.1 Regulators of development

The *sdv* genes were isolated by subtractive hybridisation from cultures grown in crossing media (low ammonium concentration) for about 2 to 3 days. At this stage the female sexual organ starts to be formed and within 5 days protoperithecia are ready to be fertilised. The mating-type gene products are thought not to be necessary for the early steps of protoperithecia formation since all the mating-type mutants produce apparently normal protoperithecia. However, for the *sdv* genes analysed in this study, transcription was regulated by mating-type genes. It is possible that expression of these genes is needed during the early stages of sexual induction but may also be necessary later in the sexual cycle. Transcripts corresponding to *asd-1* are observed in early sexual cycle (Nelson and Metzenberg, 1992) and the ASD1 protein is abundant in young asci (M. Nelson, pers. comm.). Except for *asd-1*, very little is known about the *sdv* genes, since attempts to obtain mutants through RIP failed (Nelson and Metzenberg, 1992) and their DNA sequences have not been determined.

MT A-1 is thought to function as a transcriptional activator because of its sequence similarity to the α-box of the *S. cerevisiae* **α1** mating-type protein, which, through interactions with other proteins, binds to promoters and activates α-specific and haploid-specific genes (see

Chapter 1 section 1.3.1). The similarities of the MT A-2, MT A-3 and MT a-1 mating-type proteins with other proteins and/or motifs are not strong enough to determine if these genes have activator or repressor functions. The presence of the HMG domains in MT A-3 and MT a-1 suggests that these proteins may regulate genes as either activators or repressors. Based on the MT A-2 protein sequence one cannot resolve whether *mt A-2* is an activator or a repressor. The present study suggests that the functions of mating-type proteins depend on protein-protein complexes formed between them and other proteins.

Following cellular and nuclear fusion in fungi, different developmental processes are initiated and new regulatory proteins may arise, as in the *S. cerevisiae* system. The isolation of the *rco-1*, a *TUP1* homolog (Yamashiro *et al.,* 1996) suggest that *N. crassa* and *S. cerevisiae* may have similar mechanisms of transcriptional regulation to coordinate events leading to vegetative or sexual development. As with the TUP1 protein, RCO1 is involved in several developmental processes (reviewed in Johnson, 1995; Yamashiro *et al.,* 1996). RCO1 controls the expression of conidiation genes that are involved in developmental pathways leading to the formation of the three types of *N. crassa* spores (Yamashiro *et al.,* 1996). *Asm-1* is another *N. crassa* gene that regulates conidiation and protoperithecial development (Aramayo *et al.,* 1996) and also has similarity to *S. cerevisiae* genes. The *S. cerevisiae* ASM-1-like proteins (PHD1 and SOK2) are involved in pseudohyphal growth and (at least for SOK2) may be dependent on a mating-kinase cascade (Ward *et al.,* 1995). ASM-1 has been suggested to be part of a transcription complex involved in sensing signals such as nitrogen deprivation or cAMP levels, or inducing transcription of genes essential for protoperithecia formation (Aramayo *et al.,* 1996). Thus RCO1 and ASM-1 may be required for regulation of some *sdv* genes. Further characterisation of *Asm*-1 and *rco-1* genes and isolation of other *N. crassa* regulatory genes may help uncover the

interactions of regulators of vegetative and sexual development that involve the mating-type proteins.

4.4.2 Expression of *sdv* genes and phenotype of mating-type mutants

The A^{IRIP} and a^{m1} mutants are blocked in early steps of fertilisation and any gene regulated by *mt A-1* and *mt a-1* should have altered expression patterns in these mutants. The A^{IIRIP} mutant is blocked in post-fertilisation functions. If *mt A-2* and *mt A-3* regulate a gene set involved in sexual reproduction different from the set regulated by *mt A-1,* it is expected that the pattern of expression of *sdv* genes would also be different in these mutants as compared to the wild-type.

If *mt A*-2 and *mt A-3* were redundant, as suggested by the absence of a sexual phenotype in A-2^{m1} and A-3^{m1}, the expression pattern of genes regulated by them should have been the same as wild-type in the two mutants. In addition, absence of both *mt A-2* and *mt A-3* (A^{IIRIP}) should give a different pattern of gene expression as compared to expression in the single mutants. Expression of *sdv-1* was twice as high in the A^{IIRIP} mutant as compared to the single *mt A-2* and *mt A-3* mutants. In the A-2^{m1} and A-3^{m1} mutants, expression was higher than WT. Perhaps absence of *mt A-2* or *mt A-3* caused an alteration in expression of certain genes involved in sexual development but a phenotype was not observed because the level of protein expression from those genes was below a critical threshold that did not affect sexual development. When *mt A-2* and *mt A-3* were both mutated, the levels of protein expression may have been above this threshold and post-fertilisation functions were affected. An alternative is that the particular *sdv* genes analysed here, may not be as critical for ascosporogenesis as are other genes (not yet characterised) that are regulated by *mt A-2* and *mt A-3.* In this case, even if *mt A-2* or *mt A-3* affected the expression and function of some *sdv* genes, a phenotype was not observed in the A-

2^{m1} and A-3^{m1} mutants. The *sdv* genes may also be redundant or not very important for the sexual cycle. After RIP mutagenesis of 14 *sdv* genes, *asd-1* was the only mutated gene which caused an altered sexual phenotype. These results indicated that the *sdv* genes may be redundant or not essential to sexual development (Nelson and Metzenberg, 1992).

4.4.3 Expression of *sdv* genes in *A* and *a* backgrounds and after a cross (*A/a*)

Some *sdv* genes such as *sdv-1* are transcribed to different levels in *A* and *a* backgrounds. Since the only genetic difference between an *A* and an *a* strain are the sequences at the mating-type locus and possibly the flanking sequences (Randall and Metzenberg, 1995) any difference in gene expression is due to direct or indirect mating-type regulation. The *mt A* deletion strain and the a^{m1} mutant are presumably equivalent because mating-type genes are not functional in either strains. Indeed, for *sdv*-4 and *sdv-1* the a^{m1} and the deletion strain exhibited the same trend of expression, i.e. decrease or increase, as compared to the respective WT. However, the levels of expression of *sdv* genes were different in the a^{m1} and *mt A* deletion strains, suggesting that differences in the idiomorphic flanking sequences may be important for differentiation into *A* or *a* cell-types. Mating type-specific transcripts from the idiomorph flanking sequences (Metzenberg and Randall, 1995; see Chapter 1 section 1.3.3.4) may play an important role in development.

It is clear that some genes regulated by mating-type at this stage are related to rendering the cell responsive to mating; expression of some of these genes would be distinct in the two mating-types because the pheromone and pheromone-receptors have to be mating type-specific. The final result of the developmental process (e.g. protoperithecia formation, trichogyne development), has to be similar, independent of the mating-type, since both *A* and *a* can cross efficiently as female or male. The differences observed in the expression levels of *sdv* genes in

different mating-type backgrounds may account for redundancy of these genes. If two pathways exist for the same developmental process it could be that, in *a* strains for example, a specific *sdv* gene is required in large quantities, but it is not required in *A* background.

Expression of *sdv-4* in perithecial RNA was not detected suggesting that expression of this gene may be negatively controlled after fertilisation. In the partial duplication strain *sdv-4* transcripts were also absent. It is possible that the mating-type proteins or products regulated by them interact after fertilisation and form a new regulatory complex that turns off *sdv-4* expression. Expression levels of *sdv-1* in perithecial RNA were different from both *A* and *a* levels, suggesting either a new regulatory product is formed between *A* and *a* mating-type products or that *mt A* and *mt a* products compete for the same DNA-binding site when in the same nucleus. Interestingly, the expression of *sdv-1* in the *mt* duplication strain was slightly lower than in perithecia. This result indicates that the presence of both mating-type sequences in one nucleus is not the only pre-requisite for triggering of the sexual reproduction. A complex regulation of mating-type and target genes may occur after cell and/or nuclei fusion possibly in a temporal and spatial manner, typical of developmental genes.

4.4.4 Models for regulation of *sdv* genes

Analyses of *sdv-1* and *sdv-4* genes allow the prediction of modes of regulation of the *sdv* genes by mating-type. The basis of the models for *sdv-4* and *sdv-1* regulation is similar, though variations are introduced to account for peculiarities of each set of genes. Other genes are also known to be required for expression of different *sdv* genes (such as *wc-1* and *fmf*-1) and additional putative proteins are included in the different models. Because very little is known about the *sdv* genes and the control of sexual development the models may be simplistic and involve assumptions. The first aspect for the models is the presence of a regulatory factor (or

complex) called N1, which responds to nitrogen levels, that is required for expression of the *sdv* genes and binds to one of the cis-elements (S1) of the promoter. Secondly, the mating-type proteins are expressed upon nitrogen limitation. All of the mating-type genes are expressed at the transcription level during vegetative and sexual phases (Saupe *et al.,* 1996; Chapter 2; Staben and Yanofsky, 1990) and although *mt A-2* and *mt A-3* may be post-transcriptionally regulated (Chapter 2, section 2.4) the models assume that translation of *mt A-2* and *mt A-3* is derepressed by protoperithecial initiation or nitrogen starvation. Finally, the models assume that expression of *sdv* genes is directly regulated by the mating-type proteins. However, it may be that proteins controlled by mating-type genes, rather than the mating-type products themselves, are directly involved in transcription of the *sdv* genes. The data presented here suggest that the mating-type proteins form a transcriptional complex which may bind to an upstream site in the promoter (S2). An interaction was observed with *P. anserina* FMR1 and SMR2 in the two-hybrid system (R. Debuchy pers. comm.) and perhaps interaction can also occur between the *N. crassa* homologs, MT A-1 and MT A-3. As gene regulation in the *A* and *a* backgrounds seems to be different, the two mating-types are shown separately in the diagrams of the models.

4.4.4.1 Model for regulation of *sdv-4*

Regulation of *sdv-4* seems to be similar in *A* and *a* backgrounds, because the levels of transcripts were comparable in both WT strains and in the absence of mating-type genes (a^{ml} and *A* deletion). The simplest model for regulation of *sdv-4* (Figure 4-8) is based on the fact that the most drastic effects on expression of *sdv-4* were seen in the absence of *A* and *a* mating-type products. The *A* deletion strain and a^{ml} had high levels of *sdv-4* transcripts (Figure 4-4), thus in the WT, the mating-type products contribute to repression of *sdv-4* expression. MT a-1 is responsible for partial repression of *sdv-4* transcription in *a* strains. Because the expression of

sdv-4 is different between the individual *A* mutants, each *mt A* gene may have a different effect on the expression of *sdv-4*. While in the *A* deletion strain *sdv-4* transcription is derepressed, absence of MT A-1 only (A^{IRIP}), decreases *sdv-4* transcription to undetectable levels. However, absence of either MT A-2 and MT A-3 (A-2^{m1} and A-3^{m1}) or of both genes (A^{IIRIP}) does not affect *sdv-4* transcription levels. Repression of *sdv-4* in the WT *A* may then depend ona complex formed between MT A-1, MT A-2, MT A-3. In the absence of MT A-1, MT A-2 and MT A-3 would act as strong repressors, perhaps in association with an additional repressor protein (R1). Because repression in the presence of only MT A-1 (A^{IIRIP}) is still observed (similar to WT levels) this mating-type protein may be the most important component of the transcription complex and is sufficient to maintain its stability and repress *sdv-4* transcription. An option to account for the efficiency of repression by MT A-1, would be the presence of the repressor protein R1 which would interact with MT A-1 in the absence of MT A-2 and MT A-3.

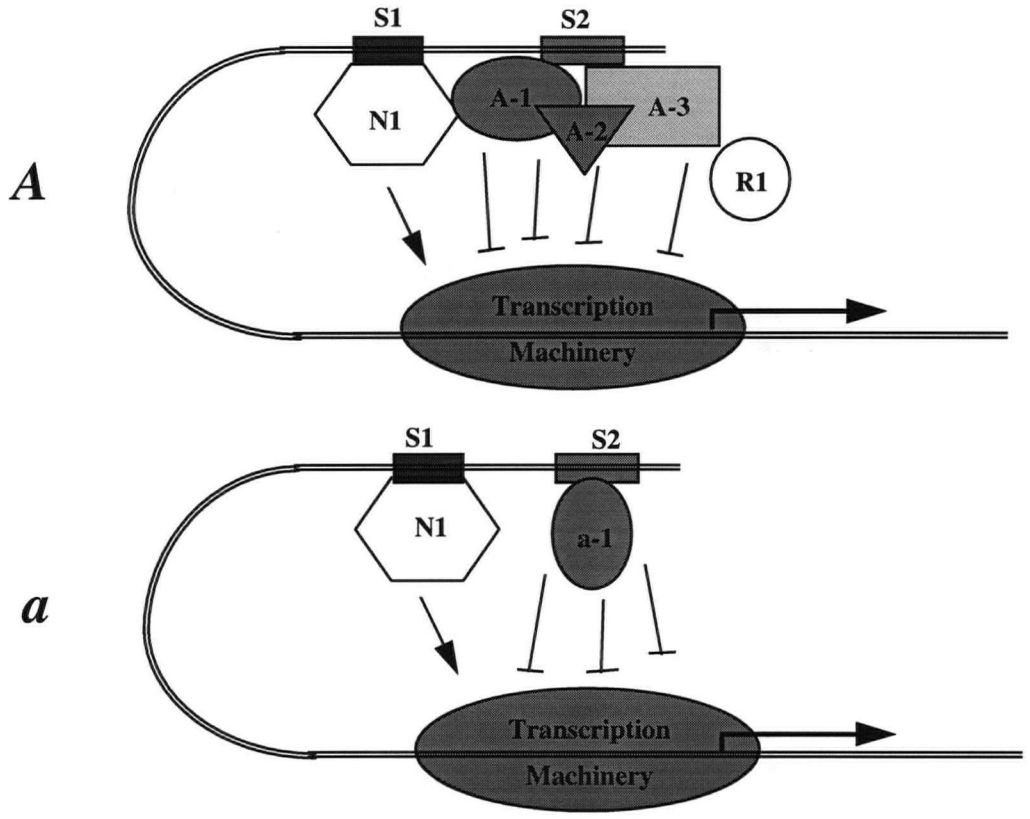


Figure 4-8 Model for regulation of *sdv-4* by mating-type genes. N1 is a transcriptional activator which is regulated by nitrogen limitation. RNA polymerase II and other transcription factors are represented by the oval shape. The mating-type proteins keep *sdv-4* transcription levels low. Function of the *mt* A proteins may switch depending on the products present in the complex. A repressor protein (R1), may be involved in regulation by interaction with the mating-type products. See text (section 4.4.4.1) for details.

In an alternative model to explain *sdv-4* regulation (Figure 4-9), MT a-1, and MT A-1/ MT A-2/MT A-3 would act as activators. However to account for the high *sdv-4* transcript levels in the *A* deletion strain and a^{m1} this model assumes the presence of a strong activator factor (N2) that can bind the cis-elements S1 and/or S2 on the promoter in the absence of the *A* mating-type complex or MT a-1. This explains why expression of *sdv-4* in the deletion strain and in the a^{m1} mutant is increased several fold as compared to the WT strains even in the absence of mating-type proteins (Figure 4-4). In the *A* background, the *mt A-1* product is the key element for

activation of transcription. In A^{IRIP}, transcription is very low because in the absence of MT A-1, MT A-2 and MT A-3 turn into strong repressors. However, if MT A-1 is present, absence of MT A-2 and MT A-3 has little effect on *sdv-4* expression levels. In WT *a,* MT a-1, together with N1, activate *sdv-4* transcription. Absence of MT a-1 allows N2 to bind to S2 and activate transcription several fold. To account for the expression of *sdv-4* when the deletion strain was grown in Vogel's medium, N2 is assumed to transcribe *sdv-4* at low levels even in the absence of the N1 factor (present only under nitrogen starvation).

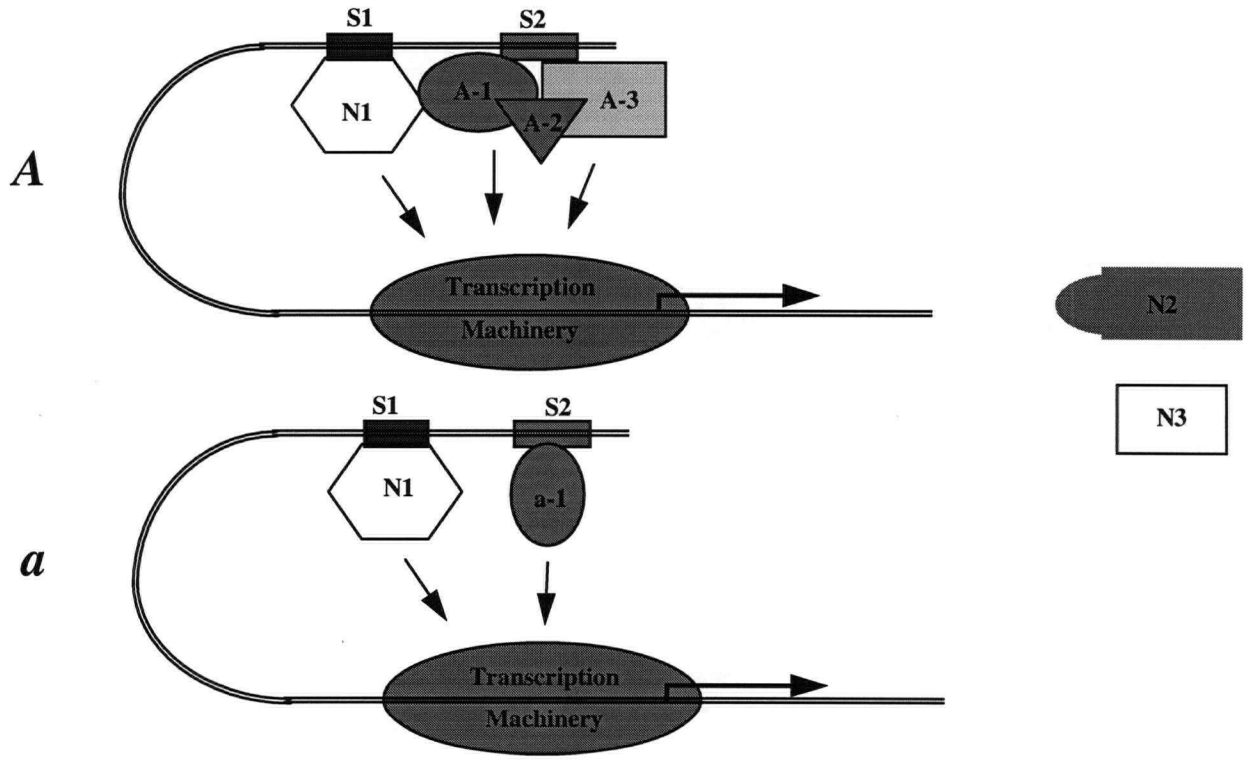


Figure 4-9 General model for regulation of *sdv*-4 and *sdv-1* by mating-type genes. N1, a general factor regulated by nitrogen starvation is necessary for transcription of *sdv* genes. MT A-1 is important for the *A* complex transcriptional activation. MT A-2 and MT A-3 function as repressors in the absence of MT A-1. Additional activators (N2 and N3) are necessary, since expression in the absence of the mating-type genes is observed. See sections 4.4.4.1 and 4.4.4.2 for details.

4.4.4.2 Model for regulation of *sdv-1*

A model, similar to that which explains regulation of *sdv-4* through activation by the mating-type proteins (Figure 4-9), can be used to explain the *sdv-1* expression pattern. Besides the mating-type gene products, the *wc-1* and *wc-2* products are required for expression of *sdv-1* (M. Nelson, pers. comm.) but not for *sdv-4* expression. In contrast to *sdv-4*, *sdv-1* seem to be regulated differently in *A* and *a* strains. In *A* strains, regulation may be more complex than in *a* strains. Because the expression of *sdv-1* in the *A* deletion strain was comparable to that of the WT *A* (Figure 4-5), the putative regulatory factor N2, in addition to mating-type proteins, is also required. The absence of MT A-1 from an *A* strain brings the levels of *sdv-1* close to zero, indicating that MT A-1 functions as an activator. Absence of *mt A-2* and *mt A-3* from the A-2^{m1} and A-3^{m1} mutants results in an increase in transcription of *sdv-1*. And the A^{IIRIP} mutant has about a 3-fold increase in the expression of *sdv-1* as compared to the WT *A*, suggesting an additive effect of MT A-2 and MT A-3. Thus MT A-2 and MT A-3 act as repressors. The *mt A-1* gene product is required for activation, otherwise A^{IRIP} would express *sdv-1* and the A^{IIRIP} mutant would have similar levels of transcription to those of the *A* deletion strain.

The transcript level of *sdv-1* in the *a* background was at least 3-fold higher than in WT *A* (Figure 4-5). Absence of *mt a-1* however did not result in the loss of *sdv-1* expression. This result suggests that in *a* strains, MT a-1 may activate *sdv-1* expression but is not essential for its expression. Other transcriptional activators may play roles in the regulation of *sdv-1* during the sexual phase. One factor may be an *a*-specific activator (N3) since the levels of *sdv-1* transcripts in the a^{m1} mutant are higher than those of the *mt A* deletion strain (see Figure 4-9). Since a^{m1} is a null mutant (Staben and Yanofsky, 1990) and no mating-type sequence is present in the *A*

deletion strain, phenotypic differences between these strains must be due to differences in the idiomorph flanking sequences.

Data from perithecial RNA analysis suggest that after cell fusion, the *A* and *a* products (or products regulated by them) interact since the expression levels of *sdv-1* are intermediate between those of the two mating-type backgrounds. However, in the *mt* duplication strain the expression of *sdv-1* is similar to that of *A*. Even though both mating-type sequences are present, regulation may be different than during a cross. Other genes required for the regulation of the *sdv* genes later in the sexual cycle may not be present. Alternatively, the control of *sdv-1* by *A* predominates over *a* regulation.

4.4.5 Expression of *asd-1* and *sdv-7*

The ASD1 protein (a rhamnogalacturonase) was found in WT asci before delimitation of ascospores (M. A. Nelson, pers. comm.). The *fmf-1* product is also required for expression of *asd-1* and thus regulation of *asd-1* may also be complex and involve several factors (Nelson and Metzenberg, 1992). Previous analysis and the results obtained in this study indicated that MT A-1 is required for *asd-1* transcription, as expression was not observed in the A^{IRIP} mutant. The a^{m1} mutant had increased levels of *asd-1* transcripts as compared to WT *a* and thus MT a-1 may repress *asd-1* transcription. The A-2^{m1}, A-3^{m1} and A^{IIRIP} mutants did not show a large variation compared to the WT *A*.

The initial results of hybridisation with *sdv-7* indicated that MT A-2 and MT a-1 may function as repressors since the levels of transcription in the specific mutants were about 10-fold higher than the WT strains. However, expression of *sdv-7* may be transient or fluctuate according to some factor, since significant variations in the expression levels occurred between RNA preparations of the same strains. Because nothing is known about the function of *sdv-7*, one

cannot resolve the relevance of the high expression levels of *sdv-7* in some cultures of the A-2^{m1} and a^{m1} mutants. However, if detection of the presence of *sdv-7* transcripts in the different cultures was due to mere serendipity, one would expect to observe the same variation in expression in a greater number of RNA preparations. However, RNA prepared from two different a^{m1} cultures exhibited high levels of *sdv-7* transcripts and in no other strain (except one preparation of A-2^{m1}) was the same high level of expression observed.

The trend of expression of *asd-1* is closest to that of *sdv-7*, indicating these two genes may be regulated in similar ways. However, more detailed analyses need to be done in order to make this correlation more clear.

4.4.6 Future directions in regulation of sexual development

Although the *sdv* genes have been known for about 5 years, only a few have been further characterised because little was known about their regulation. The present study points to some *sdv* genes that should be characterised in more detail because their regulation was shown to be influenced by all four mating-type genes. This type of study is important to focus efforts towards characterisation of genes that show similar regulation and may provide information about promoter structures of sets of sexual development genes. Also this approach could help to identify sequences that are important as DNA-binding sites for mating-type products or other related regulatory proteins.

This study is the first of its kind to indicate a molecular function for the *N. crassa* mating-type products as regulators of sexual development. Additional evidence for regulatory function of the mating-type genes may be obtained with genes that are transcribed later in the sexual development. Recently, as part of the *N. crassa* genome project, several clones specific for conidial, mycelial and sexual stages were identified (Nelson *et al.,* 1997). Expression of genes

that function at different stages of the *N. crassa* life cycle in mating-type mutants could indicate vegetative and sexual functions regulated by mating-type. Several genes have been isolated from *N. crassa* recently that appear to be transcriptional regulators of vegetative and sexual development (Aramayo *et al.,* 1996; Ballario *et al.,* 1996; Yamashiro *et al.,* 1996; Linden and Macino, 1997). Expression studies using strains with mutations at these genes may also be of help in identifying additional regulators of expression of the *sdv* genes.

5. Concluding remarks

5.1 Summary

One of the objectives of this thesis was to characterise genes of the *N. crassa A* mating-type in addition to the previously identified *mt A-1* (Glass *et al.*, 1990). Two genes, *mt A-2* and *mt A-3,* were isolated and characterised. The products of the *mt A-2* and *mt A-3* genes have characteristics of transcriptional factors, as suggested by similarities with other proteins.

Mutations at *mt A-2* and *mt A-3* revealed that these genes affect sexual development only when both are mutated. The MT A-2 and MT A-3 proteins are involved in post-fertilisation functions but do not seem to have a function during vegetative growth. The *N. crassa mt A-2* and *mt A-3* genes, although similar to the mating-type genes of the closely related species *P. anserina*, do not behave in the same manner, suggesting divergent functional evolution.

Analyses of genes preferentially transcribed during sexual development (*sdv*) suggested that all four *N. crassa* mating-type products are involved in the regulation of genes expressed under nitrogen limitation, perhaps by forming different transcriptional complexes. These analyses also suggested that if *mt A-2* and *mt A-3* are post-transcriptionally regulated, translation may be triggered by nitrogen starvation and/or protoperithecia formation.

5.2 Future directions

Even though mating-type systems in filamentous fungi seem to be more complex than in yeasts, it appears that the basic characteristics of gene regulation may be similar in the two, at least in ascomycetes. In *S. cerevisiae,* mating-type regulation depends on multiple interactions of mating-type proteins and cell type-specific products. This is coordinated by the activities of similar promoters characteristic of certain sets of genes, such as haploid-specific or cell-type

specific genes. Identification of those genes regulated by mating-type in different phases of the *N. crassa* cell cycle will likely uncover promoter sequences preferentially bound by mating-type products.

N. crassa a and *A* individuals are hermaphroditic and are indistinguishable phenotypically. The mating-type appears to be the only genetic difference between isogenic *A* and *a* strains. It seems however, that genes encoded by the idiomorph flanking sequences (Metzenberg and Randall, 1995) play a role in differentiation of the two mating-types. Thorough mutational analyses of these flanking sequences could lead to a better understanding of the importance of these sequences for mating-type regulation in *N. crassa.*

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7. Appendices

7.1 Appendix 1

7.1.1 Primer sequences of the *mt A* idiomorph

2126-2142 - 5'-GGTTTCCTTTTCGTCAG-3' (rI.1)
3363-3347 - 5'-TGTAGTCAACGGGGATC-3' (rI.2)
1454-1433- 5'- CATAACCACATCCAAGCACACC-3' (rII.1)
233-249 - 5'-GGATCAATGTAGTCGCAAGC-3' (rII.2)
630-650 - 5'-CCTCCATTTCCGCCAGTTCCC-3' (A3-836)
1790-1773 - 5'-CGACCTTAGGGCAAACG-3' (A3-1990)
1848-1864 - 5'-CGGCGAGTGACCTTGGC-3' (A2-2049)
3003-2984 - 5'-GCTCGCTGCTGACTTCGTCC-3' (A2-3200)
3105-3121 - 5'-CGCCAGCACCGACATCC-3' (CHACHA)
34-18 - 5'-CGCCGGCAGTGGACAGC-3' (ROMEO)
2196-2179 - 5'-TAGCTGGTTGCTCTGACC-3' (TANGO)
2423-2406 - 5'-GCATAGCAAGAGCTTGGC -3' (CACTUS)
1417-1435 - 5'GCAGACGGCCGACTACAGGT-3' (POLKA)
858-842 - 5'-GTACTAGCCGCCCGCGC-3' (LILI)
4032-4048 - 5'-GTTCGCCGAATCCCCGC-3' (2043)
4867-4843 - 5'-ATGGTACCTCATCTTCCACTAACCC-3'(3194)

Figure 7-1 Primers of the *mt* A idiomorph. Numbers correspond to bases complimentary to the *mt A* idiomorph sequence as deposited in GenBank (accession no. M33876).

5'-CAGACGTCGCGGTGAGTTCAG-3' (HYG)

Figure 7-2 The HYG primer. Sequences are complimentary to base 33-10 of the *hph* gene ORF.

7.2 Appendix 2

7.2.1 Analyses of variance

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
RLM 52-22	5	27062.5	5412.5	1651563
G11	5	25625	5125	8335938
A-2^{ml}	5	6625	1325	53515.63

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	52050521	2	26025260	7.775686	0.006827	3.88529
Within Groups	40164063	12	3347005			
Total	92214583	14				

Table 7-1 Analysis of Variance between A-2^{ml} mutant and parents at 8 days post fertilisation

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
RLM 52-22	5	35250	7050	1418750
G11	5	30562.5	6112.5	200000
A-2^{ml}	5	25312.5	5062.5	4925781

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	9885938	2	4942969	2.265847	0.146279
Within Groups	26178125	12	2181510		
Total	36064063	14			

Table 7-2 Analysis of Variance between A-2^{ml} mutant and parents at 11 days post fertilisation

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C(2)2-9	5	34875	6975	2370313
L22	5	15062.5	3012.5	1125781
A-3^{m1}	5	28687.5	5737.5	2948047

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	41097396	2	20548698	9.566224	0.00328	3.88529
Within Groups	25776563	12	2148047			
Total	66873958	14				

Table 7-3 Analysis of Variance between A-3^{m1} mutant and parents at 8 days post fertilisation

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C(2)2-9	5	34875	6975	2370313
L22	5	15062.5	3012.5	1125781
A-3^{m2}	5	7312.5	1462.5	817578.1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	80819271	2	40409635	28.10341	2.97E-05	3.88529
Within Groups	17254688	12	1437891			
Total	98073958	14				

Table 7-4 Analysis of Variance between A-3^{m2} mutant and parents at 8 days post fertilisation

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C(2)2-9	5	39500	7900	3415234
L22	5	31125	6225	5762891
A-3^{m1}	5	21375	4275	5563672

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	32914583	2	16457292	3.349108	0.069869
Within Groups	58967188	12	4913932		
Total	91881771	14			

Table 7-5 Analysis of Variance between A-3^{m1} mutant and parents at 11 days post fertilisation.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C(2)2-9	5	39500	7900	3415234
L22	5	31125	6225	5762891
A-3^{m2}	5	9500	1900	893750

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	95852083	2	47926042	14.27521	0.000672
Within Groups	40287500	12	3357292		
Total	1.36E+08	14			

Table 7-6 Analysis of Variance between A-3^{m2} mutant and parents at 11 days post fertilisation.

7.3 Appendix 3

7.3.1 Phosphorimager analyses of *sdv* Northern blots

strains	Blot Z21 (*crp-1*)	Blot Z6 (*tub-1*)	Blot Z13 (*crp-1*)	Blot Z18 (*crp-1*)	average	SD
A	1.001112	1	1.016026	1.004122	1.005315	0.00735
A^{IRIP}	0	0.106726		0.077292	0.061339	0.055123
A-2^{ml}	1.605337	1.969506	1.793536	1.6007	1.74227	0.176122
A-3^{ml}	1.908361	1.979125	1.448276	3.542726	2.219622	0.912926
A^{IIRIP}	3.4559	3.738734		3.341883	3.512172	0.204323
a	3.32242	3.285347		3.355596	3.321121	0.035143
a^{ml}	1.734459	2.421755		1.881155	2.503937	0.361972
Del WG	1.142211	0.322568		1.581691	1.606792	0.639055
per		1.767633			1.767633	-
Dupl		0.994861	1.085727		0.994861	-

Table 7-7 Volume of radiation counted in blots hybridised with *sdv-1*. Numbers shown are the ratio between *sdv-1* and constitutive gene control (in parenthesis) normalised according to the *A* control (ratio=1). Average and standard deviation are shown. Empty cells indicate sample was not present in the different blots.

	Blot Z4	Blot Z5	Blot Z 3	Blot Z 21	Blot Z16	Blot Z13	average	SD
A	1.001586	1.000447	1.021111	1.00514	1.001627	1.003489	1.005567	0.007793
A^{IRIP}		0	0	0			0	0
A-2^{ml}	0	0.692	1.008900	0	1.091695	2.142138	0.820972	0.801928
A-3^{ml}	0.198641	0		3.036755		0.92634	1.040434	1.389176
A^{IIRIP}	0.250085	1.337357	2.616475	1.724519			1.482109	0.980542
a	0.0238	0.539178	1.288409	2.675788			1.131794	1.152865
a^{ml}		5.704501	4.496045	10.34061			6.847052	3.085256
Del WG		7.806091	4.104593	12.65337			8.188019	4.287169
Del Vg		1.14					1.140081	-
Per.		0.044419					0.044419	-
dupl.		0	0.310482	0		0.581098	0.222895	0.280086

Table 7-8 Volume of radiation counted in blots hybridised with *sdv-4*. Numbers shown are the ratio between *sdv-1* and constitutive gene *crp-1*, normalised according to the *A* control (ratio=1). Average and standard deviation are shown. Empty cells indicate sample was not present in the different blots.

A	1.007612
A^{IRIP}	0.00013
A-2^{ml}	0.81716
A-3^{ml}	0.733607
A^{IIRIP}	0.531774
a	0.648531
a^{ml}	1.473367
Del WG	0.361101

Table 7-9 Volume of radiation counted in blots hybridised with *asd-1*. Numbers shown are the ratio between *sdv-1* and *crp-1* normalised according to the *A* control (ratio=1)

strains	Blot Z5	Blot Z3	average
A	1.000694	1.000125	1.000409
A^{IRIP}	0.437689	0.051809	0.244749
A-2^{ml}	13.82086	0	6.910429
A-3^{ml}	2.130046	0	2.130046
A^{IIRIP}	0.151567	0.928922	0.540245
a	0.444869	0.220809	0.332839
a^{ml}	7.49931	6.967692	7.233501
Del WG	0.255173	0.167383	0.211278
Del Vg	0.584538		

Table 7-10 Volume of radiation counted in blots hybridised with *sdv-7*. Numbers shown are the ratio between *sdv-7* and constitutive gene *crp-1* normalised according to the *A* control (ratio=1).

7.3.2 Northern analyses of *sdv-3, sdv-6* and *sdv-14*

The *sdv-3* gene was expressed in lower levels in the A^{m44} strain (Nelson and Metzenberg, 1992). An example of hybridisations with *sdv-3* is shown in Figure 7-3. A transcript corresponding to *sdv-3* appears in all strains with similar intensity. However, the A^{IRIP} and the A-3^{m1} mutants were not included in this experiment. Based on this and on previous analyses (Nelson and Metzenberg, 1992) it appears that *sdv-3* is an example of a gene regulated by *mt A-1* only.

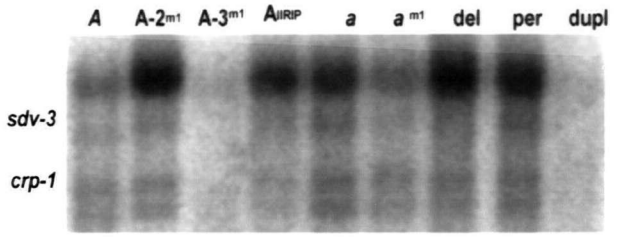

Figure 7-3 Expression of *sdv*-3.

The initial analyses of *sdv*-6 and *sdv*-14 showed an increase in levels of transcription in the A^{m44} as compared to the WT (Nelson and Metzenberg, 1992). Even though a few hybridisations were performed with *sdv*-14, all the blots had a high background and it was impossible to perform quantitative analyses (see Figure 7-4 for an example of a Northern blot with *sdv*-14). In analyses of *sdv*-6 transcripts, two bands of aproximately 2.0 and 1.7 kb were observed in most strains. In the initial studies with the *sdv* genes of Nelson and Metzenberg (1992), the size of the *sdv*-6 transcript was 2.0 kb.

However the signal yielded by probing with *sdv*-6 was very strong and might have corresponded to two transcripts. Thus Northern blots probed with the *sdv*-6 were not quantitatively analysed because it was not clear which of the bands were regulated at low nitrogen conditions as previously described.

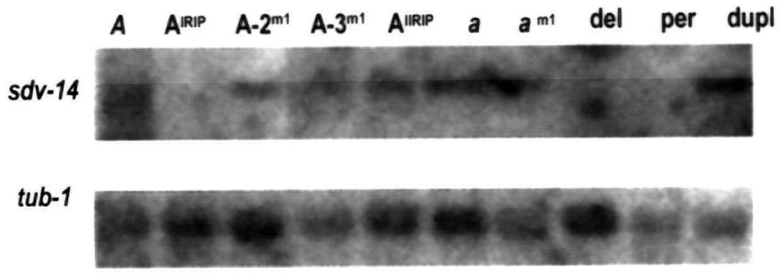

Figure 7-4 Expression of *sdv-14*