A/a INCOMPATIBILITY IN *NEUROSPORA CRASSA*--NOVEL SUPPRESSORS

AND NUCLEAR INCOMPATIBILITY

by

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ABSTRACT

The sexual functions of the mating type gene (*mt*) of
Neurospora crassa include specification of mating identity
(Shear and Dodge, 1927) and perithecial maturation
(Griffiths and DeLange, 1978; Staben and Yanofsky, 1990).
The gene also acts as a vegetative incompatibility locus, so
that *A* + *a* heterokaryons (Beadle and Coonradt, 1944) or *A/a*
duplication strains (Newmeyer and Taylor, 1967) grow poorly
or not at all.

An intriguing question regarding the mating type gene
is this: How does it control both the switch between
somatic and meiotic events and heterokaryon incompatibility?
Several research groups (Glass, *et al.*, 1990; Staben and
Yanofsky, 1990) are presently studying the sexual functions
of the mating type genes. I present a study of the
incompatibility function.

Two experiments were performed. The first was a search
for new suppressors of mating type-associated
incompatibility, which resulted in the identification of
seven new suppressors, none of which was allelic with the
one known suppressor, *tol*. The second was the comparison of
growth rates of a mating type mutant (fertile, heterokaryon
compatible) in a mixed mating type heterokaryon and in a
mixed mating type duplication to determine whether or not
cytoplasmic incompatibility is separable from nuclear

incompatibility. The results obtained suggest that the mating type mutant, a^{m33}, eliminates heterokaryon incompatibility without eliminating nuclear incompatibility.

The search for suppressors was attempted in order to define more of the genes involved in A/a incompatibility. The analysis of heterokaryon versus nuclear incompatibility was done to investigate the cellular interactions involved in A/a incompatibility.

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GENERAL INTRODUCTION

This work is a two-part investigation of the vegetative incompatibility function of the mating type gene of *Neurospora crassa*. The first part describes the generation of suppressors of *A/a* incompatibility and the second part describes the analysis of incompatibility on a cellular level. The mating type gene is involved in both the vegetative and sexual phases of the life cycle.

Life Cycle

N. crassa, a mold that grows at the sites of recent fires and in decaying vegetation, is a heterothallic ascomycete. Its life cycle is shown in Fig. 1. The haploid, partially septate mycelia grow from ascospores which are germinated by a period of heating. Growth requires the presence of inorganic salts, biotin and a utilizable carbon source (Fincham, *et al.*, 1979).

Several days after germination, the hyphal tips begin to delimit two types of conidia which, upon germination, are able to initiate new mycelia. Macroconidia are oval and multinucleate; whereas microconidia are spherical and binucleate or uninucleate (Fincham, *et al*, 1979).

Low levels of nitrogen initiate the sexual cycle. Male structures--conidia and vegetative hyphae--are already present in each single mating type colony (*A* or *a*), and
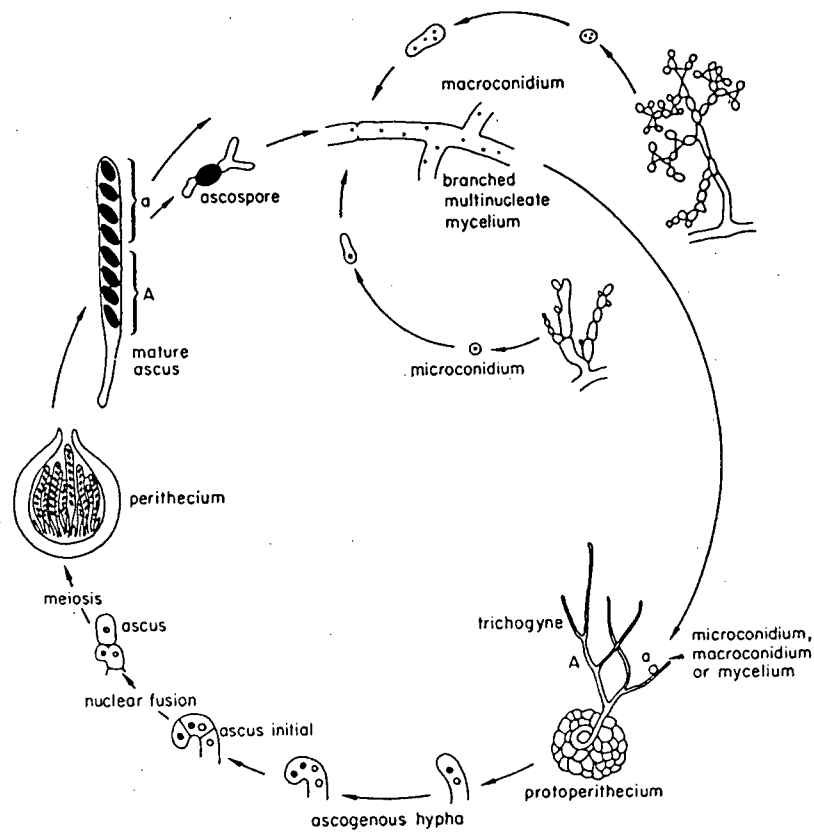
Figure 1 Life cycle of N. crassa (from Fincham, et al., 1979).

immature female structures--protoperithecia--begin to form from the same mycelium. The outer layer of the protoperithecium is a wall of hyphae. Inside is a coiled hypha, the ascogonium, from which project female reproductive hyphae, trichogynes. Although each mycelium is hermaphroditic, it is also self-sterile, and sexual fusion between male cells and protoperithecia can occur only between individuals of different mating types. Once fertilized, the protoperithecium is known as a perithecium (Fincham, et al., 1979).

Conidia emit a pheromone which directs trichogynes to grow toward them (Bistis, 1981; 1983). When contact is made between the male and female cells, plasmogamy ensues. The male nuclei, presumably under their own genetic control (Vigfusson, et al., 1971), travel down the trichogyne, where one nucleus (Sansome, 1949) enters the ascogonium and becomes associated with the female nucleus. While the perithecium darkens and enlarges, a series of synchronous nuclear divisions gives rise to a cluster of dikaryotic ascogenous hyphae (Fincham, et al., 1979).

Karyogamy occurs in the penultimate cells of the ascogenous hyphae, followed directly by meiosis plus two rounds of mitosis. Several days later, the ascospores have become multinucleate (Raju, 1980). The final products of the sexual cycle are perithecia containing many mature asci, each housing eight ascospores which are shot through an opening in the perithecial beak (Fincham, et al., 1979).

Mating Type Gene Functions

The mating type genes, *A* and *a*, are unusual, even among *Neurospora* species, in that they control two functions, mating and vegetative incompatibility. *A/a* incompatibility is not seen in either *N. tetrasperma* (Dodge, 1935) or *N. sitophila* (Mishra, 1971). Early attempts to resolve the two functions by recombination failed (Pittenger, 1957; Newmeyer, *et al.*, 1973); although later, Griffiths and DeLange (1978) reported the finding of a mating type mutant (*a^{m33}*) that was heterokaryon compatible, yet fertile.

In *N. crassa*, only strains of opposite mating types are able to cross (Shear and Dodge, 1927); so *A x a* is a successful pairing, but *A x A* or *a x a* is not. Strains of opposite mating types are vegetatively incompatible (Beadle and Coonradt, 1944); so *A + A* or *a + a* anastomose to form thriving heterokaryons, and *A + a* fuse, but the anastomosed area dies (Garnjobst and Wilson, 1956). The protoplasm of the fused, and sometimes surrounding, cells becomes granular or vacuolated. Mixed mating type heterokaryons with varying degrees of vigour can be made using forcing auxotrophic markers (Beadle and Coonradt, 1944; Gross, 1952; DeLange and Griffiths, 1975).

Protoplasmic killing, more severe than that seen between *A* and *a*, is observed in the reactions between incompatible alleles of the heterokaryon incompatibility genes *het-C/c*, *het-D/d* and *het-E/e* (Perkins, 1974). The

killing reaction can be detected *in vivo*, and also when protoplasm from one strain is microinjected into cells of an incompatible strain, at least between strains of different *het-C/c* or *het-D/d* genotypes (Wilson, *et al.*, 1961; Williams and Wilson, 1966).

Mixed mating type heterokaryons generally escape from their poor growth and start to grow at wild-type or near wild-type rates by deletion of one or the other of the mating type genes (DeLange and Griffiths, 1975). Heterokaryons heterozygous for the vegetative incompatibility genes *het-J/j* or *het-K/k* escape by deletion or mutation of the genes (Pittenger, 1964). Strains with a heterozygous duplication of the vegetative incompatibility gene, *het-6*, also escape by deletion of one of the genes (Glass, personal communication).

Not only are the mating type genes incompatible in a heterokaryon, they are also incompatible in a duplication. Strains carrying a heterozygous duplication of the mating type genes grow poorly due to the presence of opposite mating type genes in one nucleus. *A/a* duplication strains, called "dark agar" strains, produce a brown pigment when grown on glycerol complete medium and their morphology has been described as being spidery (Newmeyer and Taylor, 1967; Turner, *et al.*, 1969). Other duplications in *N. crassa* grow normally (provided the duplication does not cover any of the heterokaryon incompatibility genes) and are frequently barren, *i.e.* they produce abundant perithecia, but few

spores (Newmeyer and Taylor, 1967). A/a duplications escape from their inhibited growth and start growing at wild-type or near wild-type rates by the somatic segregation of A from a through mitotic crossing over (Fig. 2) or deletion of one of the mating type genes (Newmeyer and Taylor, 1967).

Mitotic crossing over yields a culture, barren due to the presence of duplicated genetic material, that is a mixture of mostly A or a homozygous cells. Such cultures are unstable and tend to be overgrown by one nuclear type. Deletion yields a culture, fertile due to the loss of part or all of the duplication, that is a mixture of A or a hemizygous cells. These cultures are also overgrown by one nuclear type (Newmeyer and Taylor, 1967).

Mating and Incompatibility in Other Fungi

The majority of research on yeast mating type has been done on Saccharomyces cerevisiae, also known as budding yeast because haploid cells reproduce vegetatively by budding. Mating begins with G1 arrest. Pheromone from a cells, a-factor, arrests alpha cells, and pheromone from alpha cells, alpha-factor, arrests a cells. Pairs of opposite mating type cells fuse, undergo karyogamy and then follow either of 2 paths, depending on nutritional conditions. The a/alpha diploid cells reproduce mitotically
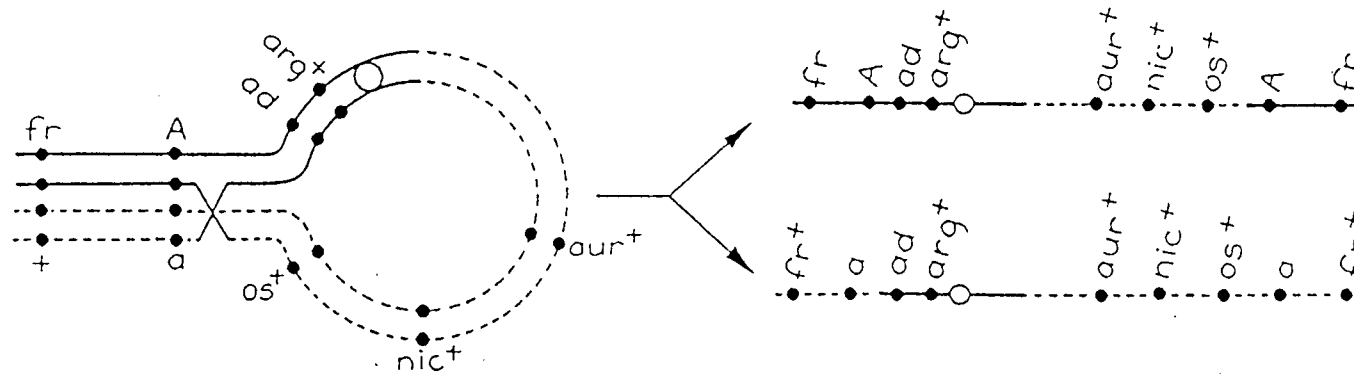
Figure 2   Example from Newmeyer and Taylor (1967) of mitotic crossover that leads to production of cells homozygous for the mating type genes. The duplication was a product from a cross to an inversion strain.

unless they are nutritionally deprived, in which case they undergo meiosis (see review by Herskowitz, 1988).

The mating types of the haploid cells are specified at the mating type locus, *MAT* (Lindegren and Lindegren, 1943), which codes for transcription factors that control the expression of genes involved in pheromone production, mating and sporulation. Cells with mating type *a* have the *MATa* allele which encodes two polypeptides, *a1* and *a2*; cells with mating type *alpha* have the *MATalpha* allele which also encodes two polypeptides, *alpha1* and *alpha2*. A portion of the *MAT* genes, called Y*a* and Y*alpha* (Nasmyth, *et al.*, 1981), is specific to *a* and *alpha* cells, respectively (Sprague, *et al.*, 1981).

Three of the mating type polypeptides, *a1*, *alpha1* and *alpha2*, are involved in the regulation of *a*-specific, *alpha*-specific and haploid cell-specific genes. The function of *a2* is unknown (Astell, *et al.*, 1981). In *a* cells, *a1* is produced (Kassir and Simchen, 1976) and *a*-specific and haploid specific genes are expressed. In *alpha* cells, *alpha1* induces the expression of *alpha*-specific genes (Sprague, *et al.*, 1983) and *alpha2* represses *a*-specific genes (Hartig, *et al.*, 1986; Wilson and Herskowitz, 1984).

In *a/alpha* diploid cells, *alpha2* carries out the same function as it does in haploid *alpha* cells, repressing *a*-specific genes (Strathern, *et al.*, 1981), but it has an additional role. A combination product of *alpha2* and *a1*

represses the expression of *alpha1*, and of haploid specific genes and stimulates sporulation (Strathern, *et al.*, 1981).

Sporulation begins when *a/alpha* diploid cells are starved of nitrogen and carbon (Esposito and Klapholz, 1981). The regulatory protein *a1/alpha2* activates meiosis by blocking the expression of *RMEI*, an inhibitor of meiosis (Mitchell and Herskowitz, 1986).

Strains of *S. cerevisiae* with the dominant allele of the homothallism gene, *HO*, are homothallic, whereas strains with the recessive allele, *ho*, are heterothallic. The *HO* gene product catalyses high frequency interconversion of mating types (Hicks and Herskowitz, 1976). Mating type interconversion occurs by the switching of the genetic information at the *MAT* locus (Nasmyth and Tatchell, 1980; Strathern, *et al.*, 1980; Hicks, *et al.*, 1977). The information comes from two transcriptionally silent genes, *HMR* and *HML*, that flank the mating type gene. Each locus contains a copy of *a* or *alpha*. The two loci are kept silent by the action of unlinked genes called *SIR* (for "silent information regulator") (Abraham, *et al.*, 1983).

The switching process begins with a double-stranded cut at *MAT* (Strathern, *et al.*, 1982) by the endonuclease encoded by *HO* (Kostriken and Heffron, 1984). A conversion-like event follows, in which heteroduplex DNA forms between the donor locus (*HMR* or *HML*) and *MAT*. The heteroduplex DNA is repaired using the donor DNA as a template (Klar and Strathern, 1984; Klar *et al.*, 1984). Switching is prevented

in diploid cells by the repression of *HO* (Jensen, *et al.*, 1983).

Mating in the fission yeast, *Schizosaccharomyces pombe*, is similar in some ways to that in *Saccharomyces cerevisiae*. Haploid cells have the mating type h^+ or h^- and propagate vegetatively by fission, not by budding. During mating, which occurs under nitrogen starvation conditions, one cell of each mating type participates in the formation of a diploid zygote. The zygote immediately undergoes meiosis and sporulation (Leupold, 1950 cited in Kelly, *et al.*, 1988).

As in homothallic strains of *S. cerevisiae*, *S. pombe* regularly switches alleles at the mating type locus (Egel, 1977; Miyata and Miyata, 1981). The mating type complex contains 3 regions--*mat1*, *mat2-P* and *mat3-M*--which control conjugation, meiosis and sporulation (Kelly, *et al.*, 1988). Two silent loci, *mat2-P* and *mat3-M* donate information to *mat1* which confers mating type, either h^+ (*mat1-P*) or h^- (*mat1-M*) (Egel, 1977; Egel and Gutz, 1981; Beach, 1983).

The complex has been sequenced (Kelly, *et al.*, 1988). Two genes are encoded by each of *mat1-P* and *mat1-M*, two of which are required for conjugation and specification of mating type, and all 4 of which are required for meiosis and sporulation (Kelly, *et al.*, 1988). Each of *mat1*, *mat2-P* and *mat3-M* contains 2 blocks of sequence homology. The silent genes, *mat2-P* and *mat3-M*, alone contain a third region of

homology which probably acts as a silencer (Kelly, *et al.*, 1988).

Like *RMEI* of *S. cerevisiae*, the protein, *ran1*, of *S. pombe* is an inhibitor of meiosis. Its action is blocked by the protein, *mei3*, which is produced in h^+/h^- cells (McLeod and Beach, 1988). *Neurospora crassa* could be like *S. cerevisiae* in that the mating type genes could act like *a1/alpha2*, combining to form a transcription factor that blocks the synthesis of a meiosis inhibitor, or it could be like *S. pombe* in that a product analogous to *mei3* could be produced in *A/a* mating diploid cells. The complex system of regulating cell type in *S. cerevisiae* must differ in *N. crassa* because each mating type idiomorph encodes but one transcript.

That the combination product, *A/a*, could act as a transcription factor is supported by the sequence similarity between the *a* idiomorph and the HMG box motif, a DNA-binding sequence (Staben and Yanofsky, 1990). The *A* idiomorph has no obvious DNA-binding motif, but it does have similarity to the MATalpha1 protein of *S. cerevisiae* and could interact with the *a* product during mating. During the vegetative state, *A* identity could be specified by *A*-specific genes, turned on by the *A* product interacting with a transcription factor that binds to their promoter regions (Glass, *et al.*, 1990). Strains containing a heterozygous duplication of the mating type region do not lose either their *A* or *a* identity,

so the combination product does not exclude mating type specificity.

Homothallism in *N. crassa* must also occur by a different mechanism than switching because each strain in the homothallic species *N. dodgei*, *N. galapagonensis*, *N. africana* and *N. lineolata* has only one copy of a sequence homologous to the *N. crassa* mating type gene, *A*. The homothallic species, *N. terricola*, has sequences homologous to the *N. crassa* mating type genes, *A* and *a*, but only one copy of each (Glass, *et al.*, 1988).

The incompatibility systems, also called breeding systems, of the 2 basidiomycetes, *Coprinus cinereus* and *Schizophyllum commune*, are tetrapolar. The mating type complex is comprised of 2 regions, *A* and *B*, each containing 2 genes, *alpha* and *beta*, with multiple alleles. Mating requires that the 2 participants differ at a minimum of one *A* gene and one *B* gene. Close linkage of *alpha* and *beta* restricts inbreeding potential by inhibiting recombination (see Koltin, *et al.*, 1972).

Cloning of the *A* factor of *C. cinereus* has revealed that the *alpha* and *beta* regions are themselves composed of a number of genes with multiple alleles, some of which are common to other alleles of *A* and some of which are unique (E. Mutasa, A. Tymon, W. Richardson, U. Kues and L. Casselton, 1991 in published abstracts from Sixteenth Fungal Genetics Conference).

Three alleles of the *A-alpha* region of *S. commune* have been cloned, sequenced and shown to contain multiple transcripts, some shared and some unique. Some of the postulated polypeptides contain homeodomain motifs (R.C. Ullrich, M.M. Stankis, H. Yang and C.P. Novotny, 1991; G. May, 1991 in published abstracts from Sixteenth Fungal Genetics Conference), implying that the products regulate the expression of other genes.

In the pathogenic basidiomycete, *Ustilago maydis*, dikaryosis between individuals from different incompatibility groups is a prerequisite for pathogenic infection. Different *a* alleles are required for fusion and different *b* alleles for pathogenicity. The two alleles of *a* have been cloned and they encode a product required for mycelial growth, a condition necessary for infection (M. Bolker and R. Kahmann, 1991 in published abstracts from Sixteenth Fungal Genetics Conference).

Ten *b* alleles have been cloned, and subsequent molecular analysis has revealed that they share a homeodomain-related motif, implying that the *b* polypeptides bind DNA, possibly to regulate sexual development (R. Kahmann, B. Gillissen, R. Schleshinger, C. Sandmann, F. Schauwecker, J. Bergemann, B. Schroeer, M. Bolker and M. Dahl, 1991 in published abstracts from Sixteenth Fungal Genetics Conference). Like the *A* and *B* regions of *S. commune* and *C. cinereus*, the *b* region of *U. maydis* is composed of 2 genes, *b-east* and *b-west*. Null mutants of the

b region are mating deficient, suggesting that the postulated *b* heterodimer formed during mating is an activator of mating genes (Kahmann, personal communication).

Studies done with expression of two of the *b* alleles have identified a 70 amino acid region responsible for allele specificity. These 2 *b* alleles are constitutively expressed in diploids and haploids, although at a lower level in the latter (L. Giasson, A. Yee and J.W. Kronstad, 1991 in published abstracts from Sixteenth Fungal Genetics Conference). Similarly, in *N. crassa* the mating type genes are expressed during sexual and vegetative phases of the life cycle, but at a lower level in the latter (Staben and Yanofsky, 1990; Glass, *et al.*, 1990).

N. crassa differs from the basidiomycetes in several ways. The mating type genes of *N. crassa* have only one ORF, so unlike the basidiomycetes, they do not function as restrictors of inbreeding. Also, in *N. crassa*, vegetative compatibility is not a prerequisite for mating. Fusion of hyphae with compatible genotypes leads to heterokaryon formation and fusion of sexual cells with compatible genotypes leads to mating. The heterokaryosis that occurs between vegetative cells is somehow different from the fusion that occurs between the trichogyne and the male cell. Mixed mating type heterokaryons on crossing medium do not exhibit the incompatibility phenotype, so perhaps it is the nutritional conditions that dictate whether the mating type genes will initiate incompatibility or mating.

The mating type gene of the yeasts is a master regulator of other genes. The mating type genes of the basidiomycetes and of *N. crassa* are also postulated to be regulatory, although the mechanisms of their actions must differ.

Each of the four major groups of fungi--phycomycetes, basidiomycetes, ascomycetes and fungi imperfecti--exhibits genetically determined incompatibility (Burnett, 1976). In some cases, *e.g. Neurospora crassa* (Fincham, *et al.*, 1979), *Podospora anserina*, under the control of the *S* locus (Esser, 1971), *Rhizoctonia solani* (Burnett, 1976) and *Endothia parasitica* (chestnut blight fungus) (Anagnostakis, 1977), the incompatibility only affects vegetative heterokaryosis; whereas in others, *e.g. Aspergillus nidulans* (Jinks, *et al.*, 1961; Grindle, 1963a; 1963b), *Podospora anserina*, under the control of the *a,b,c,v* loci or non-allelic system (Esser, 1971; Blaich and Esser, 1971), *Coprinus lagopus*, *Schizophyllum commune* (Fincham et *al.*, 1979), *Ustilago* sp. (Fincham et *al.*, 1979; Day and Cummins, 1981) and yeasts (Crandall, 1978), the genetic restrictions on fusion affect fertility.

Two species of fungus other than *N. crassa* that have mating type genes that act as heterokaryon incompatibility loci are *Ascobolus stercorarius* (Bistis, personal communication) and *Aspergillus heterothallicus* (Kwon and Raper, 1967).

Mating and Incompatibility in Other Kingdoms

Each of the five kingdoms of organisms--Monera,
Protista, Fungi, plants and animals--shows examples of
genetically controlled, intraspecific, sexual or somatic
incompatibility. Moreover, interactions *between* species,
for example, those between host and pathogen or between
symbionts, can exhibit incompatibility.

Sexual incompatibility limits mating between certain
individuals within a species. Somatic incompatibility
limits fusion between or co-existence of certain cells.
Some of the fungi demonstrate incompatible reactions which
are both somatic and sexual, in that a successful
interaction between seemingly vegetative cells leads to
mating.

In the kingdom Monera, mating in *Escherichia coli*
requires recognition of mating types; and only pairings
between cells of different mating types are compatible
(Hayes, 1952; Lederberg, 1957).

Ciliates, in the kingdom Protista, generally mate with
cells of a different mating type and fusion occurs between
"vegetative" cells (there are no cells specialized for
mating) under certain environmental and physiological
conditions (Nanney, 1977; Ricci, 1981).

In plants, there are examples of both sexual and
somatic incompatibility. Some angiosperms have the *S* locus
which controls sexual compatibility, allowing pollen to

fertilize only females with different *S* alleles (Lewis, 1954; Ebert *et al.*, 1989; Haring *et al.*, 1990). Somatic incompatibility occurs between different species of plants when tissue from one species is grafted onto an individual of another species, even from the same family (Yeoman *et al.*, 1978).

Fusion of cells in the sexual cycle of *Chlamydomonas* sp. requires recognition of opposite mating types (Wiese and Wiese, 1978; Harris, 1989) which are determined by two alleles, mt^+ and mt^-, at one locus. The alleles are believed to encode proteins that control the expression of other genes or the activity of their products, possibly by forming or causing the formation of a novel regulatory product upon cell fusion (Ferris and Goodenough, 1987).

A somatic interaction is seen in vertebrates when a graft of tissue or an organ transplant is rejected from the recipient. The reaction, in this case, is controlled by genes of the major histocompatibility complex (MHC), which are expressed in the T cells of the immune system (see reviews by Klein, 1976; Bach and vanRood, 1976). Humoral recognition occurs in the blood, and it is governed by antibodies specific for the ABO blood group antigens and the Rh factor protein (see review by Katz, 1978).

INTRODUCTION 1

A Suppressor of *A/a* Incompatibility, *tol*

Newmeyer (1970) found a recessive suppressor of *A/a* incompatibility, unlinked to *mt*, which she called *tol* for "tolerant". The new gene had no demonstrable effect on the ability of a strain to mate. It appears to be inactive during starvation because *A* + *a* heterokaryons on crossing medium do not exhibit the incompatibility phenotype. However, Johnson (1979) suggested that the gene does have a role during mating because the recessive allele, *tol*, suppresses *fmf-1*, a gene specifying female and male fertility. Crosses between *fmf-1*, *tol*$^+$ and *fmf-1*$^+$, *tol*$^+$ are sterile, whereas crosses between *fmf-1*, *tol* and *fmf-1*$^+$, *tol* are fertile. Johnson hypothesized that *tol* permits promiscuous fusion between *A* and *a* during mating, allowing transfer of *fmf-1*$^+$ product from the *fmf-1*$^+$ strain, resulting in rescue of the sterility phenotype. If *tol*$^+$ is expressed during the sexual cycle, fusion of sexual structures occurs despite the presence of *tol*$^+$. It is possible that the cytoplasm of trichogynes is modified to allow the presence of male nuclei of the opposite mating types.

Genes that suppress *A/a* incompatibility will be useful in analysing the mating type gene itself and in deciphering the process of incompatibility. Moreover, if a suppressor has its own phenotype, in addition to suppressing *A/a*

incompatibility, then other functions related to incompatibility may be revealed. For example, if a suppressor prevents incompatibility by altering cell wall structure so that it can no longer be broken down by the incompatibility reaction, it may produce a second phenotype of abnormal morphology. Two types of suppressors, extragenic and intragenic, are discussed below.

Extragenic suppressors probably interact with the mating type genes; and their detection will help in the dissection of the mechanism of incompatibility. Theories as to how *tol* affects incompatibility are considered in Discussion 1.

Other suppressors, besides *tol*, have been identified--two found in nature and two induced in the laboratory. Smith and Perkins (1972) noted that the osmotic-sensitive, reciprocal translocation strain, *cut*, suppressed *A/a* incompatibility. Newmeyer (1970) reported that the wild type strain, Panama *a*, (Fungal Genetics Stock Center #1132) segregated compatible and incompatible progeny when crossed to a duplication-generating inversion *A* tester. It is unknown whether or not this suppressor is allelic with *tol*.

Newmeyer (1970) found another suppressor, which may or may not be allelic with *tol*, in an escaped *A/a* duplication strain (called N83) from a cross between an inversion *A* strain and a normal sequence *a* strain. DeLange and Griffiths (1975) reported that 2 of their escaped mixed mating type heterokaryons, in which one component was *tol*

and the other tol^+, produced perithecia with both mating type testers, but produced ascospores with only 1. They suggested that these 2 strains arose by deletion or lethal mutation at tol^+.

Intragenic suppressors and mutants of the mating type genes will help, when mapped and sequenced, to reveal the part(s) of the mating type genes governing vegetative incompatibility. Especially useful will be those mutants in which only one of the mating type functions is defective, e.g. the fertile, heterokaryon compatible mutant, a^{m33}.

When Newmeyer discovered tol, she was using strains of *N. crassa* with *A/a* duplications, dark agars. They were meiotic segregants from a cross of a normal sequence *A* to a strain of mating type *a* carrying a pericentric inversion of a large portion of linkage group I (L.G. I) (Fig. 2). Most of the dark agars escaped from their inhibited growth by the somatic segregation of *A* from *a*. One strain, however, escaped by mutation at tol, thereby allowing both mating type genes to reside within one nucleus without causing vegetative incompatibility. The new tol gene proved to be a suppressor, not only of nuclear incompatibility, but also of heterokaryon incompatibility, allowing the vigorous growth of both duplicated *A/a,tol* strains and *A,tol* + *a,tol* heterokaryons.

Newmeyer favoured the use of a *A/a* duplication to cause inhibited growth because it eliminated the possibility that incompatibility was due to alleles at some other

heterokaryon incompatibility locus. Furthermore, if a mixed mating type heterokaryon had been used, only "dominant" alleles of suppressor genes would have allowed escape to occur.

A method similar to that used by Newmeyer (1970) has been used to generate novel suppressors of *A/a* incompatibility. The search is an attempt to chart the genetic interactions involved in mating type-associated incompatibility.

MATERIALS AND METHODS

General protocols were standard, and are described in Davis and DeSerres (1970). Crosses were made at 25°C in 15 cm test tubes or, if *fl* females were used, on petri plates.

Ascospores with *A/a* duplications were selected as meiotic segregants from crosses to strains in which the mating type gene had been translocated from L.G. I onto L.G. II. Duplication progeny contained L.G. II from the translocation parent and L.G. I from the other parent. This combination of linkage groups was selected as follows. The mating type genes were marked, one with an auxotrophic marker (*ser-3*) and the other with a temperature sensitive marker (*un-3*), and ascospores were plated on minimal medium at 32°C which permitted survival of only the duplication spores.

Strains and Markers

A list of strains and their sources is shown in Table 1. Strains were maintained at room temperature on standard media.

The marker, *un-3*, is temperature-sensitive, with strains growing poorly between 28.5°C and 30°C and not growing at all at temperatures above 30°C. It is located 0.04 to 0.1 map units to the left of the mating type gene (Perkins, *et al.*, 1982). The marker contains two mutations,

Table 1 Strains from Experiment Set 1

STRAIN	GENOTYPE	SOURCE
R601	un-3, A	C.J. Myers
R602	un-3, a	C.J. Myers
T(I->II)39311,ser,A	T(I->II)39311, ser-3, A	C.J. Myers
T(I->II)39311,ser,a	T(I->II)39311, ser-3, a	C.J. Myers
T(I->II)39311,ser,trp,tol,A	T(I->II)39311, ser-3, trp-4, tol, A	C.J. Myers
T(I->II)39311,ser,trp,tol,a	T(I->II)39311, ser-3, trp-4, tol, a	This work
trp,tol,A	trp-4, tol, A	N.L. Glass (F.G.S.C.* #2336)
fl,A		F.G.S.C. #4960
fl,a		F.G.S.C. #4961

*F.G.S.C.=Fungal Genetics Stock Center

separated by 0.1 map units, one in *cytochrome-20* and the other in *ethionine-2* (A.M. Lambowitz, unpublished results, Griffiths, personal communication). Tests for *un-3* were done on minimal medium at 25°C and at 37°C and were repeated 3 times each for positive identification. In these tests, a higher temperature (37°C) could be used than was used to select duplication progeny (32°C) because the *un-3* phenotype is more obvious at 37°C and it was not necessary to avoid killing the fungi.

The translocation T(I->II) 39311 is an insertion of an interstitial segment of the left arm of linkage group I (including *ser-3*, *un-3 and mt*) into the right arm of linkage group II, inverted with respect to the centromere (Perkins, 1972).

The gene, *ser-3*, specifies a requirement for serine and is located fewer than 2 map units to the left of the mating type gene (Perkins, *et al.*, 1982).

The gene, *trp-4*, specifies a requirement for tryptophan and is linked to *tol* by less than 1 map unit (Perkins, *et al.*, 1982).

The aconidial mutant, *fluffy* (*fl*), is highly fertile and is used as a mating type tester.

Ascospore Isolation

Ascospores were isolated as follows, unless stated otherwise. Spores were collected from the sides of the

crossing test tubes with a wire loopful of sterile distilled water and put into eppendorf tubes of sterile distilled water or 0.1% agar. Hemocytometer counts were done to determine the necessary dilution factor that would yield approximately 10-30 spores per plate. Diluted spores (1/4 mL) were pipetted and spread onto sorbose plates with a bent glass rod. The plates were placed into a 60°C oven for 30 minutes to heat shock the spores to initiate germination. Spores were left at room temperature for 4-5 hours to ensure that the mycelia were well established prior to selection. The plates were left overnight in a 32°C incubator so that the colonies grew large enough to be seen and harvested.

Individual germinated spores were collected by cutting out a square of agar containing the mycelium and placing it into a test tube containing 1 mL of Westergaard and Mitchell's liquid medium. If, after several days, the level of the liquid in the tubes dropped below half, then sterile distilled water was added to maintain the level of liquid at 1 mL.

Construction of Tester Strain (T(I->II) 39311, *ser*, *trp*, *tol*, *a*)

The strain, T(I->II) 39311, *ser*, *trp*, *tol*, a was made from a cross of a female *trp*, *tol*, A to a male T(I->II) 39311, *ser*, *a*, from which *ser*, *trp*, *tol*, *a* progeny were selected (Fig. 3). The female was inoculated onto synthetic

T(I→II)39311,ser,a x trp,tol,A
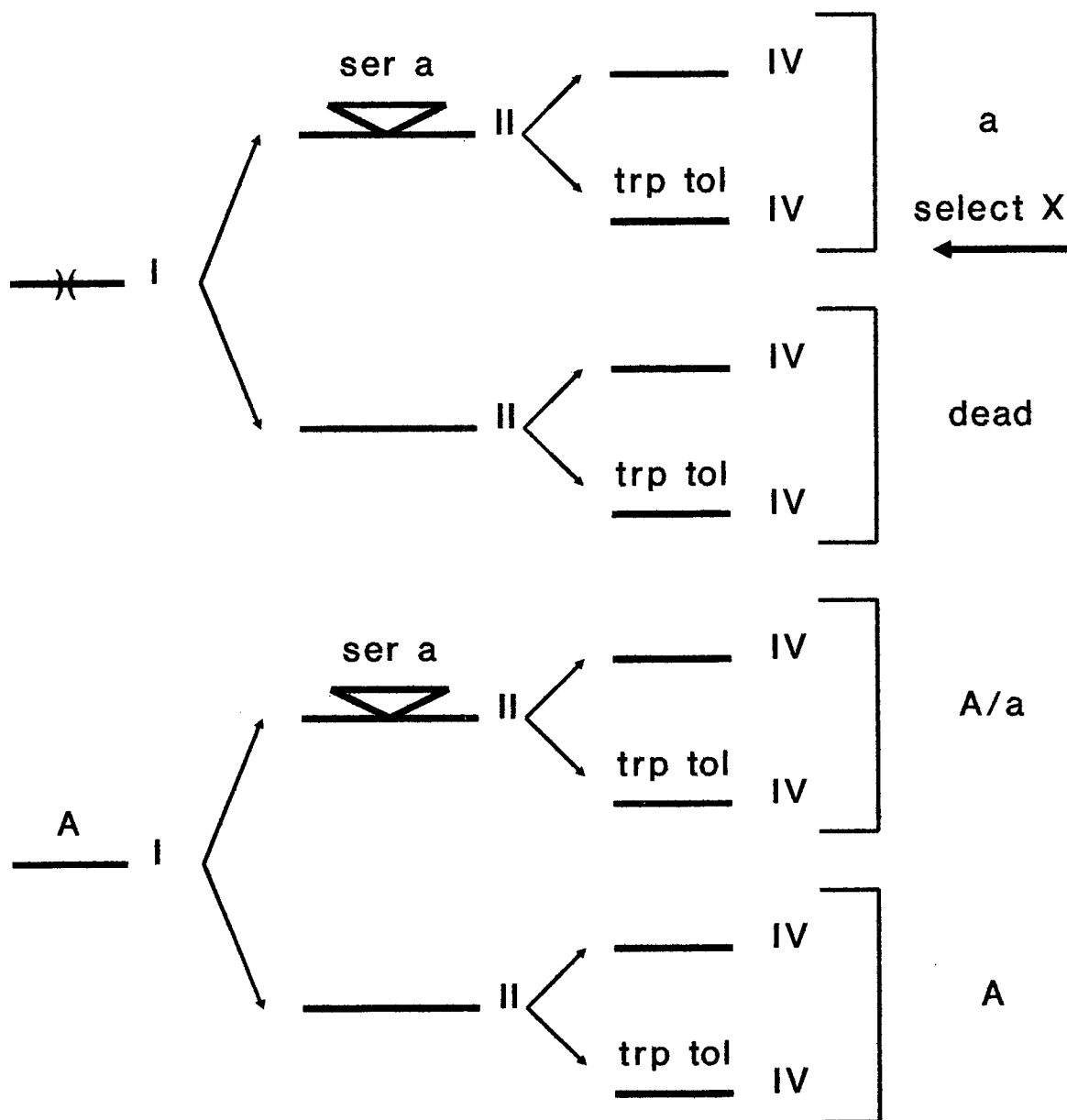
Figure 3 Construction of the strain T(I→II)39311,ser,trp,tol,a. The strain, marked X, was a product of the cross between T(I→II)39311,ser,a x trp,tol,A

crossing medium containing 10% of the normal concentration of tryptophan (*i.e.* 20 mg/L instead of 200 mg/L) because the cross was sterile on the normal concentration of tryptophan. Presumably the tryptophan was providing too much nitrogen for the sexual cycle to be initiated (Myers, personal communication).

RESULTS 1

An overall scheme of the procedure used in generating the suppressors is shown in Fig. 4. Duplication strains (F1) were selected as meiotic segregants from a set of crosses (referred to as the "first cross") between a normal chromosomal sequence parent and a translocation parent. The duplication strains escaped from inhibited growth (escaped F1) and were tested for their mating types. Those which retained both mating types (A/a escaped F1) possibly contained the desired suppressors and were crossed to normal chromosomal sequence fl strains to remove the translocation chromosome. This set of crosses is called the "second cross".

Some of these F2 progeny presumably contained suppressors. Only the temperature-sensitive F2 strains were tested for the presence of suppressors because the un-3 marker was needed to select duplication progeny in the third set of crosses. The temperature-sensitive F2 strains were crossed to tester strains containing the translocation T(I->II) 39311. Duplication progeny were selected and assessed for their compatible/incompatible phenotypes. Detailed descriptions of each step follow.

Duplication strains (labelled "F1" in Fig. 4) were created (Perkins, 1972) from a pair of crosses, reciprocal in the sense that the mating types were reversed. This set of crosses is referred to as the "first cross" and is

First Cross (to generate A/a duplications):

P   R601,un-3 x T(I->II)39311,ser,a

minimal medium, 32 degrees to select duplications

F1   A/a Duplication Strains

escape to select suppressors

Escaped F1 Strains
Choose A/a Escaped F1 (presumed suppressor strains)
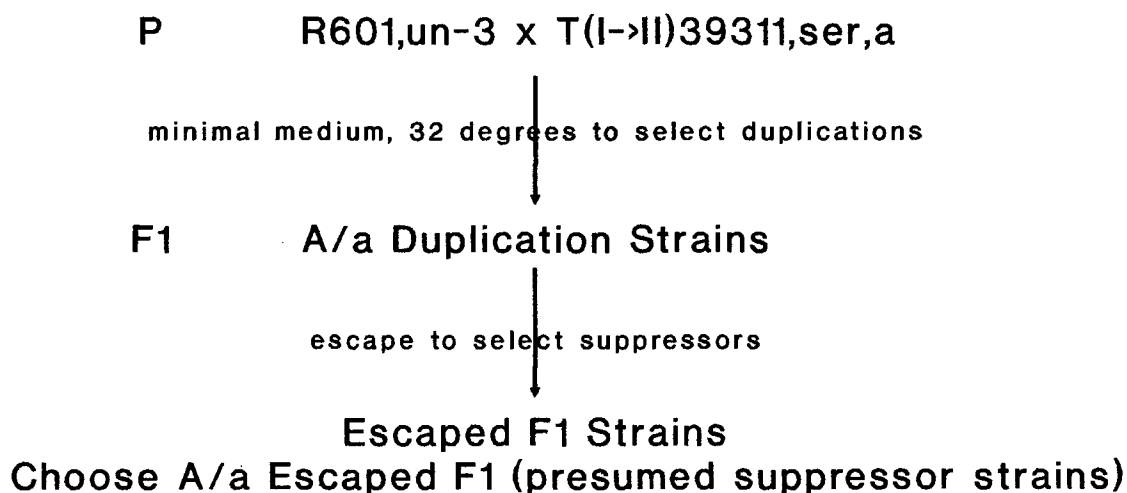
Second Cross (to eliminate duplication):

A/a Escaped F1 x fl,a and fl,A

F2
Choose temperature sensitive (t.s.) F2
(need un-3 for selection of duplications in third cross)
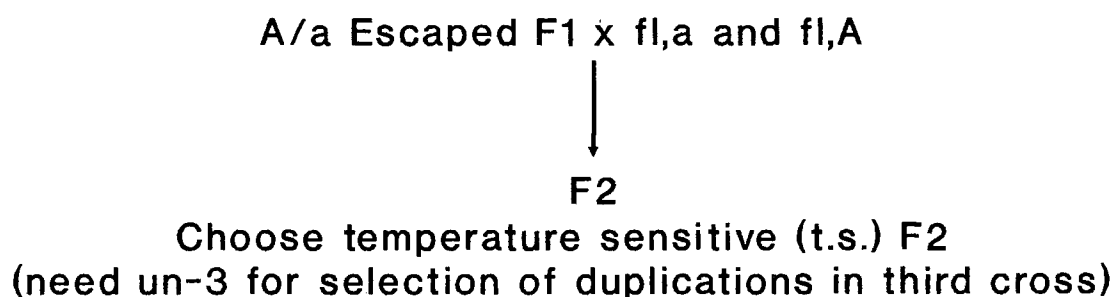
Third Cross (to confirm suppressor phenotype):

t.s. F2 x T(I->II)39311,ser,trp,tol

minimal medium, 32 degrees to select duplications

F3
(t.s. F2 strains that produced incompatible and compatible
duplication F3 progeny were the suppressor strains)
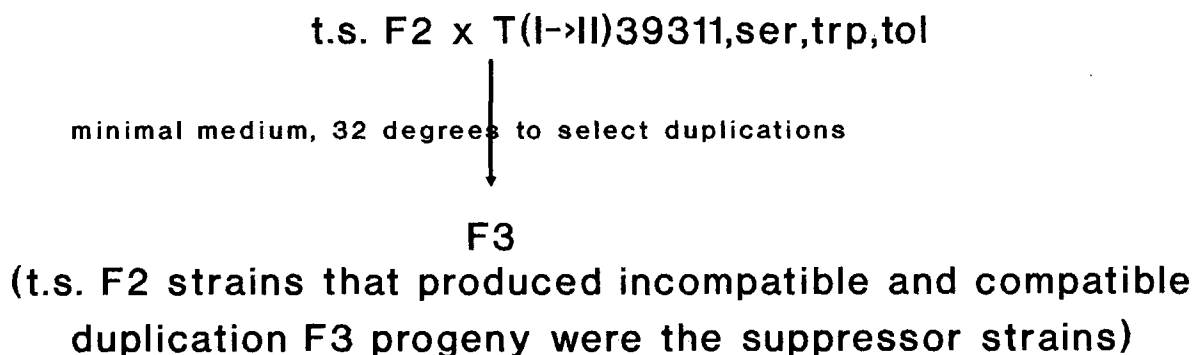
Figure 4   A summary of the selection protocol for suppressors. The reciprocal cross was R602 x T(I->II)39311,ser,A.

described in the following three paragraphs. One cross in the pair, shown in Fig. 5, was made between a normal chromosomal sequence female parent (R601) and a male parent containing a translocation (T(I->II) 39311, *ser*, *a*). In the reciprocal cross (not shown), the normal chromosomal sequence female parent was R602 and the male translocation parent was T(I->II) 39311, *ser*, *A*.

The normal sequence parents, R601 and R602 had their mating type genes, *A* and *a*, respectively, marked with the temperature-sensitive gene, *un-3*, which is less than 1 map unit to the left of *mt*. The translocation strains had their mating type genes marked with the auxotrophic gene, *ser-3*, which is less than 2 map units to the left of *mt*. The mating type genes were marked to allow selection of progeny that contained both *A* and *a*.

The germination of spores on minimal medium at 32°C selected progeny that contained L.G. I from the normal parent (*ser-3$^+$*, *un-3*, *A* or *ser-3$^+$*, *un-3*, *a*) and L.G. II from the translocation parent (*ser-3*, *un-3$^+$*, *a* or *ser-3*, *un-3$^+$*, *A*). The genotype of the selected spores was *ser-3$^+$*, *un-3*, *A/ser-3*, *un-3$^+$*, *a* or, from the reciprocal cross, *ser-3$^+$*, *un-3*, *a/ser-3*, *un-3$^+$*, *A*. Since the nuclei of the selected F1 strains contained both mating types, growth was inhibited and the cultures grew with the dark agar morphology of short hyphae growing in a tight knot (Perkins, 1972).

Figure 5 depicts only one possible pairing--that of the two L.G. I's. Although it is not shown in the figure, the
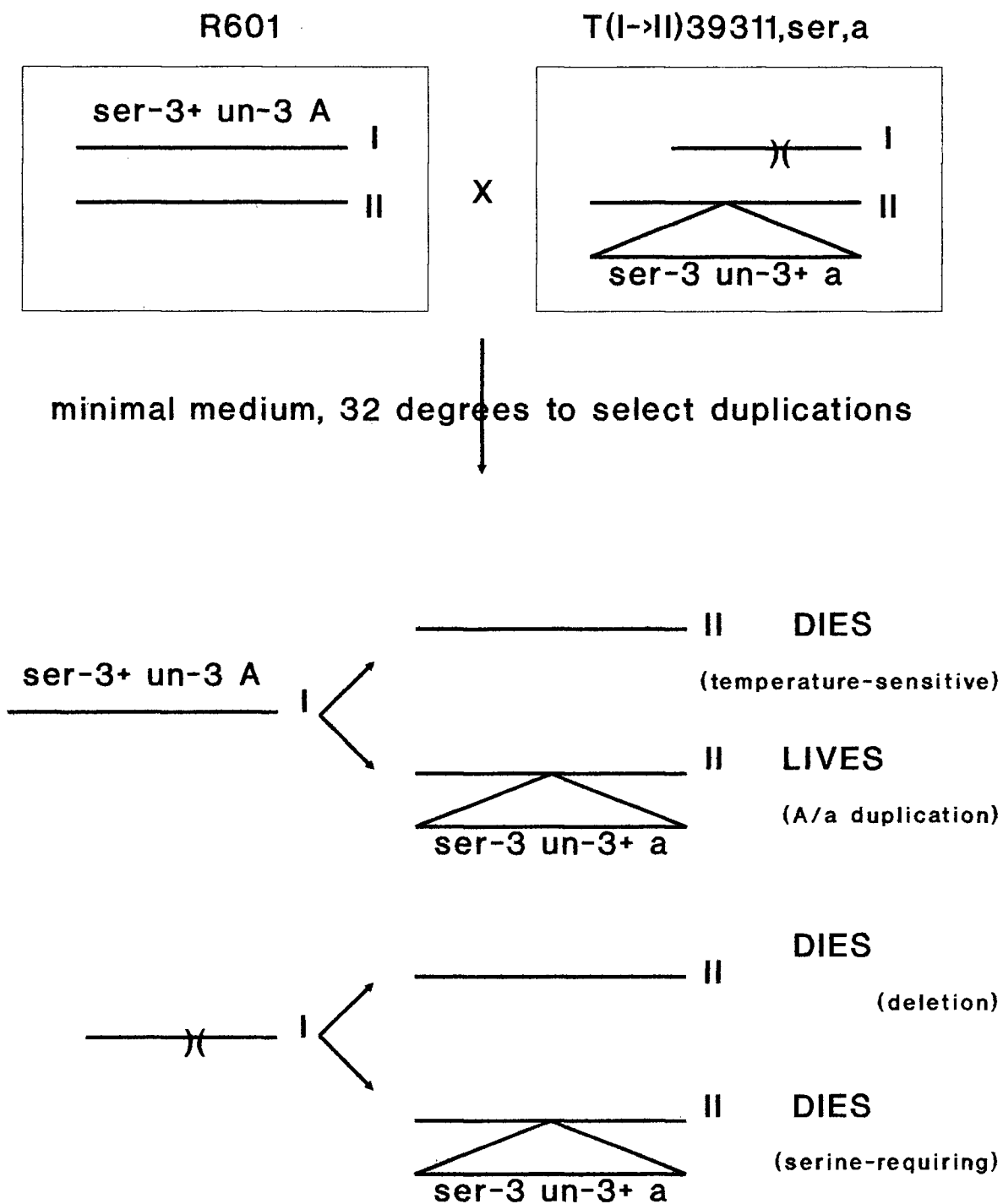
Figure 5

The first set of crosses was done to generate A/a
duplication strains. The crosses were
R601 x T(I->II)39311,ser,a and
R602 x T(I->II)39311,ser,A.

translocated portion of L.G. I is long enough to pair with L.G. I (Metzenberg, personal communication). As explained in the following paragraph, the occurrence of this pairing pattern would not have affected the experimental design.

If the translocated section of L.G. I had paired with the intact L.G. I and a single crossover had occurred in the paired region, the products would not have survived because of the formation of dicentric and acentric chromosomes. If a double crossover had occurred in the paired region, the surviving progeny would only have had one mating type, and therefore would have grown as wild type. These types of spores would not have been chosen as one of the *A/a* escaped F1 strains.

Spore cultures with standard incompatibility and compatibility phenotypes were needed as controls for comparison to the duplication progeny. Such strains were obtained as progeny from the cross between the normal chromosomal sequence female, R601 (*un-3*, *A*), and the translocation male, T(I->II) 39311, *ser*, *trp*, *tol*, *a*. Spores were germinated and grown on medium containing tryptophan at 32°C. These conditions selected progeny containing L.G. I (*ser-3*$^+$, *un-3*, *A*) from the female parent and L.G. II (*ser-3*, *un-3*$^+$, *a*) from the male parent. Half of the progeny, those containing L.G. IV (*trp-4*$^+$, *tol*$^+$) from the female, were incompatible duplications, and half, those containing L.G. IV (*trp-4*, *tol*) from the male were compatible duplications.

From the duplication-generating crosses, R601 x
T(I->II) 39311, *ser, a* and R602 x T(I->II) 39311, *ser, A*,
182 duplication ascospores were collected and maintained on
liquid medium. Each duplication culture exhibited the same
phenotype as that shown by the *A/a, tol*$^+$ incompatible
controls, growing as a small dense mass of hyphae at the
bottom of the test tube. The *A/a, tol* compatible controls
grew faster, filling the test tube after several days. All
of the spore cultures escaped from incompatibility within 2
weeks, although escape occurred at different times for each
strain. Escape was detected as a shift in hyphal morphology
from the dense growth to less dense growth and by an
increase in growth rate.

In order to eliminate strains that had escaped by
deletion of mating type genes and to identify double mating
type strains that may have escaped because of mutation to
tol or to a *tol*-like suppressor (*A/a* escaped F1 strains),
the mating type of each escaped F1 strain was tested by
spotting it on protoperithecial lawns of *fl,A* and *fl,a*
testers.

The crossing behaviours of the escaped F1 strains
allowed their division into 9 phenotypic classes, 8 of which
are described in Fig. 6. The one class not shown in the
figure was comprised of 25 strains that retained the
capacity to cross and produce ascospores with testers of
both mating types. These 25 strains (labelled "*A/a* escaped
F1" in Fig. 4) were the ones presumed to contain

REACTION WHEN CROSSED TO

	fl,A	fl,a
1	○	○
2	◍	◍
3	○	◍
4	◍	○
5	◍	▦
6	▦	◍
7	○	▦
8	▦	○

Legend

☐	No reaction
▨	Barren perithecia
▦	Mature perithecia
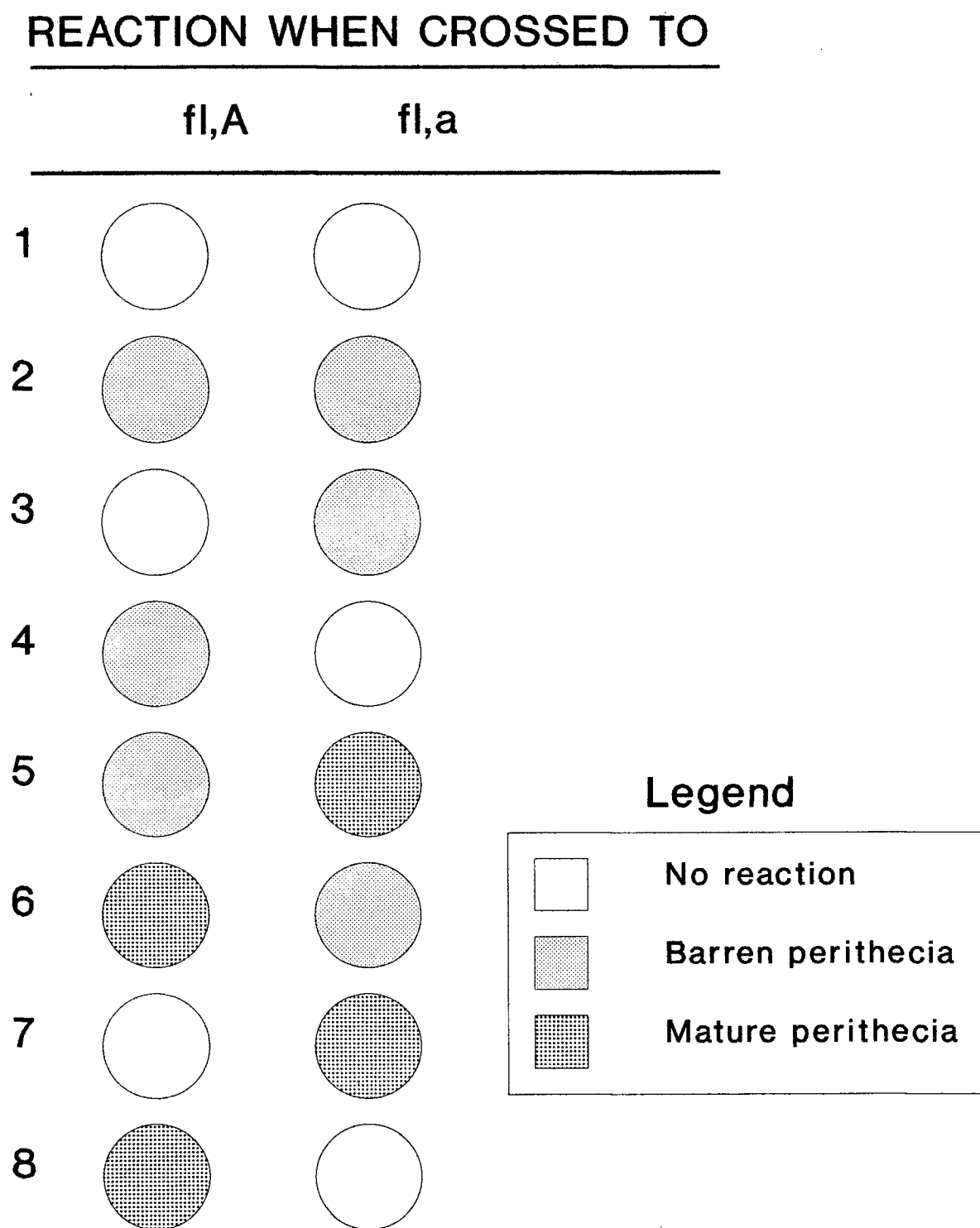
Figure 6 Eight phenotypic classes of escaped F1 strains that were no used in the experiment.

suppressors. The remaining 159 strains were not used in the rest of the experiment, but their possible origins are considered in Discussion 1.

The next set of crosses (referred to as the "second cross") was done to demonstrate that the 25 A/a escaped F1 strains actually contained suppressors. Escape in these strains had not occurred by deletion of either of the mating type genes, but it may have occurred by mitotic crossover or by some as yet unidentified mechanism other than mutation or deletion at a new suppressor locus. At the same time, the second cross served to get the suppressors into a stable background, one without duplications.

The translocation chromosome, L.G. II, was removed by crossing each A/a escaped F1 culture to fl, a and fl, A females (Fig. 7). Crosses involving a duplication strain are frequently "barren", i.e. they produce few ascospores (Newmeyer and Taylor, 1967). Some of the crosses of the A/a escaped F1 strains to fl females produced as few as 1-6 ascospores. Spores from these crosses were not collected by the plating method because many would have been lost through the procedure. Instead, they were collected individually with a tungsten needle under the microscope and placed in liquid medium. The tubes of liquid medium were heat shocked in a 60°C water bath to initiate germination of the spores, then left at room temperature. The test tubes were checked daily for the appearance of growth and the results are shown

Figure 7
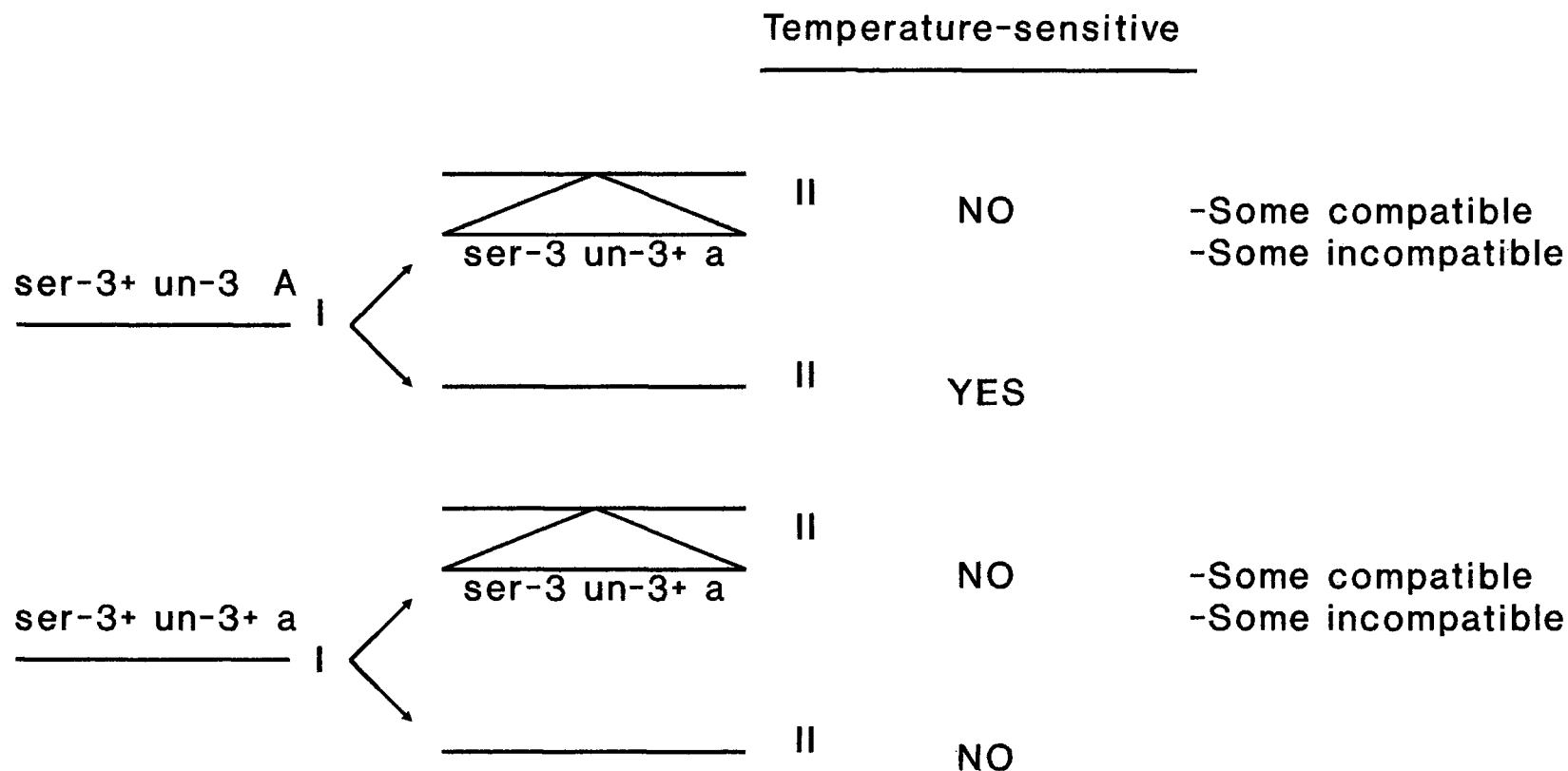
The second set of crosses was done to eliminate the
duplication from the putative suppressor strains. The
crosses were:
A/a escaped F1 derived from R601 x fl,a and
A/a escaped F1 derived from R602 x fl,A.
Temperature-sensitive progeny were chosen because un-3 was
needed as a marker in the third cross and to ensure that the
translocation chromosome was eliminated.

in Table 2. Despite careful collection of spores, there
were 10 crosses from which no spores could be collected.

Progeny from these crosses (labelled "F2" in Fig. 4)
were either incompatible duplication, compatible duplication
or single mating type strains. The compatible and single
mating type progeny were subcultured onto minimal medium
slants so that sufficient mycelia could be grown for use in
subsequent tests. Incompatible progeny were discarded for
two reasons--they were duplications and they did not contain
suppressors.

Compatible and single mating type F2 strains were
tested for *un-3* because the marker was needed in the next
cross. Furthermore, non-duplication progeny were needed for
the next cross, and so compatible duplications had to be
eliminated. They were discarded with the rest of the non-
temperature-sensitive strains. Only 18 F2 progeny out of
329 tested were temperature-sensitive. Possible reasons for
the dearth of temperature-sensitive F2 strains are presented
in Discussion 1.

The 18 temperature-sensitive F2 strains were crossed to
the tester strain, T(I->II) 39311, *ser*, *trp*, *tol*, to confirm
the presence of suppressors in the former. The temperature-
sensitive F2 progeny may not have contained suppressors for
a number of reasons.

Firstly, in the second cross, L.G.II(T I->II) was
removed, so if the mutation had occurred on this chromosome,
and had not recombined onto the homolog, it would be lost.

Table 2 Phenotypes of F2 Strains

| STRAIN | NUMBER OF F2 STRAINS WITH THE PHENOTYPE | | |
	NORMAL (INCLUDES COMPATIBLE)	INCOMPATIBLE	BLANK
A1-29	4	2	25
a1-29	3	0	10
A1-54	0	0	4
a1-54	19	8	39
A1-58	1	0	8
a1-58	13	2	25
A1-59	2	0	4
a1-59	20	2	18
A1-65	0	0	1
a1-65	15	4	21
A1-73	12	1	12
a1-73	2	4	19
A1-75	2	0	5
a1-75	5	1	20
A1-103	0	0	0
a1-103	17	14	0
A1-104	0	0	0
a1-104	17	0	23
A1-107	0	0	2
a1-107*	11	0	21
A1-113	1	2	9
a1-113*	14	0	36
A1-128	0	0	0
a1-128*	19	8	40
A2-18	0	0	3
a2-18	21	6	14
A2-20	1	0	13
a2-20	9	2	8
A2-32	3	0	10
a2-32	14	4	15
A2-37	3	2	23
a2-37	3	3	19

Table 2 continued

STRAIN	NUMBER OF F2 STRAINS WITH THE PHENOTYPE		
	NORMAL (INCLUDES COMPATIBLE)	INCOMPATIBLE	BLANK
A2-57	0	0	7
a2-57	1	1	8
A2-79	0	0	0
a2-79	0	0	0
A2-86*	11	1	0
a2-86	8	0	0
A2-95	0	0	0
a2-95	0	0	0
A2-110	0	0	0
a2-110	0	0	0
A2-117	0	0	0
a2-117	35	2	23
A2-146	2	2	16
a2-146	5	1	5
A2-155*	7	2	41
a2-155	19	4	27
A2-162	0	0	5
a2-162	10	23	17

KEY FOR STRAIN NOMENCLATURE
A or a = crossed to fl,A or fl,a in the second cross
1 or 2 = crossed to R601 or R602 in the first cross
final # = isolate # from first cross

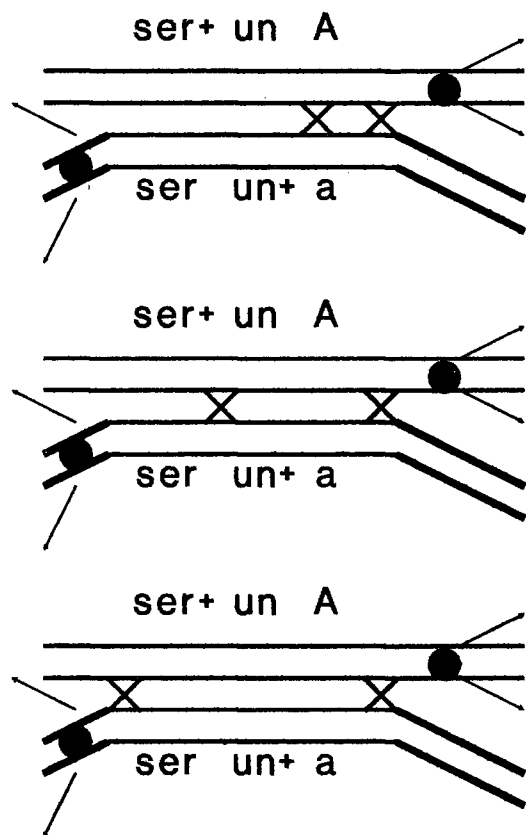*strains that had temperature-sensitive progeny

In this case, all of the duplication progeny from the crosses of temperature-sensitive F2 strains to T(I->II) 39311, *ser*, *trp*, *tol* would be incompatible.

Secondly, because of segregation, the temperature-sensitive F2 progeny may have contained the chromosome homologous to the one with the suppressor mutation. I picked as many ascospores as possible from the crosses of *A/a* escaped F1 to *fl* females, but, as previously mentioned, some of the crosses produced very few.

Finally, if the original escape event had been due to mitotic segregation, there would be no suppressor to be found. Newmeyer and Taylor (1967) did report that their *A/a* escaped strains were heterokaryons of pure *A* and pure *a* nuclei, suggesting that somatic segregation had occurred. In the system they used, there were no selective markers close to *mt* that would prevent the survival of mitotic crossover or *mt* deletion products. Figure 8 shows examples of mitotic crossovers and their products, some of which survive the selection conditions.

If a temperature-sensitive F2 strain contained a suppressor at a locus other than *tol* or *mt*, then the duplication progeny (F3) from the cross to T(I->II) 39311, *ser*, *trp*, *tol* would be of two types--compatible or incompatible. If the new mutation had occurred in the *tol* gene or in the region of the mating type gene controlling vegetative incompatibility, then all of the duplication progeny would be compatible (Fig. 9).

POSSIBLE CROSSOVERS   MITOTIC PRODUCTS

ser+ un A
ser un+ A   survives

ser+ un A

ser un+ a

ser+ un a
ser un+ a   survives

ser+ un A

ser un+ a

ser+ un A
ser un A

ser+ un+ a
ser un+ a   survives

ser+ un A

ser un+ a

ser+ un A
ser+ un A

ser un+ a
ser un+ a

Mitotic double crossovers that could occur in duplication
strains. Single crossovers result in dicentric and acentric
products. Thin lines denote L.G. I. Thick lines denote
L.G. II. (N.B. The insertion is inverted with respect to the
centromere.)

Figure 8
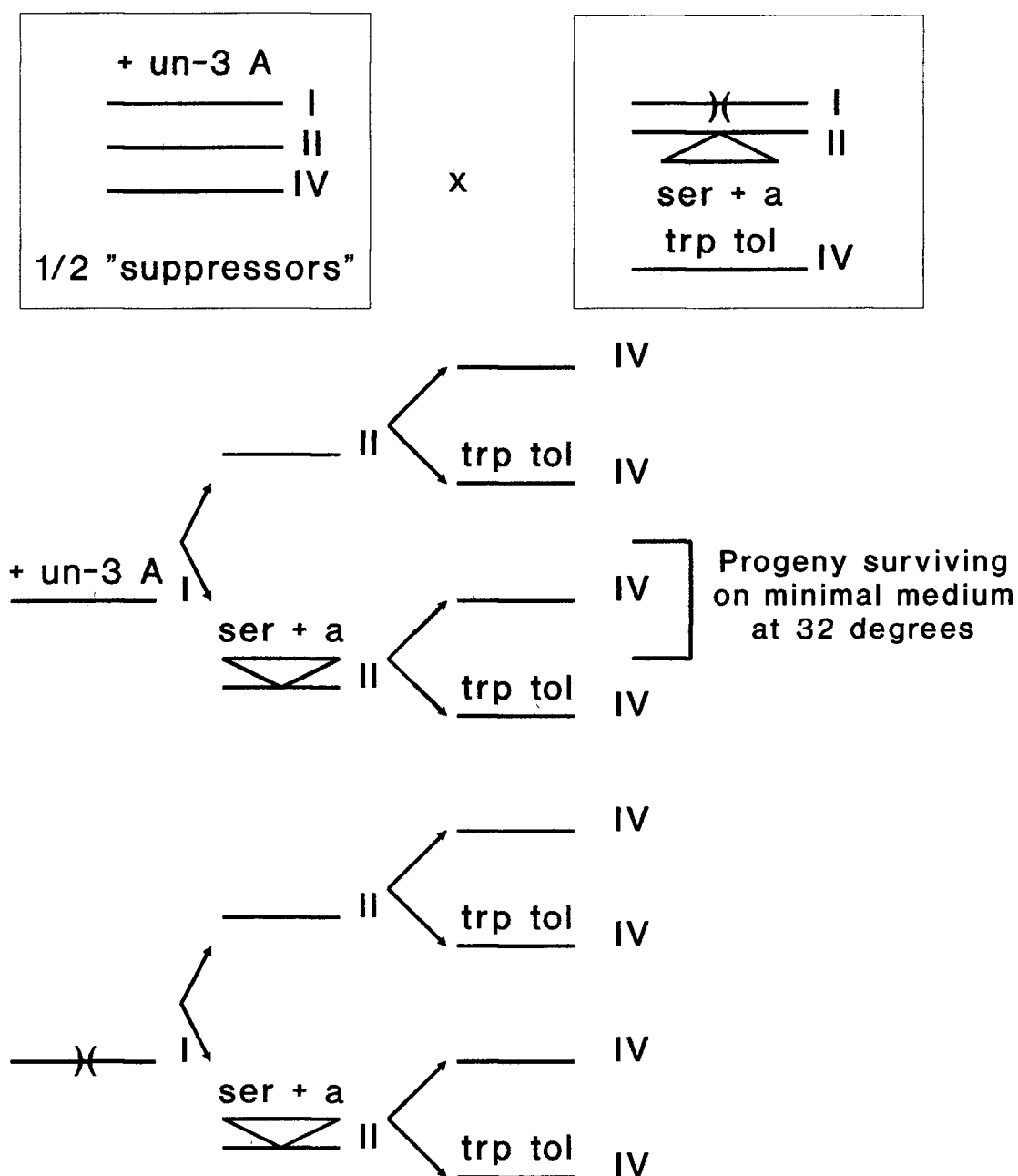
The third set of crosses was done to confirm the suppressor
phenotype of the putative suppressor strains. The crosses
were:
R601-derived suppressors x T(I->II)39311,ser,trp,tol,a and
R602-derived suppressors x T(I->II)39311,ser,trp,tol,A.
If the new mutation had occurred at mt or tol, all of the
surviving progeny would have been compatible duplications.
If the new mutation had occurred at a new "tol" locus, some
of the surviving progeny would have been compatible
duplications and some would have been incompatible
duplications.

Figure 9

Ascospores from the crosses of temperature-sensitive F2 strains to T(I->II) 39311, *ser*, *trp*, *tol* were selected as described in Materials and Methods. Table 3 shows the phenotypes of the F3 progeny from each of the 18 crosses. Seven strains segregated compatible and incompatible progeny. These were the suppressor-containing F2 strains. Ten strains segregated only incompatible progeny. These strains may have contained the chromosome homologous to the one with the suppressor. It is unlikely, however, that all 6 of the F2 progeny derived from a1-128 would have received the chromosome homologous to the one with the suppressor. There are two additional (and more likely) explanations for the lack of suppression in these six strains. The original *A/a* duplication may have escaped by mitotic segregation or by mutation/deletion at a suppressor locus on L.G. II. Since there were no temperature-sensitive F2 strains that segregated all compatible progeny, none of the original escape events could have been due to mutation at either the mating type genes or at *tol*.

One of the strains, a1-128-27, although crossed on several occasions to T(I->II) 39311, *ser*, *trp*, *tol*, produced no perithecia or ascospores. The sterility of a1-128-27 could have been unique to this cross because the strain was able to induce the production of perithecia when crossed to a female *fl*, a tester.

A sample of the compatible and incompatible F3 strains

Table 3 Phenotypes of F3 Strains

F2 STRAIN	NUMBER OF F3 STRAINS WITH THE PHENOTYPE	
	COMPATIBLE	INCOMPATIBLE
a1-113-7	0	31
a1-113-8	24	22
a1-113-9	0	6
a1-113-10	0	46
A2-155-2	7	18
A2-155-3	14	51
A2-155-5	18	28
A2-86-1	0	30
A2-86-4	0	36
A2-86-12	9	21
a1-107-9	19	18
a1-107-11	28	12
a1-128-22	0	32
a1-128-23	0	32
a1-128-24	0	25
a1-128-25	0	35
a1-128-26	0	32
a1-128-27	0	0

KEY FOR STRAIN NOMENCLATURE
A or a = crossed to fl,A or fl,a in the second cross
1 or 2 = crossed to R601 or R602 in the first cross
middle # = isolate # from first cross
last # = isolate # from second cross

was tested for mating type (Table 4). Almost all of the
compatible F3 strains (65 of 66) contained both mating
types. This is the class of progeny that show the existence
of suppressors. It is unlikely that they grew well due to
escape because of the high proportion of A/a strains.
Furthermore, the hyphae of escaped strains are wispy,
whereas these 65 compatible strains grew with a dense
morphology.

One of the apparently compatible F3 strains (one of the
progeny strains from the R601-derived parent, a1-113-8)
contained only *A*. Although escape usually does not occur
within the first 24 hours after germination of the spore, it
may have escaped earlier than normal from *A/a*
incompatibility by loss of the mating type gene from the
translocated segment. It is unlikely that the strain
resulted from a double crossover on either side of *un-3*
because of the close linkage of *un-3* to *ser-3* and *mt*. It is
also unlikely that the strain survived due to reversion of
the *un-3* mutant because the *un-3* marker contains two
mutations.

Most of the incompatible F3 strains (59 of 71) only
contained one mating type, probably because the mating type
tests were done after the incompatible strains had escaped.
Those incompatible F3 strains which contained both mating
types may have escaped by mutation at a suppressor locus or
by mitotic crossing over between the L.G. I centromere and
mt and between *mt* and *un-3*, and both types of derivative

Table 4   Mating Types of F3 Strains

TEMPERATURE-SENSITIVE F2	COMPATIBLE F3	INCOMPATIBLE F3
a1-113-8	9 A/a 1A	1 A/a 1 A 8 a
a1-107-9	10 A/a	1 A/a 9 a
a1-107-11	10 A/a	1 A 9 a
A2-86-12	9 A/a	2 A/a 6 A 3 a
A2-155-2	7 A/a	3 A/a 5 A 2 a
A2-155-3	10 A/a	8 A 2 a
A2-155-5	10 A/a	5 A/a 4 A 1 a

The presence of suppressors was implied by the production of
A/a compatible F3 progeny.

KEY FOR STRAIN NOMENCLATURE
A or a = crossed to fl,A or fl,a in the second cross
1 or 2 = crossed to R601 or R602 in the first cross
middle # = isolate # from first cross
last # = isolate # from second cross

nuclei were still present.

Most of the escaped incompatible F3 strains derived
from R601 (*un-3*, *A*) were *a*, and most of the escaped
incompatible F3 strains derived from R602 (*un-3*, *a*) were *A*.
These two types of strains could have occurred by deletion
of *mt* and *un-3* from the temperature-sensitive L.G. I
homolog. Another mechanism could have been double mitotic
crossing over, between the L.G. I centromere and *mt* and
between *mt* and *un-3*, followed by overgrowth by the *a* nuclei
in the R601-derived *a* escaped strains or by the *A* nuclei in
the R602-derived *A* escaped strains. The latter mechanism
could have given rise to the smaller classes of escaped
strains, R601-derived *A* escaped strains and R602-derived *a*
escaped strains. These classes could have arisen by mitotic
crossing over between the L.G. I centromere and *mt* and
between *mt* and *un-3*, followed by overgrowth by the *A* nuclei
in the R601-derived *A* escaped strains or by the *a* nuclei in
the R602-derived *a* escaped strains.

Although the F3 strains were selected on medium without
tryptophan, the *trp-4* gene is leaky; therefore, selection
may not have been completely restrictive. To ensure that
the compatibility was due to a novel suppressor and not to
tol, the *A/a* compatible F3 strains from each of the seven
crosses were tested for *trp-4*. None of the compatible F3
strains was tryptophan-requiring.

All of the *A/a* incompatible F3 were tested for *trp-4* to
ensure that they were correctly scored as incompatible

because they had no suppressor, and not because they grew poorly due to the *trp-4* gene. None of the incompatible F3 strains was tryptophan-requiring.

Finally, to ensure that the compatibility was due to a suppressor and not to a novel gene which increased the rate of escape, 4 hyphal tips were obtained from 2 *A/a* compatible F3 strains from each of the seven crosses and were tested for mating type. If the "compatibility" were due to early escape, then the tips would be expected to be *A* or *a*, but not both; if the compatibility were due to a suppressor, then the tips would be expected to be *A* and *a*. The results are shown in Table 5. Almost all of the tips were *A/a*, suggesting that the compatible phenotype was due to a suppressor.

Single mating type hyphal tips (all *A*) were found only in two of the suppressor strains, both derived from the same *A/a* escaped F1 strain. When this *A/a* escaped F1 strain escaped, a second mutation, in addition to the suppressor may have occurred. The second mutation could be one that causes instability of duplications. The single mating type mitotic segregants could have arisen by deletion of *a* during somatic growth or mitotic crossover between the L.G. I centromere and *mt* and between *mt* and *ser-3* (only *A* products would have survived).

Table 6 summarizes the findings described in Results 1.

Table 5 Mating Types of Hyphal Tips of
A/a Compatible F3 Strains

COMPATIBLE F3 TIP DERIVED FROM STRAIN:	MATING TYPES OF TIPS
a1-113-8	8 A/a
a1-107-9	8 A/a
a1-107-11	8 A/a
A2-86-12	8 A/a
A2-155-2	8 A/a
A2-155-3	4 A/a, 4 A
A2-155-5	6 A/a, 2 A

Most of the hyphal tips were A/a, suggesting that the suppressors do not function by increasing the rate of escape.

Table 6 Summary of Results 1

A/a ESCAPED F1	# SPORES FROM SECOND CROSS	# TEMPERATURE-SENSITIVE	TEMPERATURE-SENSITIVE F2	# SPORES FROM THIRD CROSS	# SPORES COMPATIBLE
1-29	9		a1-107-9	37	19
1-54	27		a1-107-11	40	28
1-58	16				
1-59	24		a1-113-7	31	0
1-65	19		a1-113-8	46	24
1-73	19		a1-113-9	6	0
1-75	8		a1-113-10	46	0
1-104	17				
1-107	11	2			
1-113	17	4	a1-128-22	32	0
1-128	27	6	a1-128-23	32	0
2-18	27		a1-128-24	25	0
2-20	12		a1-128-25	35	0
2-32	21		a1-128-26	32	0
2-37	11		a1-128-27	0	0
2-57	2				
2-79	0		A2-86-1	30	0
2-86	20	3	A2-86-4	36	0
2-95	0		A2-86-12	30	9
2-103	31				
2-110	0				
2-117	37		A2-155-2	25	7
2-146	10		A2-155-3	65	14
2-155	32	3	A2-155-5	46	18
2-162	33				

KEY FOR A/a ESCAPED F1
1 or 2 ▪ crossed to R601 or R602 in the first cross
second # ▪ isolate # from first cross

KEY FOR TEMPERATURE-SENSITIVE F2
A or a ▪ crossed to fl,A or fl,a in the second cross
1 or 2 ▪ crossed to R601 or R602 in the first cross
middle # ▪ isolate # from first cross
last # ▪ isolate # from second cross

DISCUSSION 1

Seven strains containing novel suppressors of *A/a* incompatibility have been isolated. The strains were derived from 4 strains that had undergone escape events (strains #1-107, 1-113, 2-86 and 2-155), suggesting that there may be 4 novel suppressor mutations. None of the suppressors is allelic with *tol* or with the mating type gene. The seven strains produced *A/a* compatible progeny when crossed with a duplication-generating translocation strain. The *A/a* compatible progeny were probably duplications, not heterokaryons, because hyphal tips isolated from these strains, in general, contained both mating types. The compatible phenotype observed in these *A/a* progeny is due to new mutations that suppress *A/a* incompatibility, not to new mutations that increase the rate of escape from *A/a* incompatibility by increasing the rate of deletion or mitotic crossing over.

There were surprisingly few temperature-sensitive F2 progeny from the 50 crosses of the second cross (18 of 329 tested). Two types of crosses produced no temperature-sensitive F2 progeny: *fl, A* x *A/a* escaped F1 strains derived from R601 (*un-3, A*) and *fl, a* x *A/a* escaped F1 strains derived from R602 (*un-3, a*). In these 2 types of crosses, L.G. I from the *fl* parent had the same mating type gene as L.G. I from the temperature-sensitive parent; so perhaps the L.G. I homologs paired with the translocated segment which

has the opposite mating type (Fig. 10). When such pairings occur, fewer temperature sensitive progeny are produced. The L.G. I homologs probably did not pair with each other. If they did, temperature-sensitive progeny would have been produced.

The alternative pairing hypothesis explains the absence of temperature-sensitive progeny from the two aforementioned types of crosses. When L.G. I from the *fl* parent pairs with the translocated DNA, no temperature sensitive progeny are produced. If there is a single crossover in the paired region, acentric and dicentric chromosomes are produced, and these, presumably, do not survive. If there is a double crossover in the paired region, the *un-3* allele always segregates with an *un-3*$^+$ allele. When L.G. I from the temperature-sensitive parent pairs with the translocated DNA, very few temperature sensitive progeny are produced. If there is a single crossover in the paired region, acentric and dicentric chromosomes are produced. If there is a double crossover in the paired region, only one of the 6 possible types of double crossover--one crossover on each side of the mating type gene--gives temperature sensitive progeny. This type of double crossover is rare because of the proximity of *un-3* to the mating type gene.

If one disregards the two types of crosses that yielded no temperature-sensitive F2 progeny, the proportion of temperature-sensitive F2 strains is still low (18 of 165). In the two types of crosses that did give temperature-

PAIRING   SEGREGATION

IIa, Ia

IIb, Ib

IIa, Ib

IIb, Ia
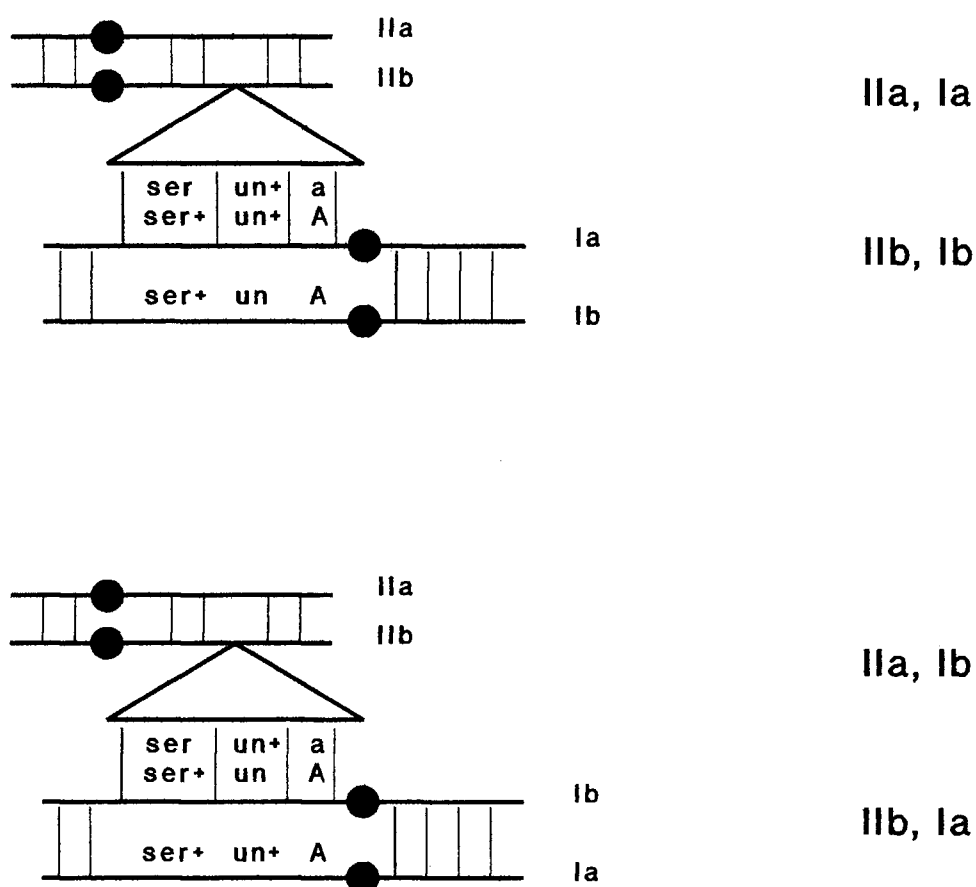
Figure 10

One explanation for the low number of temperature-sensitive
F2 strains is the alternative pairing hypothesis. Either of
the L.G.I homologs could have paired with the translocation.
Thin vertical lines indicate regions of pairing.

sensitive F2 progeny (R601-derived escaped strains x *fl*, *a* and R602-derived escaped strains x *fl*, *A*), perhaps pairing of the L.G. I homologs with each other, as depicted in Fig. 7, did not always occur. If either of the L.G. I homologs had paired with the translocated DNA, fewer temperature-sensitive progeny would have been produced.

Two additional explanations for the low number of temperature-sensitive progeny are possible. Duplication strains undergo a process called RIP ("Repeat-Induced Point mutation", previously "Rearrangements Induced Premeiotically") during mating, with the result that duplicated genes are often mutated (Selker and Stevens, 1985; Selker, *et al.*, 1987; Selker and Garrett, 1988; Cambareri, *et al.*, 1989; Grayburn and Selker, 1989; Selker, 1990; Cambareri, *et al.*, 1991). RIP of either *un-3* or *un-3*[+] could have produced *un-3* nulls, which are lethal (Lambowitz, unpublished results, Glass, personal communication). Finally, germination of *un-3* spores may occur less frequently than germination of wild type spores (Glass and L. Stenberg, personal communication), possibly because of the heat shock required to initiate germination.

A/a duplication strains escaped from incompatibility by several different means other than the generation of suppressors. The 8 phenotypic classes of escaped F1 strains, shown in Fig. 6, could have been the results of deletion/mutation at the mating type locus, RIP, mitotic

crossing over or low fertility due to the presence of duplicated genetic material.

Any of the 8 phenotypic classes of escaped F1 strains could have arisen by deletion/mutation at one or both of the mating type loci. Experiments performed by DeLange and Griffiths (1975) on escape from *A/a* incompatibility suggested that mixed mating type heterokaryons often escaped by deletion of one mating type. Similar events could have occurred in mixed mating type duplication strains.

The 8 classes of escaped strains could have arisen by RIP. *A/a* duplications are not really duplications because *A* and *a* are idiomorphs, not alleles (Metzenberg and Glass, 1990), *i.e.* the central portions of the DNAs are completely different from each other (Glass, *et al.*, 1988). The flanking regions, however are virtually identical (Glass, *et al.*, 1988) and RIP can sometimes occur in unique sequences close to the duplicated ones (Foss, *et al.*, 1991).

The single mating type escaped strains, classes 7 and 8, could have been generated by mitotic crossing over. Once the mating type genes had segregated into different nuclei, one type of nucleus could have outgrown the other and the resultant culture would only have had a single mating type reaction.

The barren phenotype associated with duplications in *N. crassa* could account for classes 2-6. Duplication strains sometimes produce abundant perithecia, but very few

ascospores (Newmeyer and Taylor, 1967). If this occurred, the spores simply may have been overlooked during scoring.

What is *tol*?

It is not possible to formulate a comprehensive statement regarding the biological significance of incompatibility in all of the fungi, for it manifests itself in many different forms. Incompatibility can be heterogenic, preventing fusion between strains of different genotypes, either vegetative fusion (*e.g.* in *N. crassa*), sexual fusion (*e.g.* in *Sordaria fimicola* (Olive, 1956) and *Ceratostomella radicicola* (El-Ani, *et al.*, 1957)) or both vegetative and sexual fusion (*e.g.* in *Aspergillus nidulans* (Jinks, *et al.*, 1961) and *Podospora anserina* (Esser, 1971)); or it can be homogenic, preventing fusion between strains of similar genotypes, either sexual fusion (*e.g.* in *N. crassa*) or both vegetative and sexual fusion (*e.g.* in *Schizophyllum commune* (Raper, 1966 cited in Burnett, 1975)). The significance of each incompatibility system, because of the diversity, must be considered separately.

Vegetative incompatibility controlled by the mating type genes of *N. crassa* may be biologically unrelated to incompatibility controlled by the *het* genes. This idea is supported by the existence of the gene, *tol*, which suppresses *A/a* incompatibility without affecting *het-C/c* or *het-E/e* incompatibility (Newmeyer, 1970; Perkins, 1974).

Vegetative incompatibility seems to be an intrinsic function of the mating type genes. Genetic and molecular data support this idea and suggest that compatibility mechanisms evolved secondarily. Metzenberg and Ahlgren (1973) introgressed the mating type genes of *N. tetrasperma* into a largely *N. crassa* background. The resultant strains demonstrated incompatibility in mixed mating type heterokaryons and in heterozygous duplications, suggesting that the *N. tetrasperma* genes had the ability to instigate the incompatibility reaction. In their own environment, in *N. tetrasperma*, the mating type genes must either be suppressed for the incompatibility function or lack target genes or both.

A recent study by D. Jacobson (personal communication) suggests that one way in which *N. tetrasperma* tolerates *A/a* ascospores is by the presence of the suppressor, *tol*. He introgressed sequences of *N. tetrasperma* corresponding to *tol* of *N. crassa* into a *N. crassa* background. The resultant strain of *N. crassa* behaved as if it were *tol*, not *tol*[+].

The mating type genes of *N. sitophila*, like *N. tetrasperma*, are able to produce the incompatibility phenotype in a *N. crassa* background (Perkins, 1977). *N. sitophila* mating type genes were introgressed into *N. crassa*, whereupon the *N. sitophila* genes exhibited incompatibility in *A/a* duplications. Even more interesting was the observation that the suppressor gene, *tol*, eliminated the incompatibility, indicating that *tol* is

unable to detect any significant difference between *N. crassa* and *N. sitophila* mating type genes.

A result with similar implications was obtained by Glass (personal communication). Replacement of the *N. crassa A* gene with the *N. africana A* gene did not alter the ability of the transformant to initiate the incompatibility reaction, again implying that the mating type gene from a homothallic species is capable of orchestrating vegetative incompatibility.

Incompatibility controlled by the vegetative incompatibility genes may have developed independently of *A/a* incompatibility. There are many theories regarding the biological benefits of heterokaryon incompatibility. Caten (1972) suggested that incompatibility systems exist to limit the spread of infectious viruses or cytoplasmic determinants. Later studies indicated, however, that plasmids can cross incompatibility barriers within a species (Collins and Saville, 1990) and even between species (Griffiths, *et al.*, 1990), presumably during brief periods of unstable fusion.

Hartl, *et al.* (1975) suggested that incompatibility prevents the exploitation of an adapted mycelium by a less well adapted one growing in the same niche. Although this hypothesis could apply to a fungus like *N. crassa*, in which a defective homokaryon can survive by fusion with another mycelium, it does not seem a likely explanation for

incompatibility in fungi like the basidiomycetes in which fusion leads to mating.

It has been suggested that incompatibility serves to distinguish individuals, which may be important in the maintenance of fine-tuning between nuclei and organelles. Considering that nuclei are associated with different organelles every time mating occurs, this does not seem to be a likely explanation for the existence of incompatibility.

J. Begueret (personal communication) succeeded in creating a novel incompatibility group via mutation in *Podospora*, which led him to propose that incompatibility genes are nothing more than mutations without biological significance. Jinks, *et al.* (1961) made a similar suggestion in their study of incompatibility in *Aspergillus nidulans*. They maintained that heterokaryon incompatibility is a consequence of genetic diversity, not a cause. This idea would only apply to fungi like *N. crassa* in which the incompatibility reaction does not affect mating. If a novel allele arose in a population, it would be selected against because the cell containing it would die if it fused with an unlike type. A novel allele would not be selected against if it arose in a spore that established a separate population before encountering incompatible fungal types. In the basidiomycetes, however, a novel incompatibility group would be able to mate with all other existing groups, allowing it to spread quickly through a population.

The gene, tol^+, and the new suppressors could be mating
type target genes, controlling different steps in the
pathway to *A/a* incompatibility, and when they are mutated,
the reaction fails to occur. Their relationships to each
other in terms of where they fit in to the pathway are
unknown at present. They could be mutants in sequential
reactions or reactions that occur simultaneously.

The suppressors could be enzymes required for
vegetative growth, normally turned off by the *A/a* product
(directly or indirectly) during incompatibility. The
mutants could be altered such that they are no longer
recognized by the regulating product and are, therefore, not
repressed and the incompatibility reaction does not occur.
The suppressors could be required for recognition of
vegetatively growing *A* or *a* hyphae. The mutants, in this
case, could be defective for vegetative recognition, thus
eliminating the incompatibility reaction. The suppressors
could produce toxic metabolites in the presence of the *A/a*
product. These mutants could be defective for toxin
production itself or for regulation so that the
incompatibility reaction does not occur.

The existence of other genes affecting the
incompatibility reaction raises a question regarding *N.
tetrasperma.* As previously mentioned, one strain of *N.
tetrasperma* appears to contain the recessive allele of *tol*
(Jacobson, personal communication). What is the state of
the other suppressor genes? As long as a strain has *tol*, it

will be heterokaryon compatible, so the alleles of the other
suppressors would not affect the phenotype. Until more is
known about the relative functions of the new suppressors,
it is not possible to predict the suppressor genotype(s) of
N. tetrasperma.

Perhaps there are suppressors that, unlike *tol*,
suppress both *A/a* incompatibility and mating. If so, it
would mean that mating and *A/a* incompatibility act through
at least one common step. Perhaps there are *A/a*
incompatibility suppressors that also suppress
incompatibility controlled by one or some of the *het* genes.
If so, it would imply that incompatibility is effected
through one pathway and that *tol* acts before or after the
common part(s) of the pathway. The number of different
suppressor genes may give an indication of the number of
steps involved in generating the incompatibility reaction.

INTRODUCTION 2

A molecular picture of the mating type genes is emerging with the aid of mating type mutants (Griffiths and DeLange, 1978; Griffiths, 1982; Griffiths, personal communication) and molecular biological techniques (Glass, et al., 1988; Staben and Yanofsky, 1990; Glass, et al., 1990).

The A idiomorph, as defined by its region of non-homology with a, is 5301 base pairs in length (Glass, et al., 1990), and a, by the same definition, is 3235 base pairs in length (Staben and Yanofsky, 1990) (Fig. 11). All of the A^m mutants (Griffiths, 1982) that have been sequenced map within the single ORF, called $A-1$. All of the A^m and a^m mutants of Griffiths and DeLange (1978) that have been sequenced map within the exons in the ORFs (Glass, et al., 1990; Staben and Yanofsky, 1990). Some examples follow.

One of the mutants, a^{m1}, has a frameshift due to the deletion of a single base pair. The insertion of 212 base pairs, which characterizes the mutant a^{m30} causes a premature transcription stop. The unique compatible, fertile mutant, a^{m33}, has a base pair substitution which is located farther downstream than either of the other two mutations.

Species in other genera have idiomorphs instead of alleles at their mating type loci, e.g. Saccharomyces
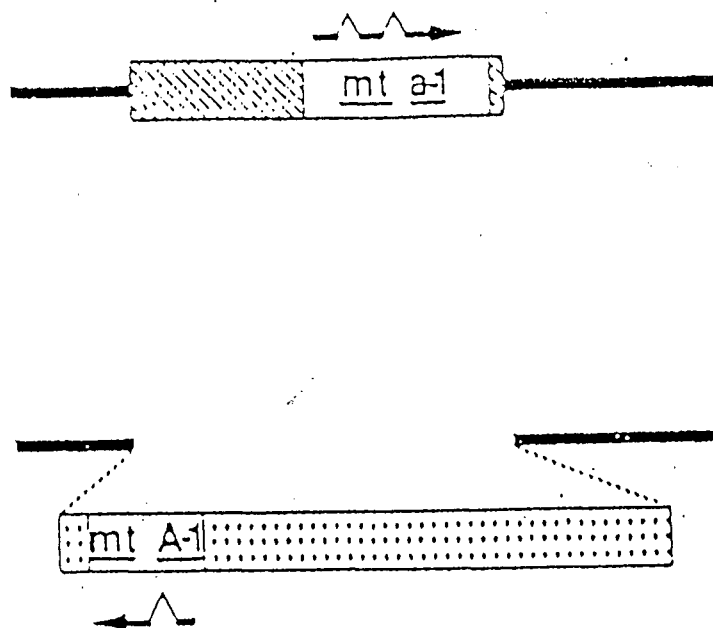
LEGEND
Thick lines = identical flanking sequences
Filled boxes = idiomorphs
Open boxes = postulated ORFs
Arrows = presumed transcripts with introns

Figure 11 Mating type regions of N. crassa (from Staben and Yanofsky, 1990).

cerevisiae (Strathern, et al., 1980; Nasmyth and Tatchell,

1980), *Schizosaccharomyces pombe* (Kelly, et al., 1988),

Ustilago maydis a gene (M. Bolker and R. Kahmann, 1991, in

published abstracts from Sixteenth Fungal Genetics

Conference) and *Podospora anserina* (Coppin, 1991, in

published abstracts from Sixteenth Fungal Genetics

Conference).

The mating type genes of *N. crassa* are incompatible not

only in a heterokaryon, but also in one nucleus. Newmeyer

(1970) used nuclear incompatibility to induce *tol*, which

suppresses both types of incompatibility. Glass, et al.

(1990) observed a 100-fold reduction in transformation

efficiency of the ORF, *A-1*, into *a* spheroplasts, compared to

the transformation efficiency into *A* spheroplasts; and Glass

(personal communication) observed a similar reduction of

transformation efficiency of *a* sequences into *A*. Staben and

Yanofsky (1990) also reported a decrease in the frequency of

A transformants when the donor DNA was ORF, *a-1*, as compared

to when the donor DNA was a portion of the *a* idiomorph not

including *a-1*.

Nuclear incompatibility may be separable from

heterokaryon incompatibility. Glass and Griffiths (personal

communication) created a *A*-duplication strain by

transforming a strain of mating type *A* with the ORF of the

sterile, compatible mutant, A^{m64}. In order to RIP the *A*

gene, they crossed the transformant to a strain of mating

type *a*. Surprisingly, some of the progeny displayed the

incompatibility phenotype. Growth occurred as a small wispy knot of mycelia with no aerial hyphae. All of the cultures escaped from slow growth within one week, and then showed a mating type a reaction when tested. If the ORF had segregated from mt as an independent locus, then one half of the a spores would have contained A^{m64} and it is possible that these were the incompatible strains (Fig. 12).

If the incompatible strains were A^{m64}/a, it appears as though A^{m64} has lost *heterokaryon* incompatibility while retaining *nuclear* incompatibility. At first it seemed possible that the incompatible phenotype was due to residual heterokaryon incompatibility specified by A^{m64}, but this hypothesis has been discarded because a mixed mating type heterokaryon of A^{m64} grew as fast as a positive control strain.

To test the possibility that mutations at mt can eliminate heterokaryon incompatibility without eliminating nuclear incompatibility, duplication strains that contained the fertile, compatible mutant, a^{m33}, and A were examined for their morphological characteristics and growth rates. A/a^{m33} duplication strains were made by crossing 3 different a^{m33}-containing strains to A strains containing a translocation of the mating type gene. The mutant, a^{m33}, is known to be compatible in a heterokaryon with A, although the growth rate of an a^{m33} + A heterokaryon has not been measured precisely before now. It was measured to determine if a^{m33} had any residual heterokaryon incompatibility.

1/2 A $\Big\langle$ 1/2 Am64

NORMAL OR RIP

1/2 a $\Big\langle$ 1/2 Am64   INCOMPATIBLE?

NORMAL a
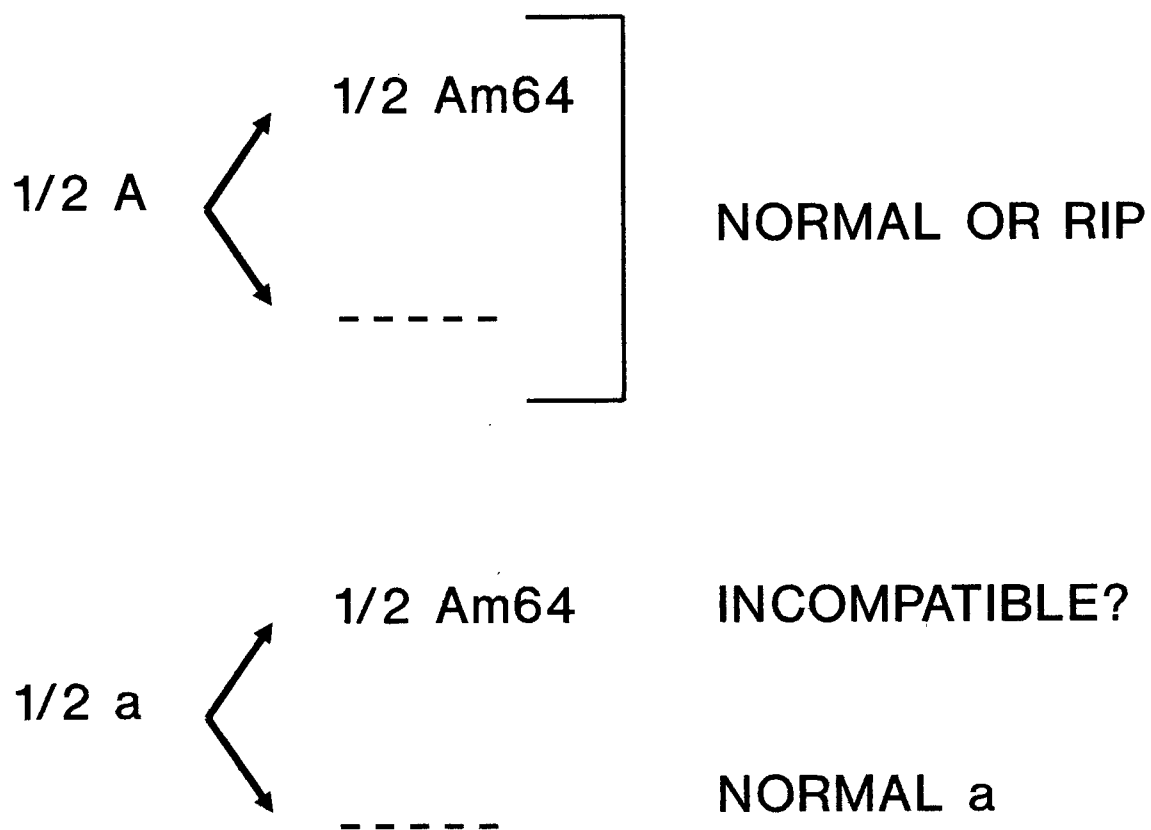
Figure 12 Segregation of Am64 ORF and resulting phenotypes.

RESULTS 2

The growth rate of the sterile, compatible mutant, A^{m64}, in a mixed mating type heterokaryon, A^{m64} + 7 (ad-3B, a), was measured to determine if A^{m64} had residual incompatibility. The growth rate was compared to that of the component strains alone and to that of an incompatible mixed mating type heterokaryon, 153 (ad-3A, nic-2, A) + 7 (ad-3B, a). The heterokaryon, A^{m64} + 7 (ad-3B, a), grew as fast as the component strain 7 (ad-3B, a), and faster than the incompatible mixed mating type heterokaryon, 153 (ad-3A, nic-2, A)+ 7 (ad-3B, a) (Table 7 and Fig. 13), suggesting that the mutant, A^{m64}, has lost its heterokaryon incompatibility function completely .

The following section describes a series of tests done to study heterokaryon and nuclear incompatibility in another mating type mutant, a^{m33}.

The growth rate of a^{m33} in a mixed mating type heterokaryon (a^{m33}, ad-3B + I-22-83 (ad-3A, nic-2, un-3, A)) was measured to determine if the mating type mutant had residual incompatibility. The growth rate was compared to several controls: (1) an incompatible mixed mating type heterokaryon (51-2 (ad-3B, cyh-1, a) + I-22-83 (ad-3A, nic-2, un-3, A)); (2) a mating type homokaryon--a^{m33}, ad-3B(128) paired with 51-2, a strain of mating type a (ad-3B(114), cyh-1, a); (3) a compatible mixed mating type heterokaryon in which both components had tol (I-9-57

## Table 7   Strains and Media Used in the Measurement of Growth Rate of Am6 in a Mixed Mating Type Heterokaryon

STRAIN	GENOTYPE		
	mt	AUXOTROPHIC GENES	OTHER
153 *	A	ad-3A, nic-2	un-3, cyh-1
7	a	ad-3B	
Am64	Am64	ad-3A, nic-2	un-3, cyh-1

	MINIMAL MEDIUM +
POSITIVE CONTROLS Am64 7	adenine, nicotinic acid adenine
NEGATIVE CONTROLS Am64 7 153 + 7	-- -- --
EXPERIMENTAL Am64 + 7	--

ad-3A ▪ adenine-requiring (complements ad-3B)
ad-3B ▪ adenine-requiring (complements ad-3A)
nic-2 ▪ nicotinic acid- or nicotinamide-requiring
un-3 ▪ temperature-sensitive
cyh-1 ▪ cycloheximide-resistant

* 153 was the strain used to generate Am64.
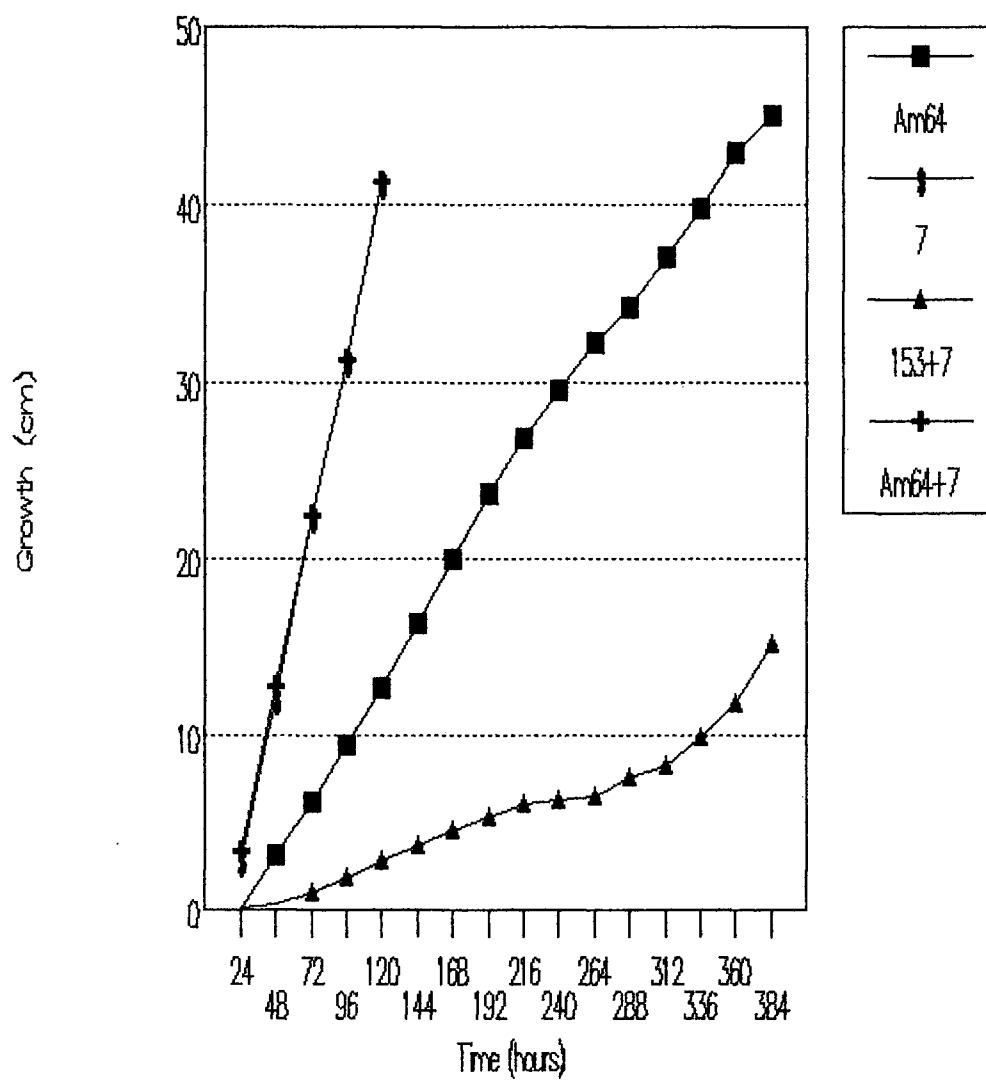
Growth rate of Am64 in a mixed mating type heterokaryon.
Each line represents the average of a minimum of 3
measurements of growth rate.

Figure 13

(*ad-3B(128)*, *tol*, a)+ I-9-3 (*ad-3B(114)*, *tol*, A)); and (4)
the following component strains on supplemented media:
I-22-83; a^{m33}, *ad-3B*; and 51-2. The heterokaryon, a^{m33} +
I-22-83, grew as fast as the mating type homokaryon, a^{m33} +
51-2 and the compatible mixed mating type heterokaryon,
I-9-57 + I-9-3; and faster than the incompatible
heterokaryon, 51-2 + I-22-83 (Table 8 and Fig. 14),
suggesting that the mutant, a^{m33}, has lost its heterokaryon
incompatibility function completely.

To determine the phenotype shown by a^{m33} in the same
nucleus with A, duplication progeny were obtained from the
following crosses. Three a^{m33}-containing strains, a^{m33}, *ad*;
R1-14 and R1-29 (Table 9) were crossed to the translocation
strain, T(I->II) 39311, *ser-3*, A, to generate duplication
progeny. The crosses are diagrammed in Figs. 15A and 15B.
Single spores were viewed through a dissecting microscope
and collected with a tungsten needle. Individual spores
were placed into slants of supplemented medium (minimal
medium + adenine for the a^{m33}, *ad*-derived spores; and
minimal medium + nicotinic acid and pantothenic acid for the
R1-14- and R1-29-derived spores). The test tubes were
placed into a 60°C water bath for 30 minutes to initiate
germination of the spores, which were examined after 3 days.

The progeny from each of the 3 crosses expressed one of
two growth phenotypes. One phenotypic class, called
"inhibited", grew slightly less vigorously than the other,
"healthy". The inhibited phenotype was distinct from the

Table 8 Strains and Media Used in the Measurement of Growth Rate of am33 in a Mixed Mating Type Heterokaryon

STRAIN	GENOTYPE
I-22-83	A, ad-3A, nic-2, un-3
51-2	a, ad-3B(114), cyh-1
I-9-57	a, ad-3B(128), tol
I-9-3	A, ad-3B(114), tol

	MINIMAL MEDIUM +
POSITIVE CONTROLS	
I-9-57 + I-9-3*	--
am33, ad + 51-2	--
I-9-3	adenine
am33, ad	adenine
51-2	adenine
I-22-83	adenine, nicotinic acid
I-9-57	adenine
NEGATIVE CONTROLS	
51-2 + I-22-83**	--
I-9-3	--
am33, ad	--
51-2	--
I-22-83	--
I-9-57	--
EXPERIMENTAL	
am33, ad + I-22-83	--

ad-3A = adenine-requiring
ad-3B(114) = adenine-requiring
ad-3B(128) = adenine-requiring
un-3 = temperature-sensitive
cyh-1 = cycloheximide-resistant

* The alleles, ad-3B(114) and ad-3B(128), complement.
** The genes, ad-3A and ad-3B, complement.
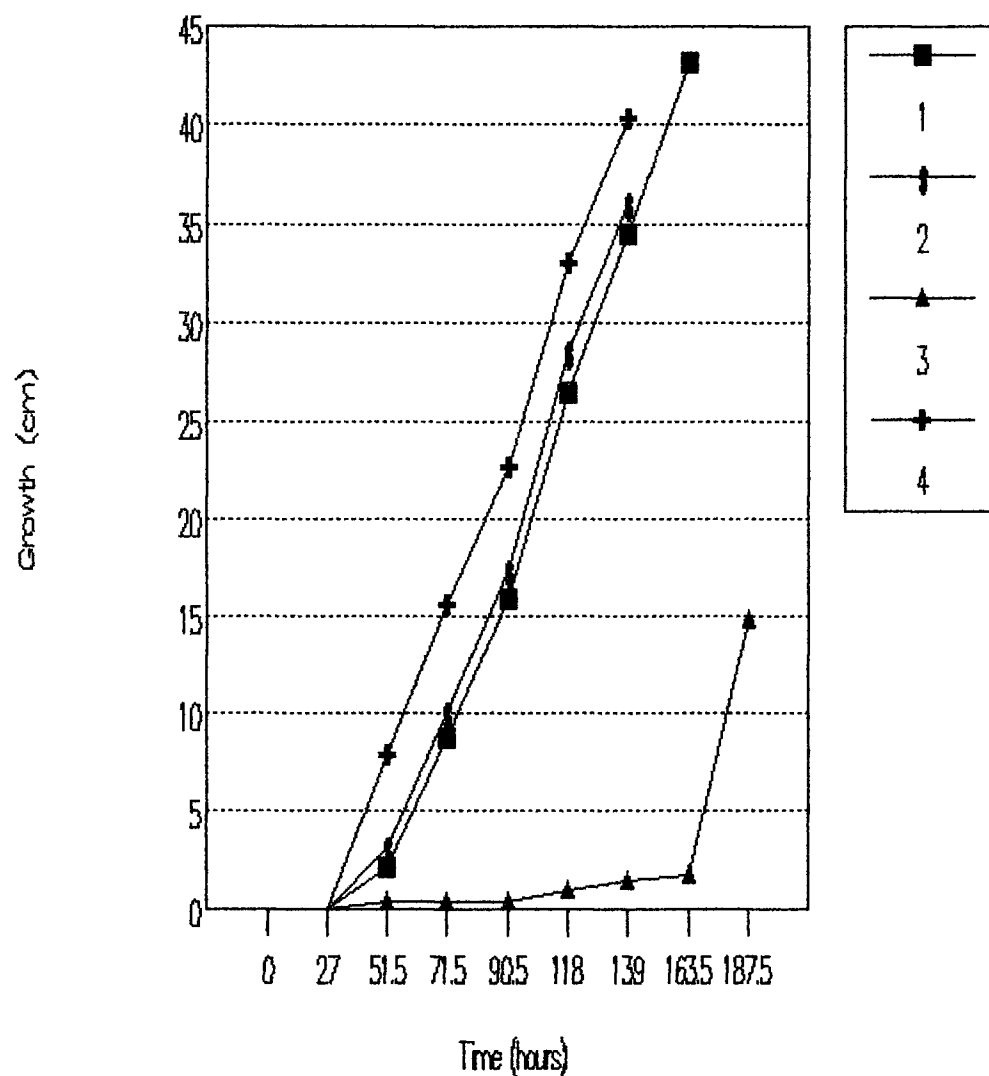
LEGEND

1 = I-9-57 + I-9-3
2 = am33,ad + 51-2
3 = 51-2 + I-22-83
4 = am33,ad + I-22-83

Figure 14

Growth rate of am33 in a mixed mating type heterokaryon.
Each line represents the average of 2 measurements
of growth rate.

Table 9 Genotypes of am33 Strains

STRAIN	GENOTYPE	SOURCE
am33, ad	am33, ad-3B (128)	A.J.F.G.
R1-14	am33, nic-3, pan-1, al-1 (#43)	N.L.G.
R1-29	am33, nic-3, pan-1, al-1 (#29)	N.L.G.

am33 = fertile compatible mating type mutant
ad-3B (128) = adenine-requiring (allele #128)
nic-3 = nicotinic acid- or nicotinamide-requiring
pan-1 = pantothenic-acid requiring
al-1 = albino
#43 = isolate # of N.L.G.
#29 = isolate # of N.L.G.
A.J.F.G. = A.J.F. Griffiths
N.L.G. = N.L. Glass
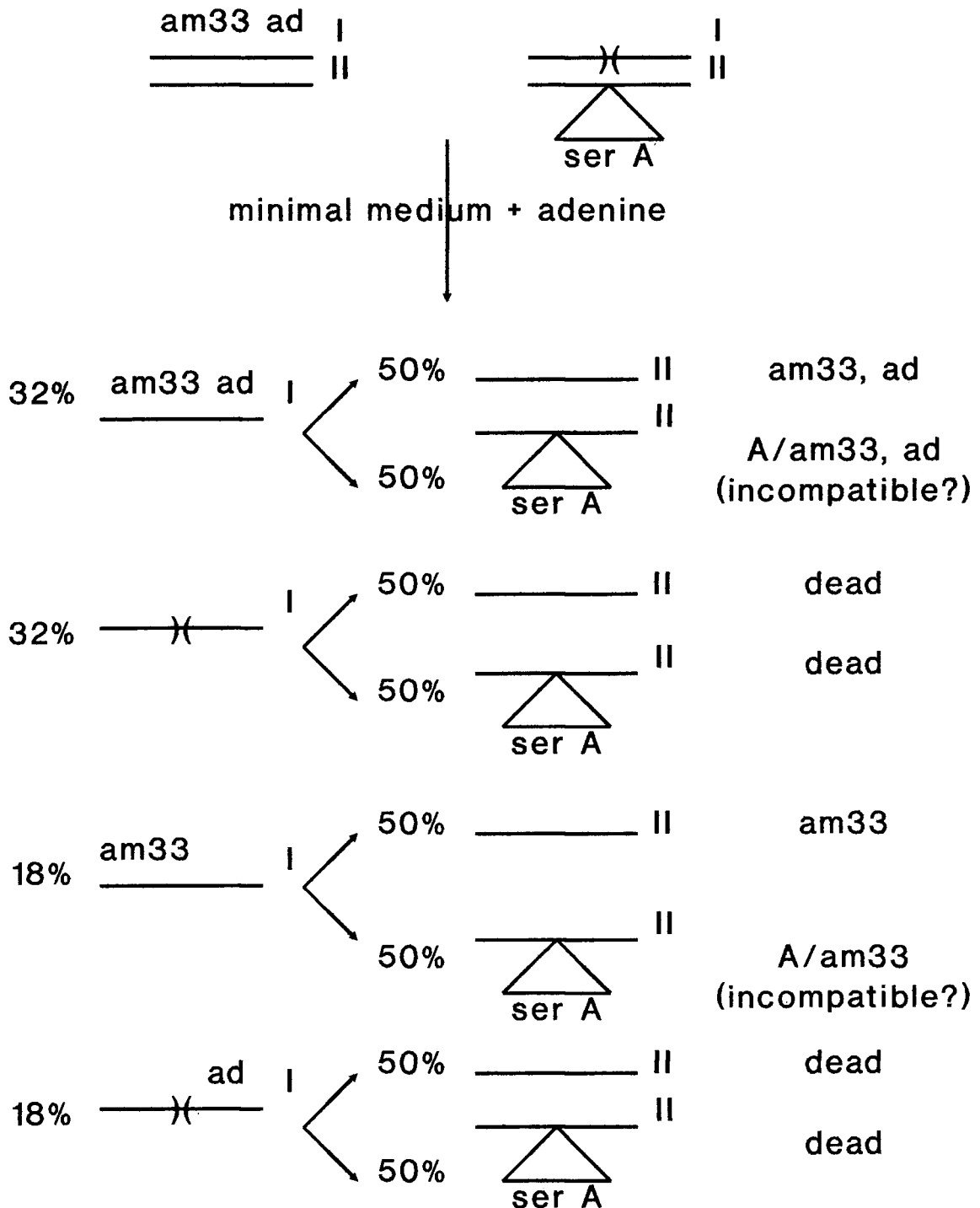
am33, ad-3B x T(I→II)39311, ser-3, A

Figure 15A Cross made to generate A/am33 duplications. Genetic
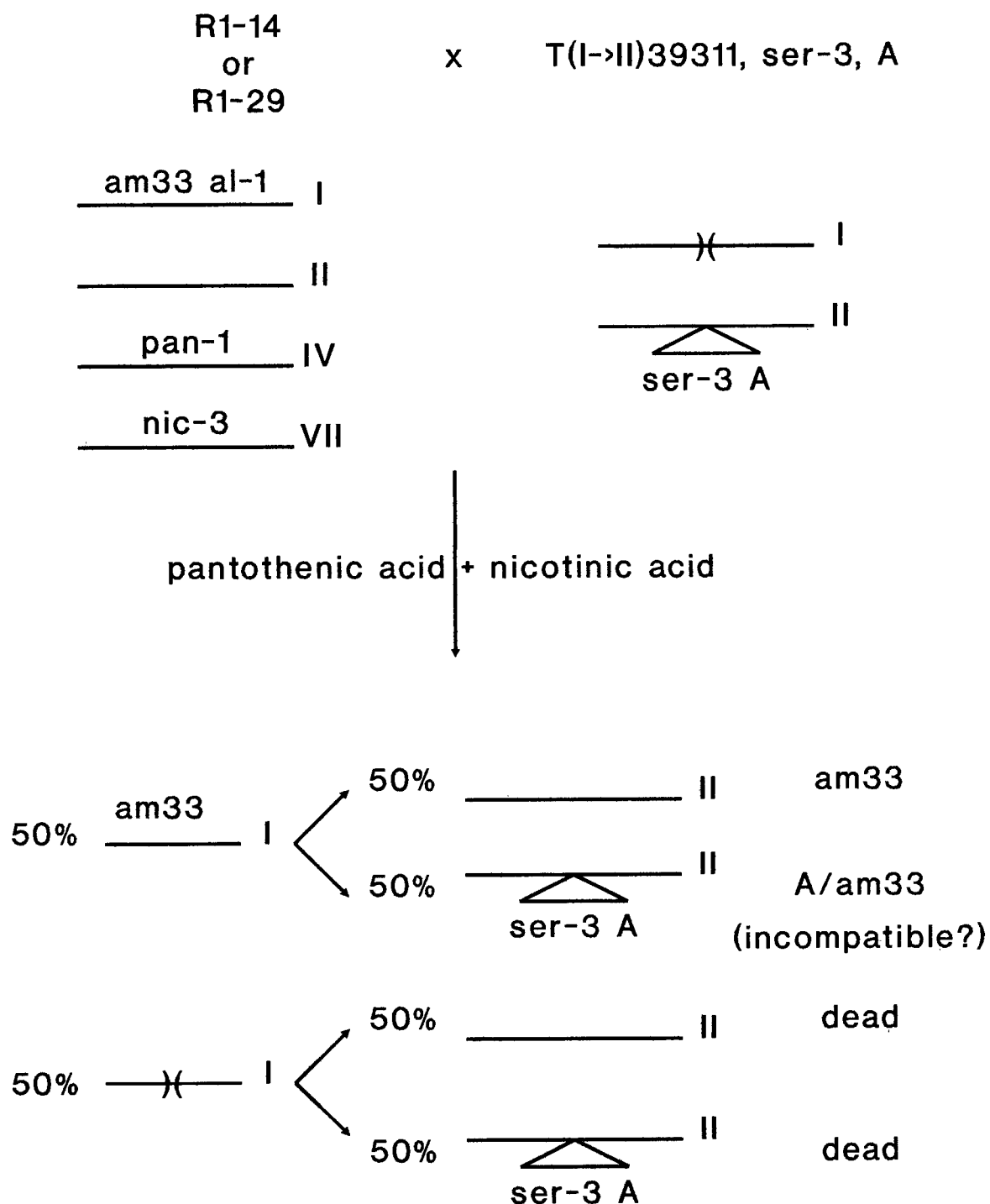distance between ad-3B and mt is 36%
(Perkins, et al., 1982).

R1-14
or
R1-29

x   T(I->II)39311, ser-3, A

am33 al-1 I

II

pan-1 IV

nic-3 VII

I

II

ser-3 A

pantothenic acid + nicotinic acid

50% am33 I

50% II am33

50% II A/am33 (incompatible?)

ser-3 A

50% I

50% II dead

50% II dead

ser-3 A

Figure 15B   Crosses done to generate A/am33 duplications.

standard incompatibility phenotype (dark agar) in that the growth was more luxuriant.

Mating type tests were done on all of the isolates (Table 10). If perithecia were produced with the *fl* tester, the reaction was scored as positive. Crosses, therefore, could have been barren. The healthy progeny from all three crosses were either *a*, which was expected (Fig. 15A and 15B) or *A/a*, which was unexpected. The inhibited progeny were mostly *A/a*, which was the result predicted in the hypothesis, although the predicted phenotype was dark agar. One inhibited isolate could have contained both mating types, but the *a* mating type reaction was difficult to confirm. One inhibited isolate reacted as only *a*. The significance of the four types of progeny is considered in Discussion 2.

In order to verify the difference in phenotypes, sample progeny from each cross were inoculated onto plates. The difference between the phenotypes became more evident under these growth conditions. As a control, a *A/a*, *tol* duplication strain, which has a phenotype known as "square agar" (Newmeyer, 1970), very slightly different from wild type, was also inoculated onto a plate.

All of the healthy progeny tested, including the single and double mating type isolates, and the square agar control grew to cover the plate with an even layer of mycelia. Most of the inhibited progeny (22 of 30) grew in a dense mat in the centre of the plate with a few hyphae extending beyond

Table 10 Mating Types of Progeny

CROSS	SPORE TYPE	MATING TYPE
am33,ad-3B x T(I-›II)39311,ser,A	17 h	12 A/a 5 a
	12 i	11 A/a 1 A/?
R1-14 x T(I-›II)39311,ser,A	9 h	6 A/a 3 a
	11 i	11 A/a
R1-29 x T(I-›II)39311,ser,A	7 h	3 A/a 4 a
	7 i	6 A/a 1 a

h = healthy (i.e. vigorous growth)
i = inhibited (i.e. less vigorous growth)

The A/a healthy isolates were unexpected.

the central mat (Table 11). Some of the progeny originally scored as inhibited (8 of 30) grew evenly on plates. Since the plating test was done several months after the initial spore isolation, it is possible that these progeny had escaped. Strains were kept in the freezer (-20°C) during most of this time, which could explain why all of the inhibited progeny had not escaped.

The growth rates of all of the isolates were measured and compared to incompatible duplication (*A/a,tol$^+$*) and compatible duplication (*A/a,tol*) controls. Fungi were inoculated at one end of a 50 cm growth tube containing 30 mL of supplemented medium for the isolates and minimal medium for the controls. The mycelial fronts were marked at regular intervals. Results are shown in Figs. 16-29. The slopes of the graphs are shown in Table 12.

All of the healthy isolates grew at the same rate as their siblings, as fast as the compatible controls, and considerably faster than the incompatible controls. The growth rates of the inhibited strains varied widely. Only one strain, the single mating type inhibited strain, 29-i-7, grew as fast as the compatible controls and healthy strains. The rest grew at rates intermediate between those of the compatible and incompatible controls. The significance of these results is considered in Discussion 2.

The following test was done to determine whether the mating types of the *A/a* strains had segregated into different nuclei. Conidial samples from the crosses with

Table 11 Phenotypes of Progeny

STRAIN	# OF ISOLATES WITH PHENOTYPE OF	
	DENSE MAT	EVEN GROWTH
a-i-x	8	4
a-h-x	0	17
14-i-x	10	1
14-h-x	0	9
29-i-x	4	3
29-h-x	0	7

KEY
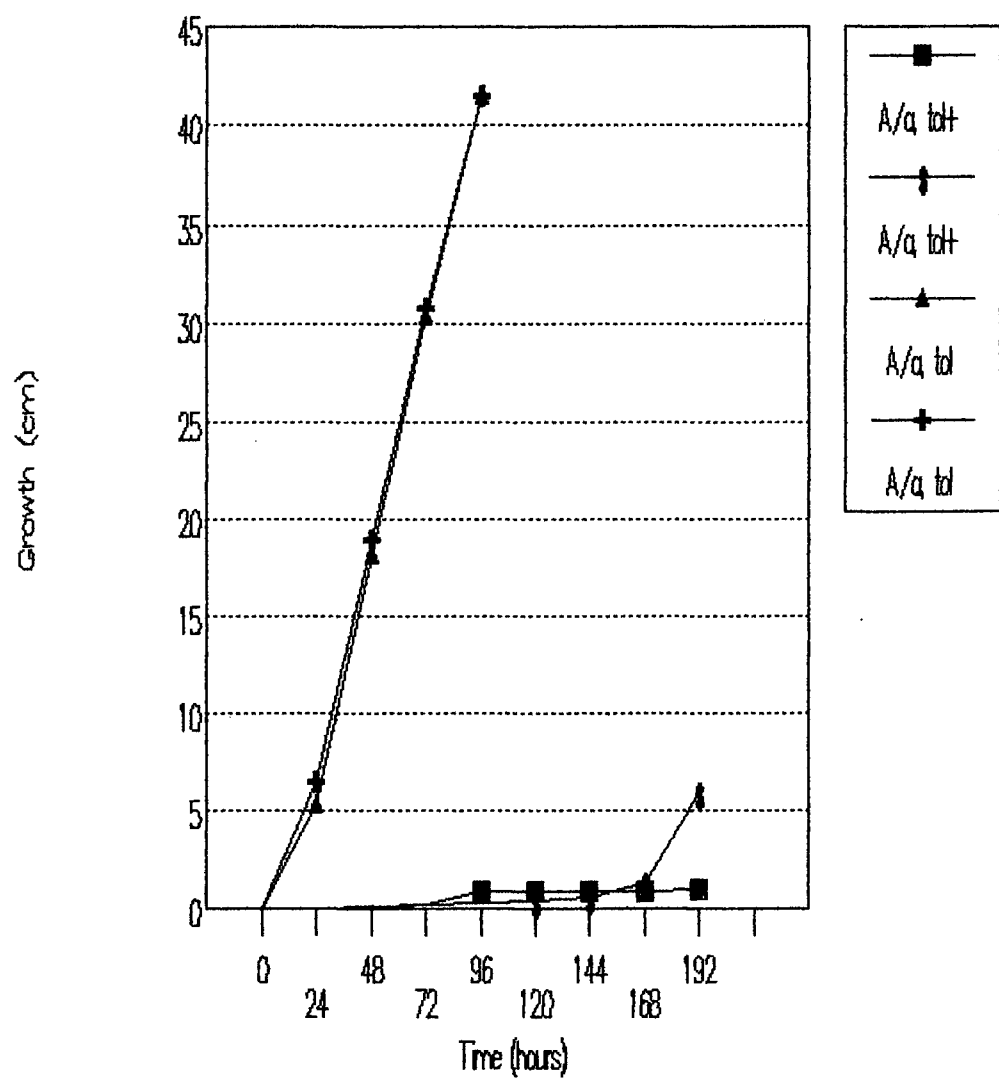a, 14 or 29 = strains am33,ad; R1-14 or R1-29
h or i = healthy or inhibited
x = isolate #

Inhibited strains with even growth were probably escapes.

the females, R1-14 and R1-29, were plated on sorbose plates containing pantothenic acid and nicotinic acid. Single conidial colonies were cut out of the agar, grown and tested for their mating types. All of the inhibited isolates tested contained both mating types, suggesting that the mating types had not segregated mitotically. The healthy isolates of strains which were originally *A/a* were all *a* (Table 13). The significance of these results is discussed below.

Growth Rates of Controls

LEGEND FOR FIGURES 16-29

a, 14 or 29 • strains am33, ad; R1-14 or R1-29
h or i • healthy or inhibited
x • isolate #

Figure 16
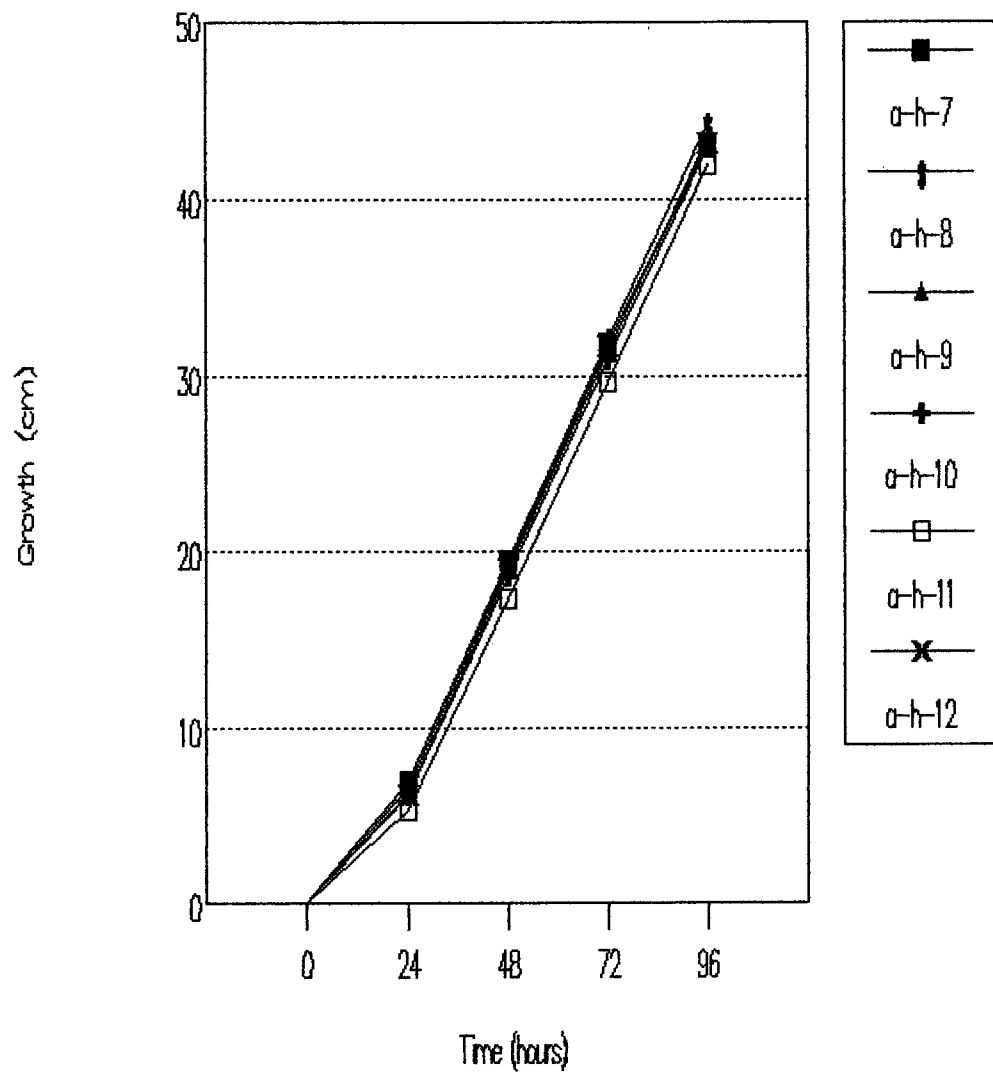
Growth Rates of a-h-x
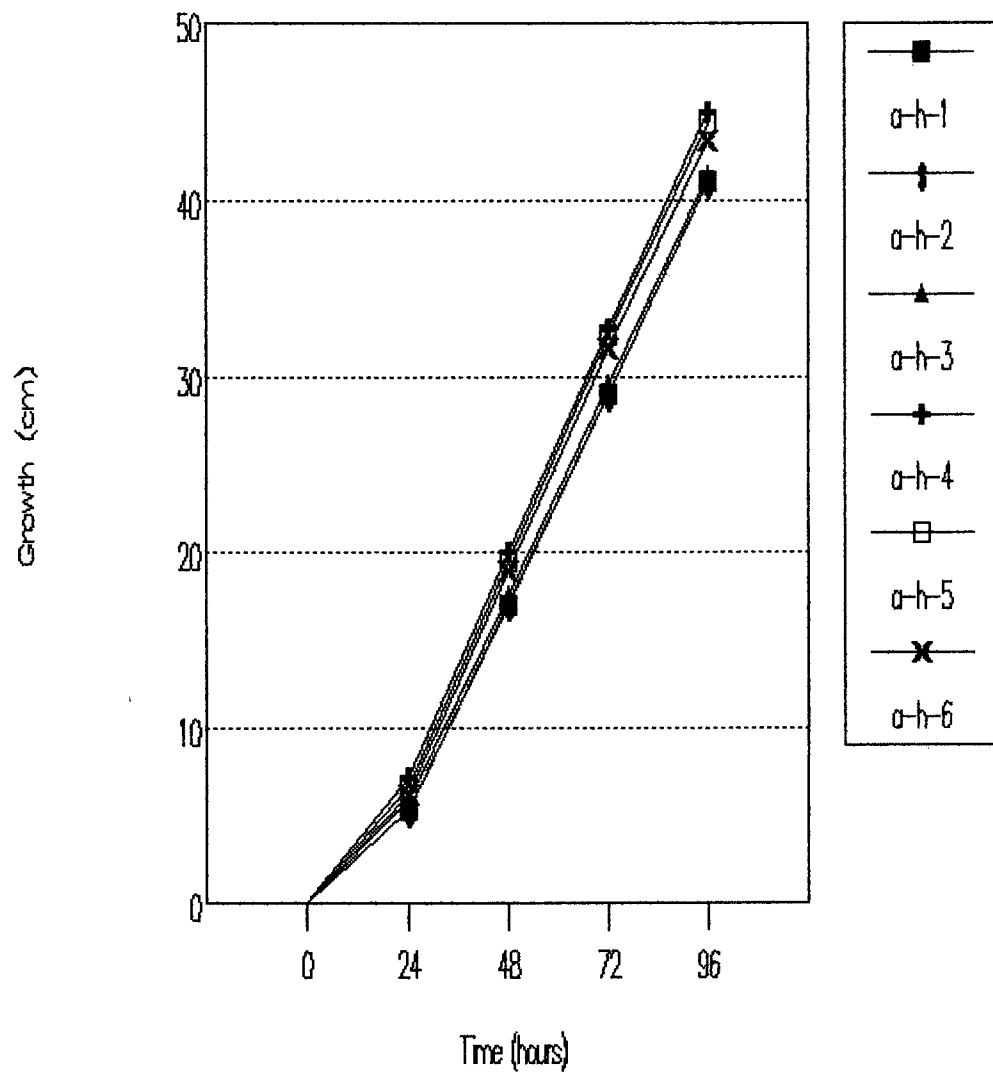
Figure 17
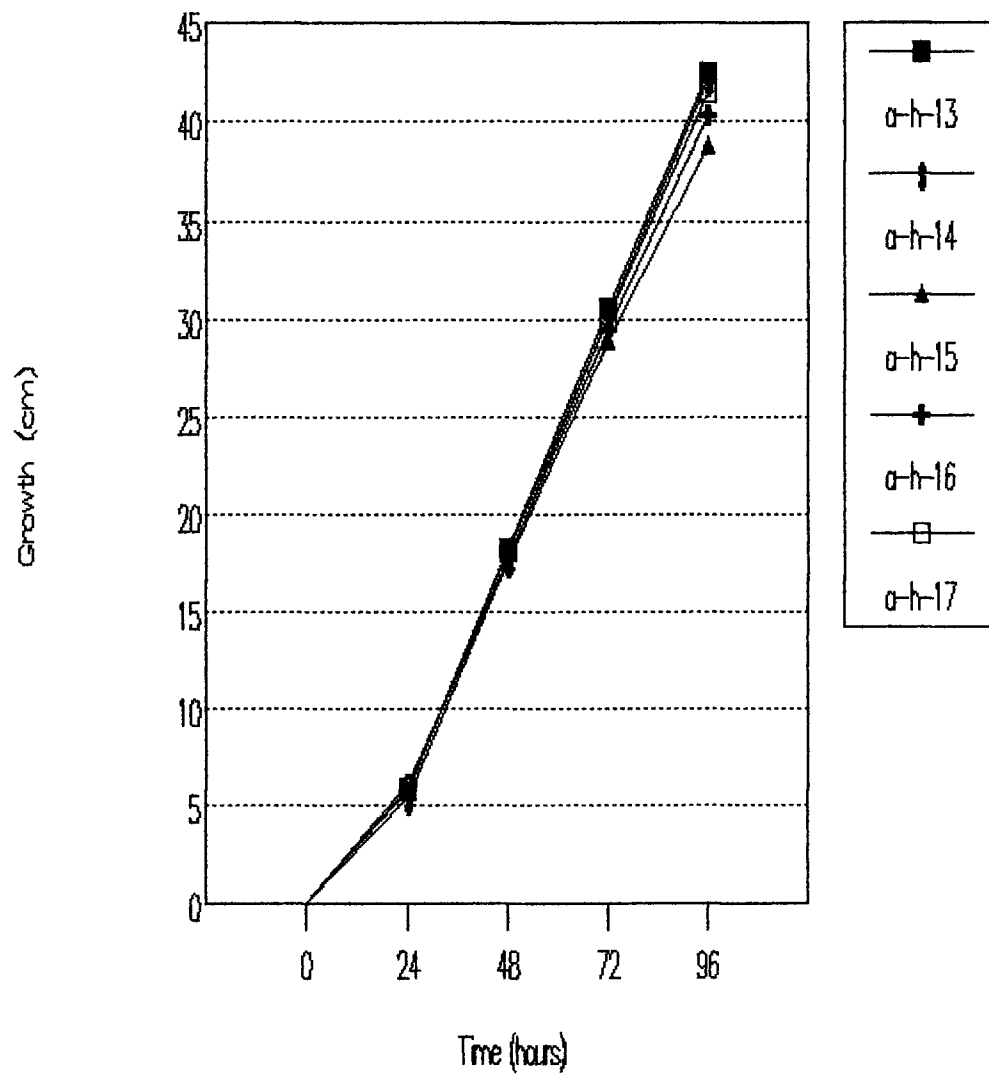
Growth Rates of a-h-x

Figure 18
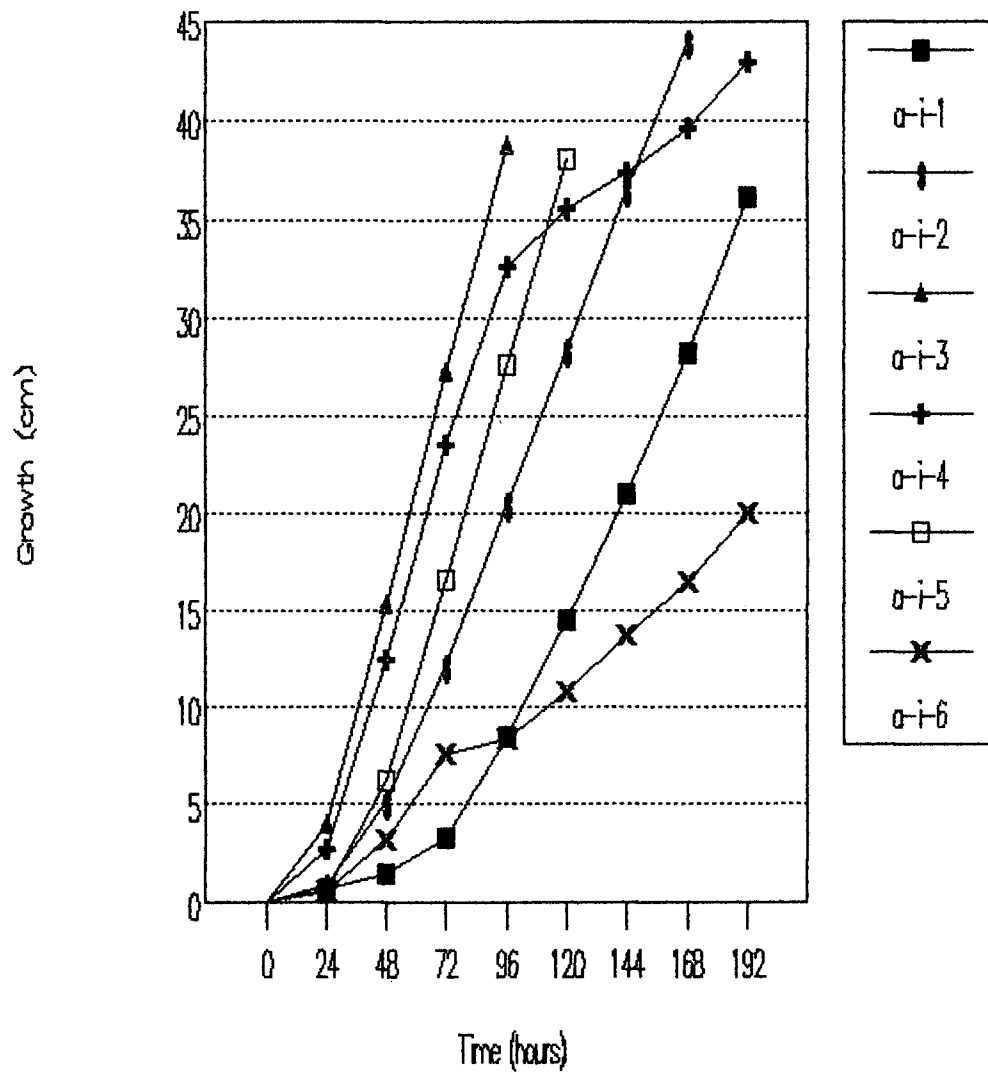
Growth Rates of a-h-x

Figure 19

Growth Rates of a-i-x

Figure 20

Growth Rates of a-i-x

Figure 21
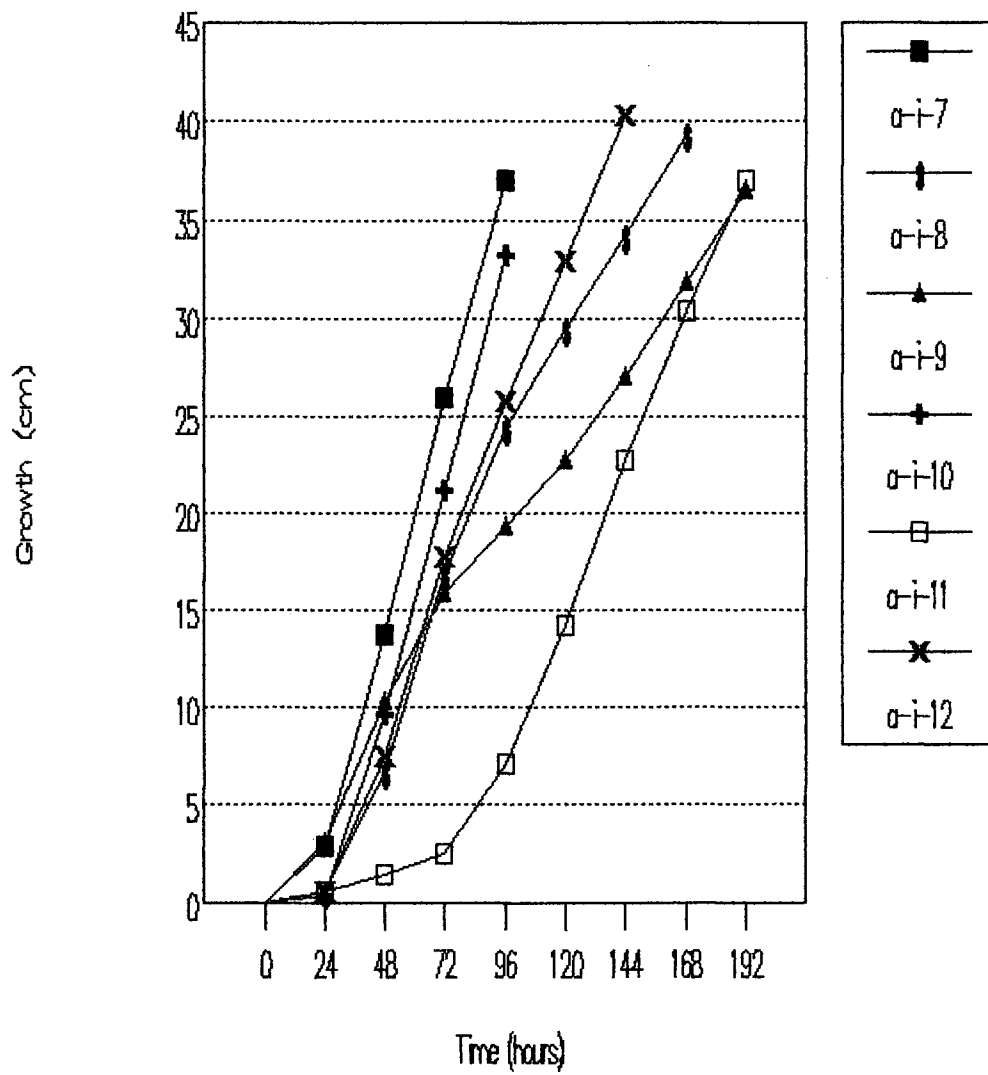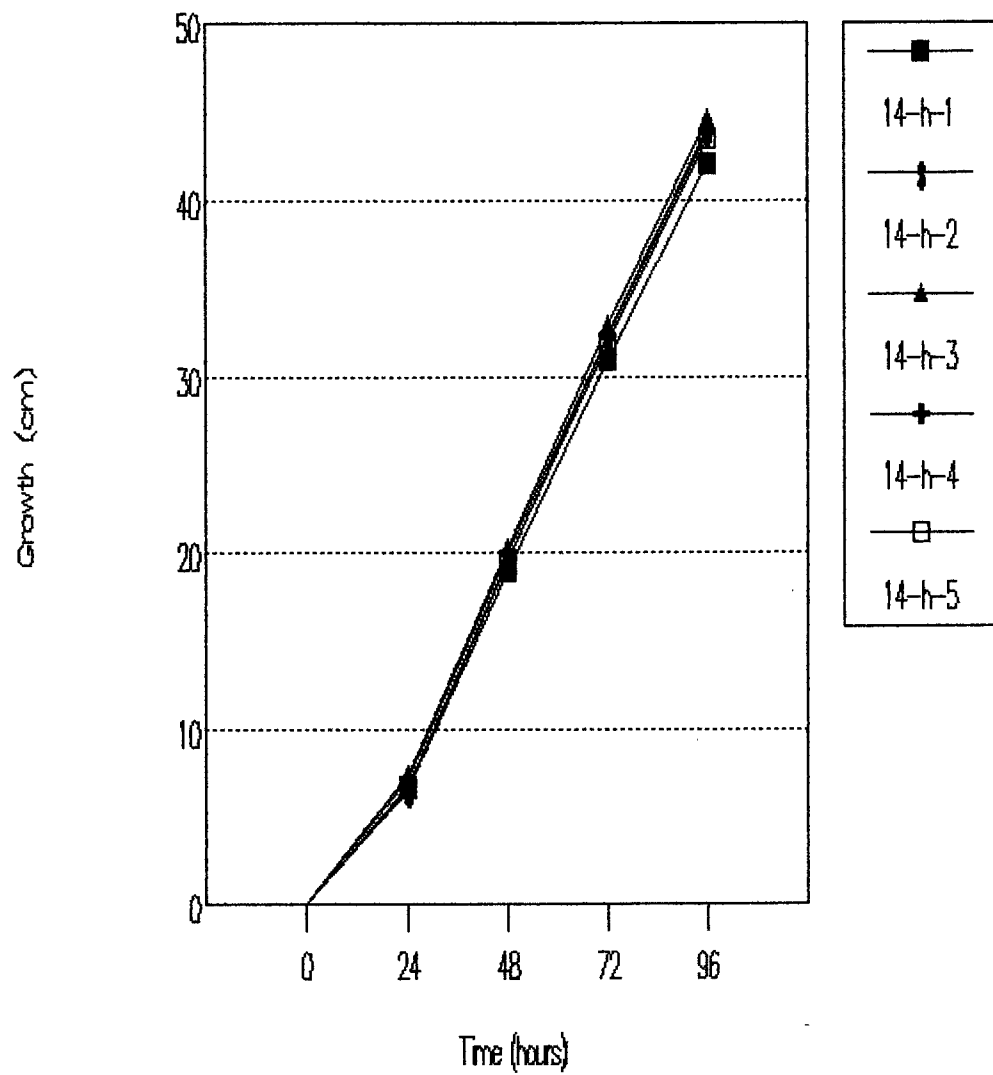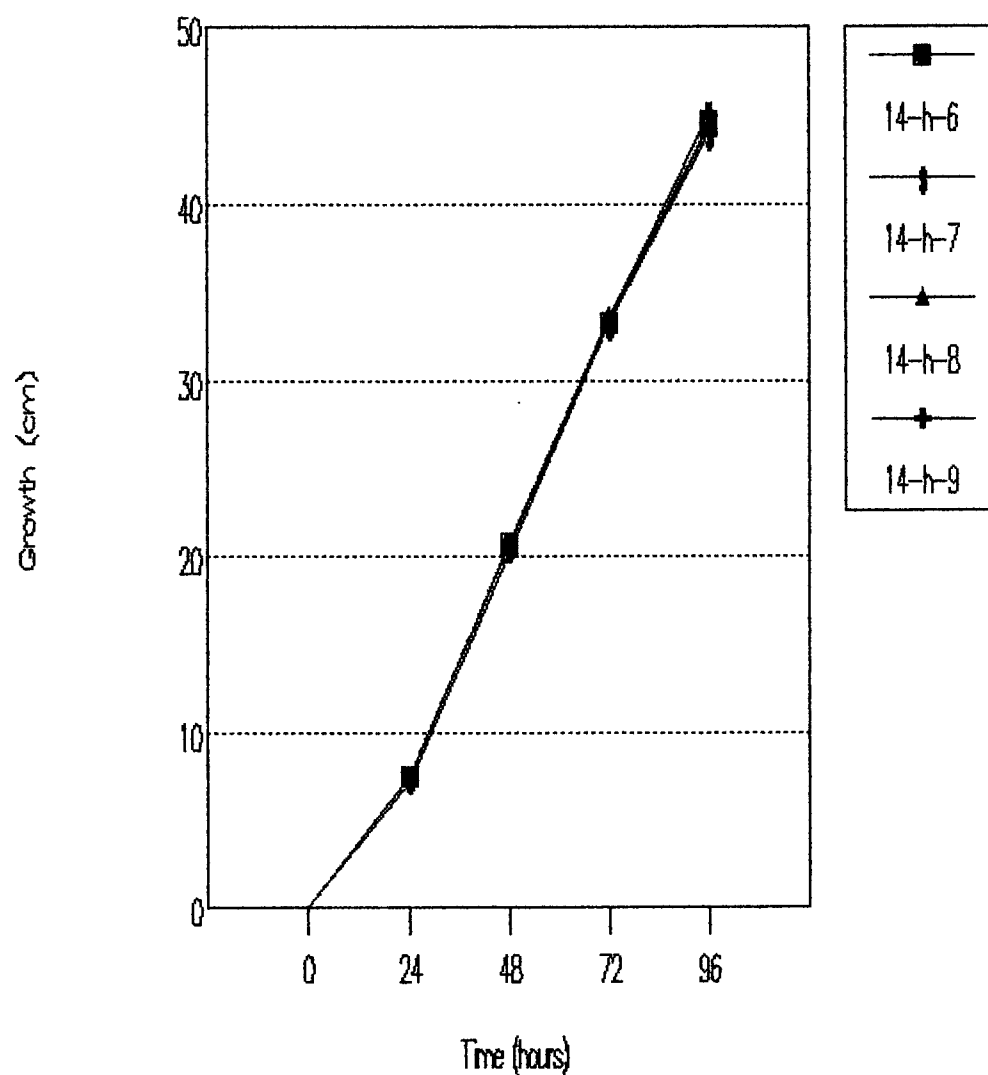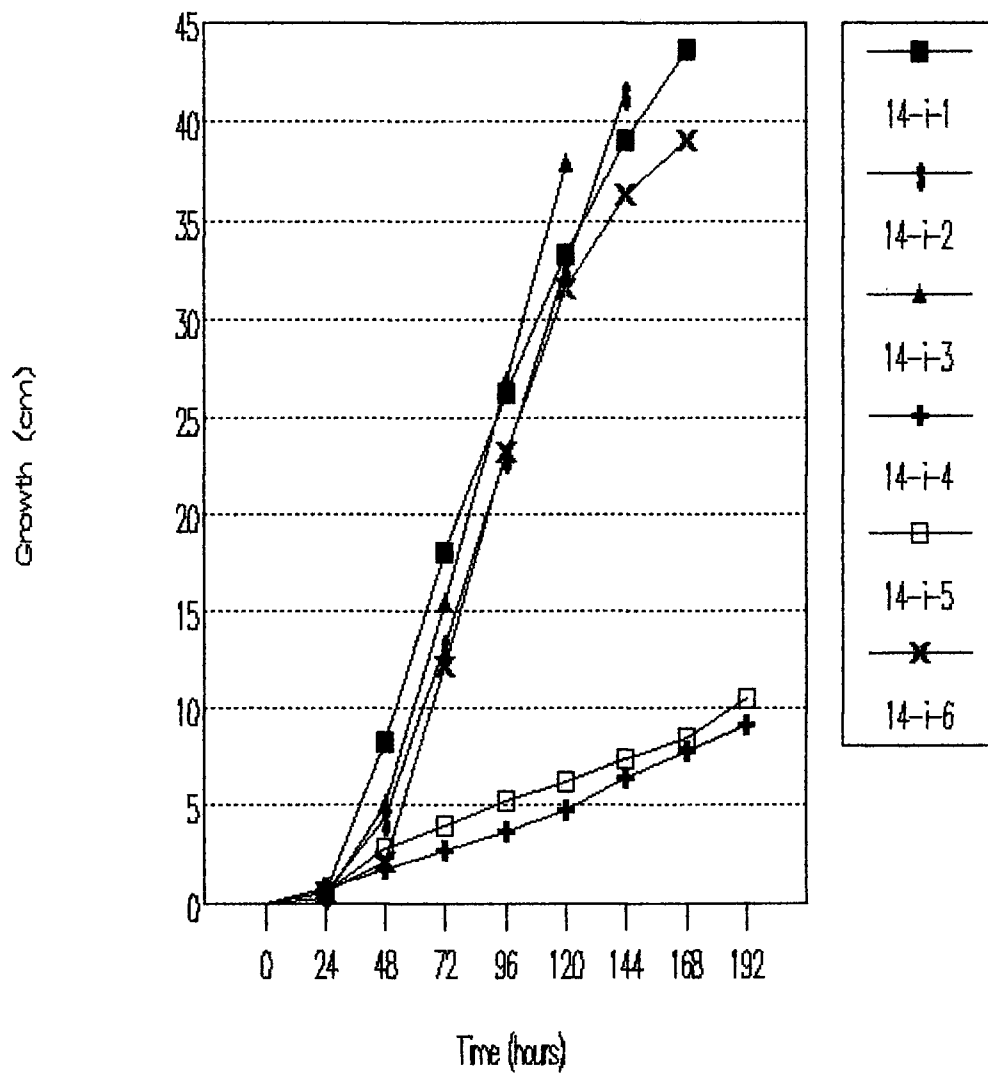
Growth Rates of 14-h-x

Figure 22

Figure 23

Figure 24
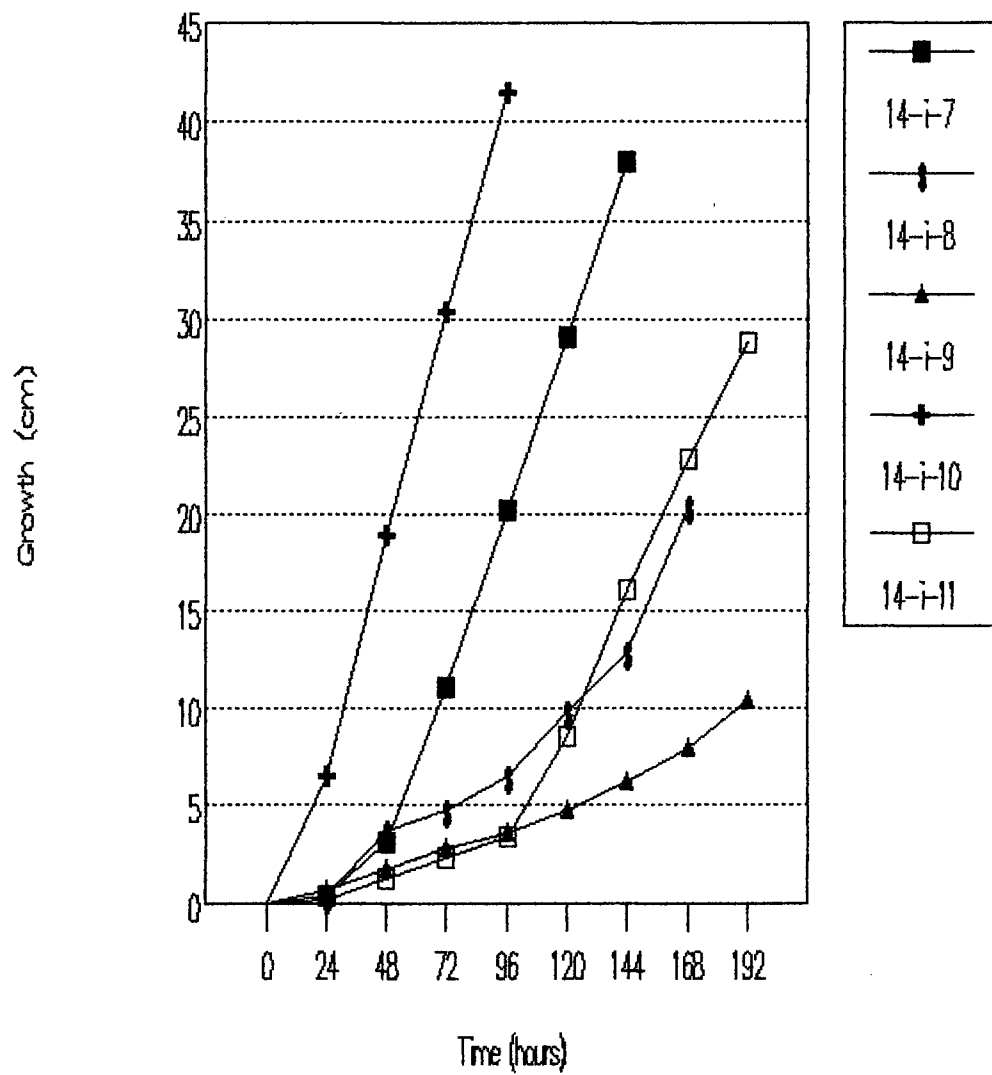
Growth Rates of 14-i-x

Figure 25

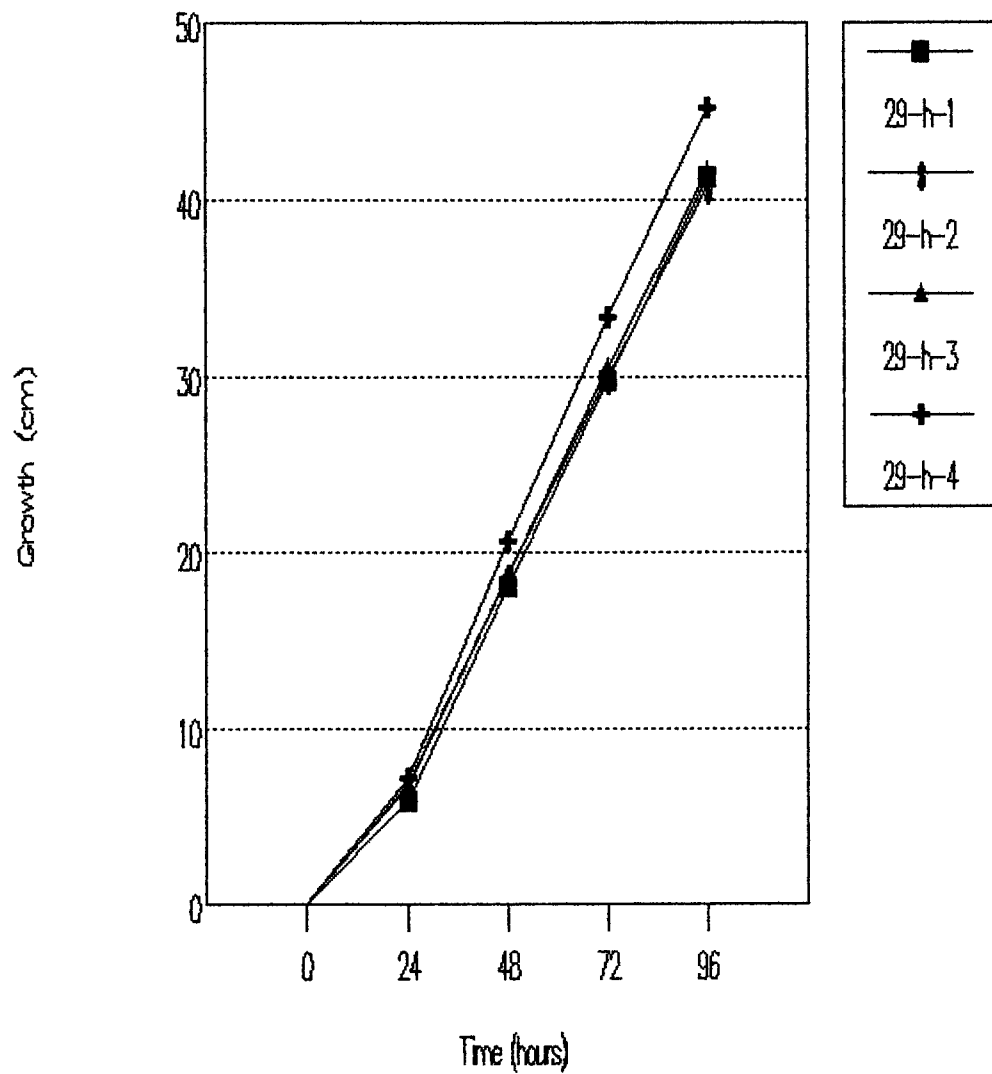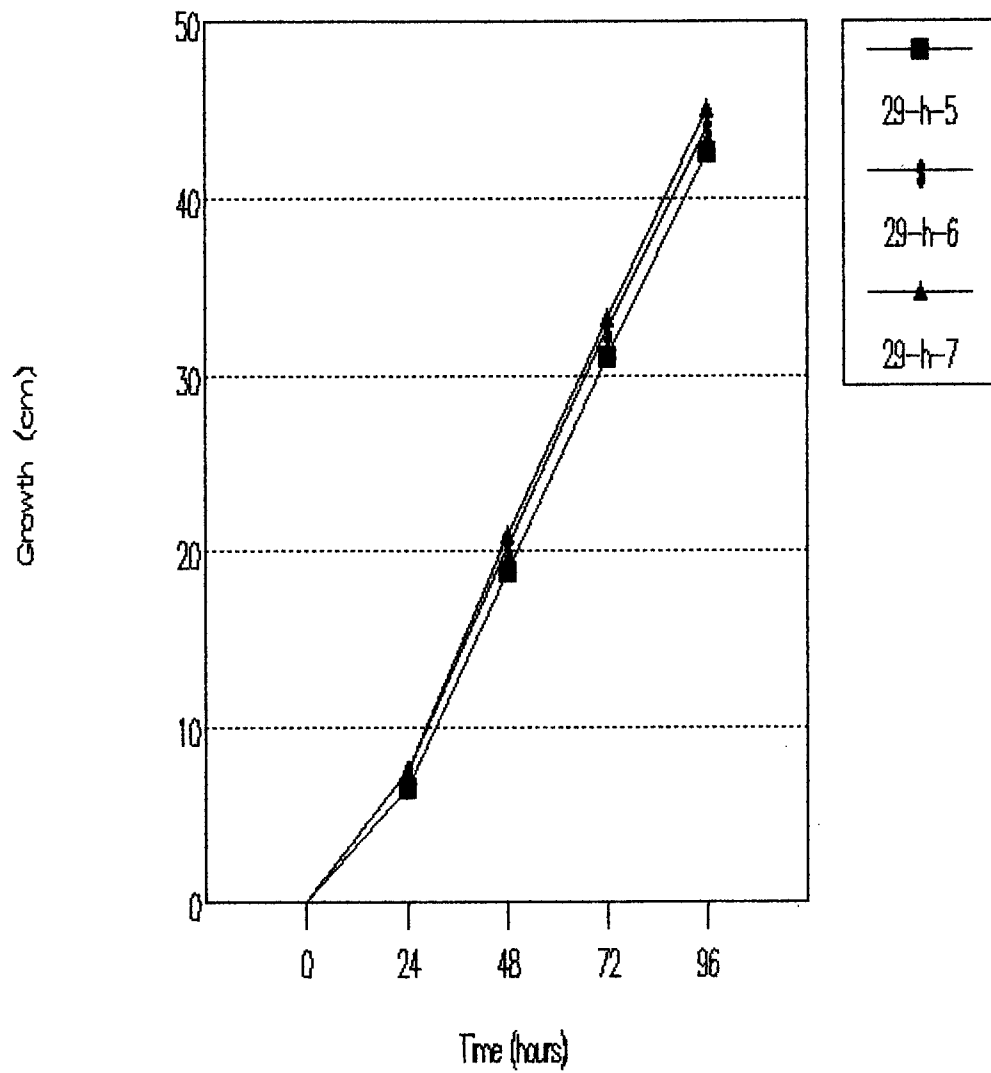# Growth Rates of 29-h-x

Figure 26

Growth Rates of 29-h-x

Figure 27
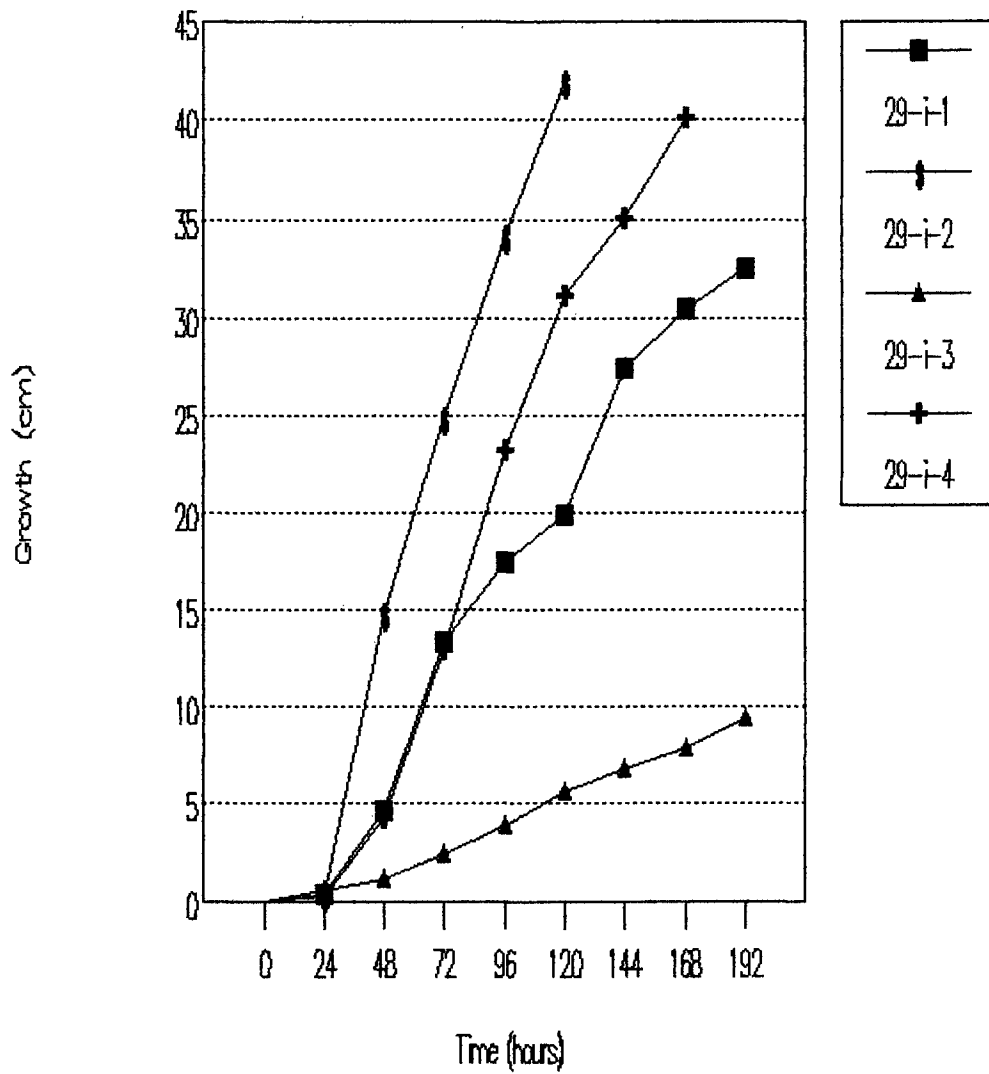
Figure 28

Growth Rates of 29-i-x
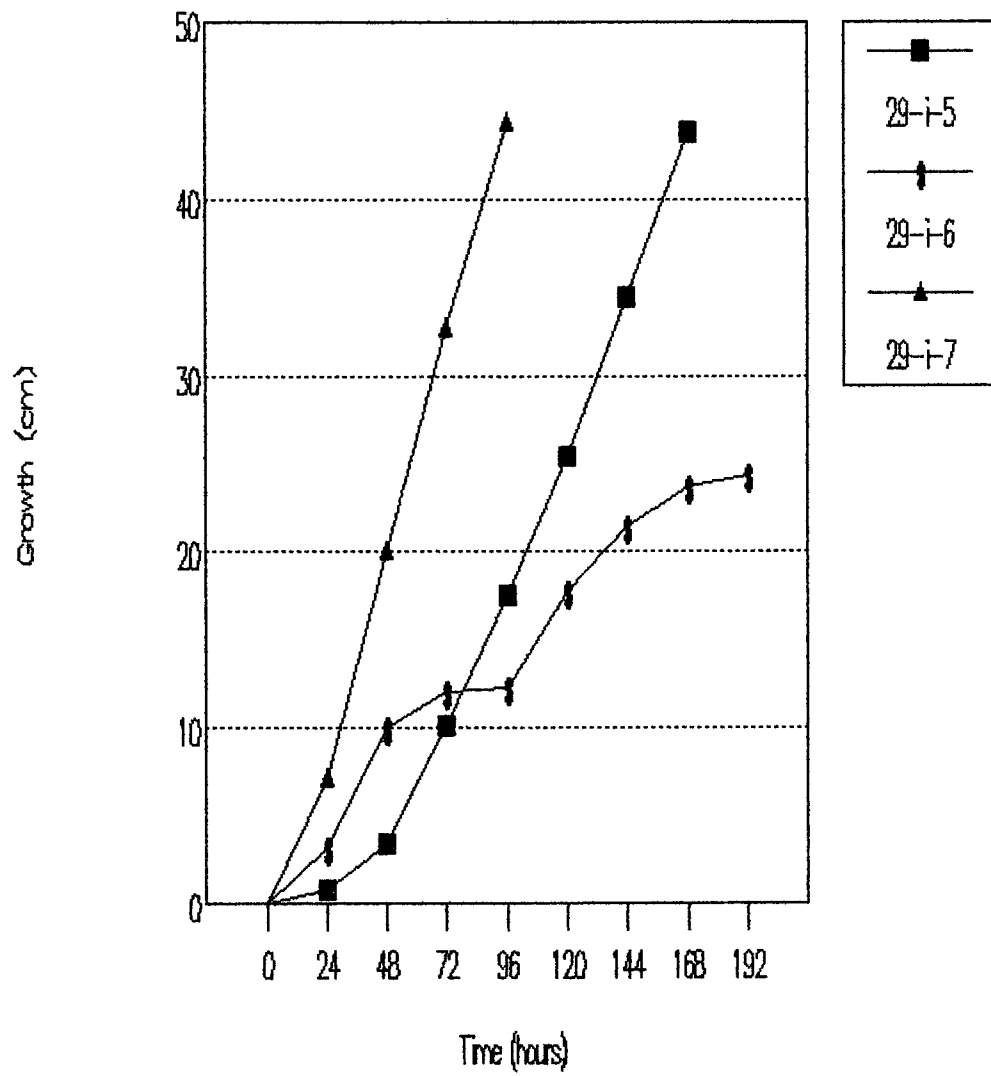
Figure 29

Table 12 Slopes of Growth Rates of Progeny

STRAIN	SLOPE*
A/a, tol+ (average of 2 strains)	.0035
A/a, tol (average of 2 strains)	.50
a-h-x (average of 17 strains)	.50
a-i-1	.27
a-i-2	.33
a-i-3	.48
a-i-4	.42, .11
a-i-5	.44
a-i-6	.12
a-i-7	.47
a-i-8	.23
a-i-9	.38, .17
a-i-10	.46
a-i-11	.31
a-i-12	.34
14-h-x (average of 9 strains)	.51
14-i-x (average of 14-i-1, 2, 3, 6, 7)	.39
14-i-x (average of 14-i-4, 5, 9)	.056
14-i-8	.096
14-i-10	.49
14-i-11	.044, .26
29-h-x (average of 7 strains)	.50
29-i-1	.19
29-i-2	.43
29-i-3	.053
29-i-4	.37
29-i-5	.34
29-i-6	.13
29-i-7	.52

* Slopes were measured at straight regions of the graph, over no fewer than 72 hours (except for the first slope for a-i-9, which was measured over 48 hours).

All except 1 (29-i-7) of the inhibited strains grew more slowly than the healthy strains and the positive control strains (A/a, tol).

Table 13   Mating Types of Single Conidial Isolates of Progeny

STRAIN	MATING TYPE
14-h-4-1	a
14-h-4-2	a
14-h-4-3	a
14-h-5-1	a
14-h-5-2	a
14-h-5-3	a
14-h-6-1	a
14-i-3-1	A/a
29-h-5-1	a
29-h-5-2	a
29-h-6-1	a
29-h-6-2	a
29-h-6-3	a
29-h-7-1	a
29-h-7-2	a
29-h-7-3	a
29-i-1-1	A/a
29-i-1-2	A/a
29-i-1-3	A/a
29-i-3-1	A/a

14 or 29 = strain R1-14 or R1-29
h or i = healthy or inhibited
first # = spore isolate #
last # = conidial isolate #

Healthy strains were a; inhibited strains were A/a.

DISCUSSION 2

The findings of Results 2 present evidence to support
the idea that at least 2 mating type mutants completely
deficient in heterokaryon incompatibility, A^{m64} and a^{m33},
retain nuclear incompatibility. The growth rates of the two
mutants in mixed mating type heterokaryons equalled those of
positive controls and surpassed those of negative controls,
so it appears that neither mutant has residual heterokaryon
incompatibility. The nuclear incompatibility seen,
therefore, was not due to residual heterokaryon
incompatibility.

Four phenotypes were seen in the progeny from the three
crosses made to generate A/a^{m33} duplications: (1) single
mating type healthy (12 isolates), (2) double mating type
healthy (21 isolates), (3) single mating type inhibited (1
isolate) and (4) double mating type inhibited (28, or
possibly 29, isolates). The only phenotype that was
expected (Fig. 15A and 15B) was the first phenotypic class,
single mating type healthy, which were normal segregants
from the cross. The phenotype of the duplicated progeny was
unknown, although it was predicted to be dark agar.

The fourth phenotypic class, the double mating type
inhibited strains, is the one providing evidence that
nuclear incompatibility can exist in the absence of
heterokaryon incompatibility. All of the A/a inhibited

strains grew more slowly than the healthy strains. The reduced growth rates could have been due to the presence of a^{m33} and A in the same nucleus. Four of the seven A/a inhibited strains that grew evenly on plates (a-i-3, a-i-7, 14-i-10, 29-i-2) had the highest growth rates of all of the A/a inhibited strains, supporting the idea that they had escaped and were growing at rates higher than the inhibited strains, yet lower than the healthy strains. Two of the seven strains (a-i-4, a-i-9) had growth rates that were high at first, but suddenly dropped. One of the seven strains (29-i-6) had an irregular growth pattern.

The growth rates of the A/a inhibited strains were highly variable. The variant rates could be characteristic of nuclear incompatibility itself. They could also have been produced by escape, either by a number of different mechanisms or by the same mechanism producing different results. Perhaps the inhibited strains showed various growth rates because they were in different stages of escape by somatic segregation and overgrowth by a nuclei.

The second phenotypic class, the A/a healthy isolates was not expected. Metzenberg (personal communication) suggested that these strains grew vigorously because the two mating types had segregated somatically into separate nuclei producing $A + a^{m33}$ heterokaryons, which are known to be compatible. The translocation is long enough to sustain a double crossover. To test his hypothesis, single conidial isolates were obtained from healthy and inhibited strains

and tested for their mating types. If Metzenberg's hypothesis were correct, conidia derived from healthy strains would have been A or a, whereas conidia derived from inhibited strains would have been A/a. These results were observed, with the exception that there were no A conidial isolates from the healthy strains.

If the healthy phenotype were due to mitotic segregation, the cultures would have contained both mating types shortly after germination of the spores. After a period of time, it is conceivable that the a nuclei overgrew the A nuclei because the latter contained the $ser-3$ marker which is closely linked to the mating type gene. Overgrowth by a nuclei may have been slowed by cross-feeding of the serine-requiring A nuclei by the a nuclei.

The healthy strains grew as fast as the compatible controls (Figs. 16-19, 22, 23, 26 and 27). The compatible controls were A/a duplications containing tol. The evidence presented above suggests that the healthy strains were simply a^{m33} strains.

The third phenotypic class, the 1 unexpected inhibited strain that reacted with only one mating type (29-i-7), could have escaped from the inhibition by deletion of A. It grew evenly on a plate, and had a growth rate that exceeded some of the healthy strains, suggesting that it had escaped from inhibited growth.

An explanation is needed for why some A/a^{m33} strains (the healthy ones) escaped early, while others (the

inhibited ones) escaped later or not at all. Would all of the inhibited strains have escaped eventually? Was the time of escape the only difference between the healthy and inhibited strains or was there a difference in the mechanisms of escape? Was it significant that escaped inhibited strains did not grow as fast as healthy strains? Was there a gene segregating that caused early/late escape by mitotic crossing over in the A/a healthy strains and the A/a inhibited strains? The first of the three crosses segregated half A/a healthy and half A/a inhibited progeny. In the other 2 crosses, the sample sizes were probably too small to reflect accurate ratios. These questions can only be addressed by further study.

Perkins (personal communication) has observed that A/a^{m33} duplications grow with an abnormal morphology which he calls "square" because Newmeyer (1970) called the morphology associated with A/a, tol duplications "square". Perkins' observations are not inconsistent with those presented above, except that Newmeyer's square strains grow at wild type rates, whereas Perkins' square strains, assuming they exhibit the same growth rates as those discussed here, grow at sub-wild type rates. For this reason, A/a^{m33} duplications should be referred to by some name other than square.

Molecular Model

One current model of the molecular interactions that occur during mating is as follows (Metzenberg and Glass, 1990; Glass, personal communication). A combination product of A and a effects incompatibility during the vegetative cycle, when the mating type genes are expressed at low levels, and the same product instigates mating functions during the sexual cycle, when the mating type genes are expressed at higher levels.

Nuclear incompatibility can be explained in the context of this model. There is obviously a difference in the function of the mating type products during the vegetative and sexual cycles. Perhaps the mutant, A^{m64}, is defective for mating, but not incompatibility, as previously believed. It, and the other mutant studied here, a^{m33}, could be defective in terms of the stability of their products. Both mutants are functional for nuclear incompatibility and defective for heterokaryon incompatibility. In terms of the stability hypothesis, heterokaryon incompatibility requires more stable mating type products than nuclear incompatibility. These results can be explained as follows.

In a mixed mating type heterokaryon, the A and a products are synthesized in different nuclei. When the products encounter one another, the combination product enters the nucleus and initiates incompatibility. In a A/a duplication strain, the A and a products are made in the

same nucleus, so they would encounter each other with greater speed because of their proximity, and would, therefore, require less stability. In the mutants, A^{m64} and a^{m33}, heterokaryon incompatiblity may be eliminated because the products degrade too quickly to find the opposite mating type product with which to combine.

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