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Research Focus

Aspergillus genomes: secret sex and the secrets of sex

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The genomic sequences of three species of *Aspergillus*, including the model organism *A. nidulans* (which is homothallic: having no differentiated mating types, a strain being able to cross with itself), suggest that *A. fumigatus* and *A. oryzae*, considered to be asexual, might in fact be heterothallic (having two differentiated mating types, a strain being able to cross only with strains of opposite mating type). The genomic data have implications for the understanding of the evolution and the mechanism of sexual reproduction in this genus. We propose a model of epigenetic heterothallism to account for the reproductive patterns observed in *Aspergillus nidulans*.

In 1953 Pontecorvo published a compendium of the genetics of the ascomycete fungus *Aspergillus nidulans* [1]. This beautiful and sophisticated system went on to be used in the analysis of many metabolic and cellular processes. It suffices to point out the identification by Herb Arst and coworkers of the crucial actors of nitrogen-metabolite and carbon-catabolite repression [2], the work of Ron Morris and his school on mitosis and the cell cycle [3], which matches the Nobel Prize winning work of Paul Nurse and Lee Hartwell, and the dissection of conidial development initiated by Bill Timberlake with the introduction of the methodology of cascade hybridisation [4]. The taxonomic and evolutionary relationships of the *Aspergilli* are described in Box 1.

Other *Aspergilli* are useful in biotechnological processes, from the production of soy sauce and sake (*A. soyae*, *A. oryzae*) and the production of citric acid (*A. niger*) to the production of heterologous proteins. *A. fumigatus* used to be a rare pathogen but is now one of the most common causes of fatal hospital-acquired infection in immunosuppressed patients. *A. flavus*, a noxious contaminant of food-stuffs, produces aflatoxin, the most powerful carcinogen known. *A. sydowii* is an insidious pathogen of gorgonian corals.

A recent issue of *Nature* carries three articles describing the genomic sequences of *A. nidulans* [5], *A. oryzae* [6] and *A. fumigatus* [7]. The sequence differences between *A. nidulans* and the other two *Aspergilli* are as great as the differences found between fish and humans, which are separated by ~450 million years. This probably reflects a rapid divergence within the genus rather than an ancient separation of the species [8]. The *A. oryzae* genome encodes 12 074 predicted proteins, compared with 9926 for *A. fumigatus* and 9541 for *A. nidulans*. The extra *A. oryzae* putative genes are mainly involved in secondary metabolism and might be the result of horizontal gene transfer, including from prokaryotes. The number of proteins listed for the three species in sequence databases has increased since publication of the sequences, as the splicing programs originally used [5–7] resulted in several spurious gene fusions.

A. nidulans is homothallic (see Box 2), but there are heterothallic species of *Aspergillus*, whereas no sexual cycle has been described for *A. fumigatus* or *A. oryzae*. Phylogenetic studies place the *Aspergilli* in a monophyletic genus in which sexual and asexual species are sister groups [9]. It is commonly agreed that asexual species can arise repeatedly within the same taxon by loss of genes essential for the sexual process.

In heterothallic ascomycetes, mating type is determined by the alternative presence of either of two genes. Opposite mating type genes do not show any sequence similarity to each other, even if they occupy the same place (locus) in the chromosome, and thus they are properly called idiomorphs. I shall refer to the opposite mating type genes according to the names of their conserved motifs: α and *HMG*. In the homothallic *A. nidulans* both the α and *HMG* genes are present, occupying unlinked loci in the genome [5]. In the sequenced genomes of *A. oryzae* and *A. fumigatus* we find only the α gene (*A. oryzae*) or only the *HMG* gene (*A. fumigatus*) [5,10,11]. These occupy the same chromosomal position in a region of 1.7 Mb synteny. The *HMG* locus of *A. nidulans* is in a region of 409 kb synteny with one flank of the mating type locus of the other two species, and the α locus conserves a 34 kb region of synteny on the opposite flank (Figure 1a).

These results present several fascinating evolutionary and physiological problems. Firstly, is heterothallism or homothallism primitive in the sexually reproducing *Aspergilli*? The genomic evidence favours a heterodox scenario of primitive homothallism [5,9] (see Box 2). A hypothetical homothallic species with closely linked mating loci would originate by chromosome translocation, the situation seen in *A. nidulans*, and by loss of each of the mating loci that are found in *A. fumigatus* and *A. oryzae* [5] (Figure 1b). A scenario in which the pattern seen in *A. nidulans* is primitive is equally consistent with the genomic data (Figure 1c).

Secondly, is the presence of mating loci in *A. fumigatus* and *A. oryzae* a sign of covert sex? In the genomes of the three *Aspergilli*, 215 genes putatively involved in sexual reproduction have been identified. A few of these genes

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Box 1. Ascomycetes

The fungi are a large eukaryotic kingdom (1.5 million species estimated) considered to be the sister taxon to the metazoans. About 40% of all fungal species belong to the phylum ascomycetes; these include industrially useful organisms, pathogens of plants, animals and humans, and favourite model organisms such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Neurospora crassa* and *A. nidulans*. The phylum is defined by the fact that the products of meiosis are contained in a sac, the 'ascus'. Mushrooms belong to another major phylum, the basidiomycetes. Other fungal phyla are the chytrids, zygomycetes and glomeromycota. Old classifications included a group called the deuteromycetes or 'fungi imperfecti', which lack sexual reproduction. This is not a natural taxon; each of these 'imperfect' organisms can be grouped with 'perfect' (sexually reproducing) species on the basis of morphological and molecular data (see Ref. [9] for perfect and imperfect *Aspergilli*). Even more irritating is the fact that perfect and imperfect forms belonging to what turned out to be to the same species were given different names. Strictly speaking, *A. nidulans* is the name of the imperfect form of *Emericella nidulans, Aspergillus* being by definition an imperfect genus. However, geneticists since Pontecorvo have tended to ignore this scholastic pedantry and use the name *A. nidulans*. A very simplified phylogeny of some familiar ascomycetes is shown in Figure I.

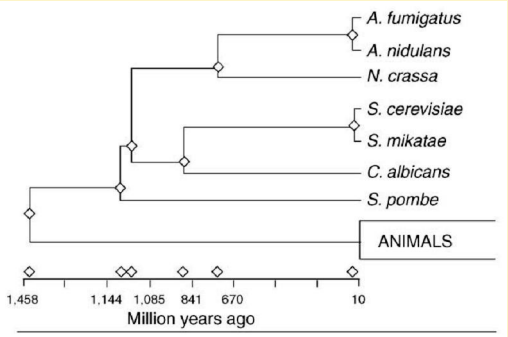


Figure I. Redrawn from Ref. [8].

Box 2. Homothallism and heterothallism, and somatic and germinal diploids in the ascomycetes

Homothallism and heterothallism

In yeasts such as *S. cerevisiae* and *S. pombe*, single haploid cells of opposite mating types fuse to give a zygote that undergoes karyogamy without any previous cell division. The resulting diploid can divide indefinitely; meiosis is induced by environmental signals to give four haploid ascospores contained in each ascus. The diploids of these (evolutionarily distant) yeasts and their relatives can be considered to be both somatic and germinal (see later). In all the sexually reproducing filamentous ascomycetes (such as *N. crassa* and *A. nidulans*), zygotes arise only in specialized structures (fruiting bodies, called perithecia and cleistothecia, respectively in *N. crassa* and *A. nidulans*. In heterothallic species, only nuclei of opposite mating types (see main text) can produce zygotes, whereas in homothallic species, fusion of genetically identical nuclei can produce zygotes. Heterothallism has traditionally been considered the primitive condition and homothallism the derivative that has appeared independently many times (but see text and Ref. [9]).

Somatic and germinal diploids

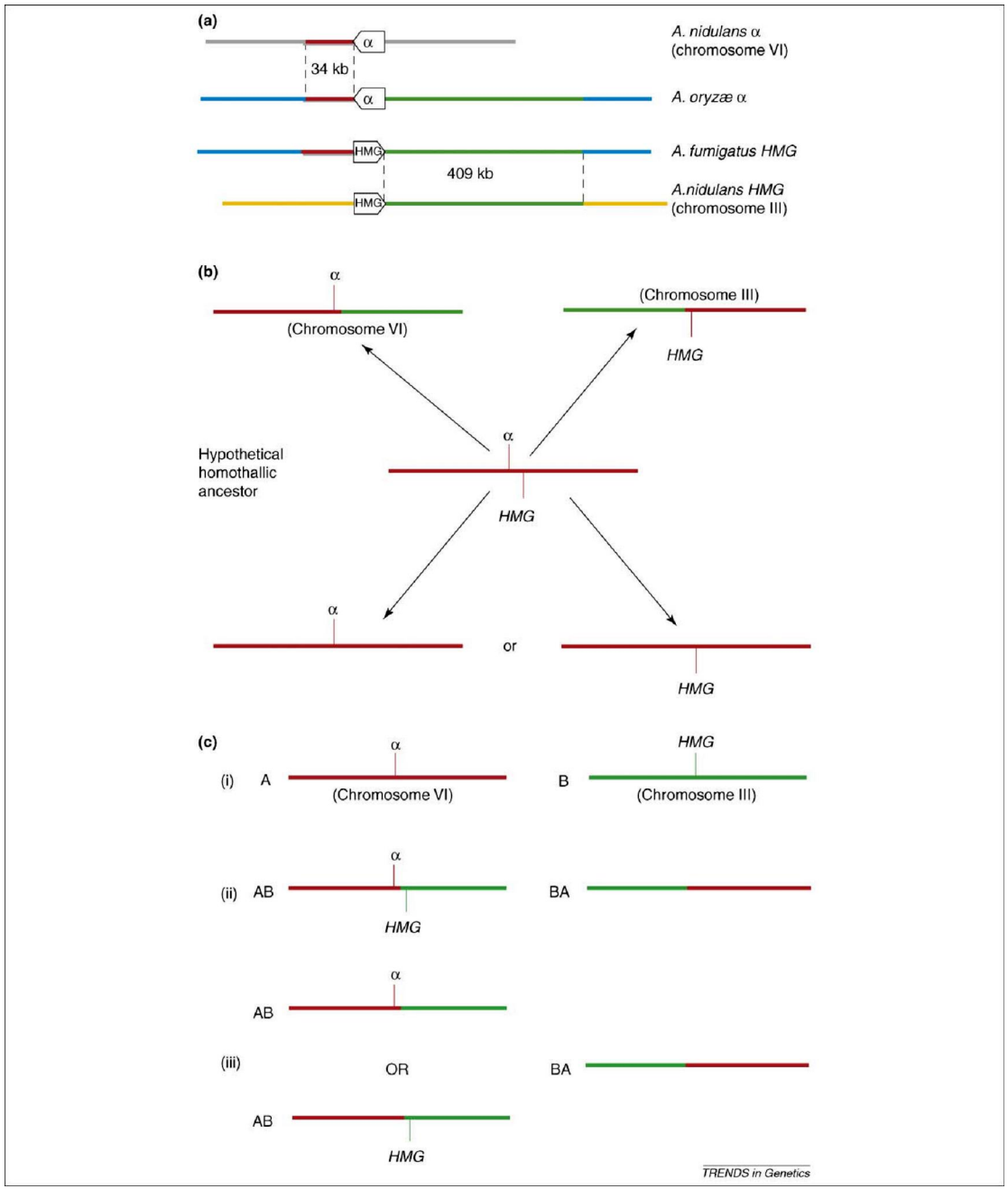
In both heterothallic and homothallic species the paired haploid nuclei divide synchronously as dikaryons. After an undetermined number of synchronous divisions the two nuclei of the dikaryon fuse to form a diploid. The fusion occurs in specialized cells within the fruiting body, the penultimate crozier (hook) cell of the ascogenous hyphae. This diploid is the 'germinal diploid', also called meiocyte [14]. An essential characteristic of these diploid nuclei is that they never undergo a standard mitosis; rather, they are committed to meiosis, which occurs shortly after karyogamy, giving four meiosis products that usually undergo an additional mitosis, resulting in an ascus containing eight ascospores.

In every filamentous ascomycete for which the experiment has been attempted, it is possible to obtain heterokaryons, which contain nuclei originating from two different strains. Mycelia of the same species can fuse, and if the strains carry complementary recessive genetic markers it is very simple to select for heterokaryotic growth. Uniquely among the filamentous ascomycetes, in the heterokaryons (and presumably also in homokaryons) of *Aspergilli* and the *Penicillia*, nuclei can fuse in the vegetative mycelium to give diploid nuclei. These nuclei are not committed to meiosis and can divide indefinitely as diploid nuclei. The availability of these diploids is the basis of the parasexual cycle, where chromosomes segregate as whole units, and enables mapping of markers in relation the centromere by mitotic crossing-over. These stable diploids are 'somatic' not 'germinal' diploids. Although the parasexual cycle has been described by Pontecorvo [1] and in the classical reviews of Etta Käffer [15–17], to my knowledge a conceptual difference between 'somatic' and 'germinal' diploids and the use of these terms in this context has not been proposed before.

aspect of sex in *A. nidulans*. All the others were identified by homology with genes involved in the sexual cycle of other fungi, including yeasts, and their relevance to sex in *A. nidulans*, let alone in other *Aspergilli*, remains to be demonstrated.

The presence of the *HMG*, rather than the α, mating type in *A. fumigatus* is an artefact of the strain chosen for genomic sequencing. Equally frequent and mutually exclusive *α* and *HMG* genes occur in natural isolates of *A. fumigatus* [12]. At least one *α* strain contains a closely linked C terminus of the *HMG* gene, which strongly supports a scenario of primitive homothallism (Figure 1). Population studies are consistent with the hypothesis that a small amount of sex is indulged in by *A. fumigatus* in nature. *HMG* strains of *A. oryzae* have also been reported [12]. These results have an obvious bearing on the interpretation of epidemiological data and on the evolution of sex in fungi. Yet, only the isolation of cross-fertile strains can settle the question of sex in 'asexual' *Aspergilli*. Very recently, complete genomic sequences of *A. niger*, *A. terreus* and *A. flavus* have become available. No sexual cycle has ever been described for these organisms. In the sequenced *A. niger* strain, an *HMG* mating type gene is present but not an *α* gene. Surprisingly, in *A. terreus* and *A. flavus* both mating types are present; moreover, they are unlinked, as in *A. nidulans*. This is a particularly challenging observation to interpret, as *A. oryzae* is supposed to be a domesticated

Figure 1. Schematic representation of the arrangement of the mating type genes *HMG* and *α* in the *Aspergilli* and hypothetical scenarios of the origin of these arrangements. **(a)** In *A. nidulans*, the two mating type genes are on different chromosomes. In the sequenced strains of *A. oryzae* and *A. fumigatus* only *α* or *HMG* are present, respectively, on chromosomes that are syntenic for ~1.7 Mbp flanking the mating type genes. Downstream of the *α* gene on chromosome VI of *A. nidulans* there is a 34 kb region of synteny with the 'sex' chromosomes of *A. oryzae* and also *A. fumigatus*; downstream of the *HMG* gene there is a 409 kb region of synteny between chromosome III of *A. nidulans* and the 'sex' chromosome of *A. fumigatus*. Syntenic regions are shown in the same colour. The regions are drawn schematically and not to



scale. Within the syntenic region of the 'sex' chromosome of *A. oryzae* and *A. fumigatus*, the mating type genes are in opposite orientations. **(b)** A scenario (simplified and redrawn from Ref. [5]) in which a hypothetical homothallic ancestor in which the α and *HMG* genes are closely linked generates (by a reciprocal translocation, top) the situation found in *A. nidulans* and (by loss of one of each of the mating types, bottom) the situation found in the sequenced strains of *A. oryzae* and *A. fumigatus*. **(c)** An alternative scenario, equally compatible with the genomic data [5] in (a). Here, the 'primitive' situation (i) is that found in *A. nidulans* and the hypothetical heterothallic strain (ii) with closely linked mating type loci is an intermediate derived through a reciprocal translocation. A and B are two chromosomes carrying the *HMG* and α mating types (corresponding to chromosomes VI and III in *A. nidulans*, respectively), and AB and BA are products of the reciprocal translocation. (iii) Deletion of alternative mating types in different strains of the same species leads to heterothallism. The upper 'AB' chromosome represents the situation found in the sequenced *A. oryzae* strain, the lower 'AB' chromosome that found in the sequenced *A fumigatus* strain. Data from Ref. [12] show that, at least for *A. fumigatus* and probably for *A. oryzae*, both situations are extant. Note that in scenarios (b,c) the α and *HMG* mating type genes do not occupy exactly the same position, an observation that is consistent with data of Ref. [12].

Box 3. Genomic databases for the *Aspergilli*:

- *A. nidulans* (http://www.broad.mit.edu/annotation/fungi/aspergillus/)
- *A. fumigatus* (http://www.tigr.org/tdb/e2k1/afu1/)
- *A. oryzae* (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao)
- *A. niger* (http://genome.jgi-psf.org/Aspni1/Aspni1.home.html)
- *A. flauvus* (http://www.aspergillusflavus.org/genomics/)
- *A. terreus* (http://www.broad.mit.edu/annotation/genome/aspergillus_terreus/Home.html)

strain of *A. flavus* [6]. The URLs of the genomic databases of the *Aspergilli* are indicated in Box 3.

The development of a classical genetic system for 'asexual' *Aspergilli* would be interesting in its own right, but I doubt that it would have an important role in elucidating, for example, the pathogenic proclivities of *A. fumigatus* [5]. The genetic system of *A. nidulans*, with its hundred of markers, is the fruit of more than fifty years of toil and one of its strengths is the high isogenicity of the laboratory strains. It is unlikely that this background work will be repeated for the other *Aspergilli*. The reverse genetics methodologies newly developed for *A. nidulans* are being extended to other species, and classical genetic analysis of other *Aspergilli*, even if possible, would generally be bypassed altogether.

Last, but not least, genomic data can lead to the solution of other old and extant problems on the nature and mechanism of homothallism. In all sexually reproducing filamentous ascomycetes, diploids are transient. The first division of meiosis follows immediately and inevitably after karyogamy (the fusion of haploid nuclei). Germinal-line diploids (as opposed to somatic diploids, see later and Box 2) of *A. nidulans* follow this pattern of meiosis immediately after karyogamy, the process of dikaryon and diploid formation (see Box 2) leading to meiosis occurs only in specialized structures, the fruiting bodies (proto-cleistothecia). Maternally determined characters and mitochondrial markers show clearly that there is a male and a female nucleus ([13] and references therein) and that generally all the asci in one cleistothecium derive from the same original fertilization event ([1], but see Ref. [14] for exceptions to this rule). We have no idea whether 'maleness' and 'femaleness' are random events or whether they are epigenetically determined.

A consequence of homothallism is that when two nearly isogenic strains of *A. nidulans* differing only a few known markers (lets call the strains A and B) are crossed, three types of cleistothecia are expected. When the dikaryons that give rise to the germinal diploids (see Box 2) are formed by two A or two B nuclei, the cleistothecium is called 'selfed', and progeny is all identical to either parent, whereas when the dikaryon is formed by one A and B nucleus, the cleistothecium is called 'crossed'. Obviously only crossed fruiting bodies are useful for Mendelian genetics. In any one cross, cleistothecia are expected to appear in a ratio of 1A:A to 2A:B to 1B:B. The fact that in many crosses, the A:B class of cleistothecia are far more frequent than expected (and in some cases both the A:A and/or B:B classes are completely absent) has been called 'relative heterothallism' [1,14]. Sometimes A:B cleistothecia are

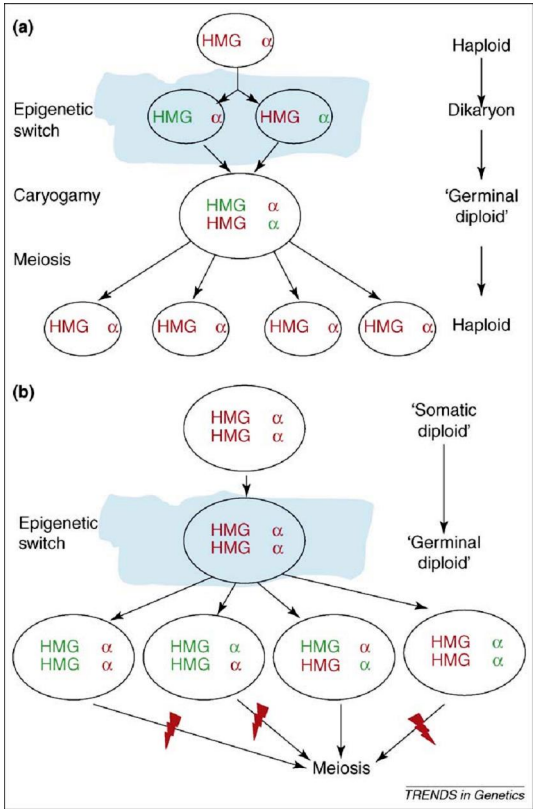


Figure 2. A model of epigenetic heterothallism in the homothallic *A. nidulans*. (a) Two genetically identical nuclei switch to opposite mating types in a fruiting body (indicated by the blue shading). The epigenetic switch occurs at the dikaryon stage, before karyogamy. This mimics the situation seen in heterothallic species, in which dikaryons form only when nuclei are of opposite mating types. This leads to karyogamy and normal meiosis, followed (in *A. nidulans*) by two mitotic divisions leading to eight binucleate ascospores within each ascus. **(b)** When a somatic diploid nucleus, which has already two presumably epigenetically inactive copies of each α and *HMG* genes, finds itself in a proto-cleistothecium, nine different switching patterns are possible (including failure to switch), of which only four are shown for simplicity. Only those nuclei in which both α and *HMG* are epigenetically heterozygous result in a normal meiosis, leading, in agreement with Ref. [18], to rare eight-spored asci containing haploid viable ascospores. Other combinations will lead to sterile or aberrant meiosis, including the 16-spored asci described in Ref. [1]. Red, inactive mating genes; green, active mating genes. Red arrows, blocks in meiosis or ascospore maturation. The aberrations described in Refs [1,18] could well correspond to different abnormal configurations of active and inactive mating genes.

Again, we do not know what determines the strikingly variable proportions of the three classes of cleistothecia.

Uniquely among the filamentous ascomycetes, in the *Aspergilli* and the related *Penicillia* somatic diploids can be obtained in vegetative mycelia (see Box 2). This implies that in these fungi nuclei fuse in the absence of the developmental signals present in fruiting bodies. These diploids are relatively stable and, unlike germinal diploids, can be maintained for an indefinite number of divisions. Such somatic diploids are the basis of the parasexual cycle, one of the tools of *A. nidulans* genetics that is also useful in its non-sexed relatives [15–17].

A final question concerns the epigenetic mark that seals

somatic diploids. Fruiting bodies can form in somatic diploid strains of *A. nidulans*. However, most of the ascospores they contain are sterile, and some asci contain sixteen rather than the standard eight ascospores. This has been interpreted as a tetraploid meiosis in which two diploid nuclei had fused and then undergone meiosis [1], but cytological observations failed to detect nuclear fusions and are incompatible with this neat model [18]. Thus, somatic diploids can 'switch' in the right developmental environment to germinal diploids, which will engage instantly in meiosis. Why this meiosis should be aberrant at all and sometimes lead to 16 ascospores asci remains a mystery.

These old problems can now be examined through new eyes. I propose that *A. nidulans* is epigenetically heterothallic. In this model, within specialized structures and preceding karyogamy, alternative mating type loci would be activated in the pairs of nuclei destined to fuse, leading to diploids that are epigenetically heterozygous at each of the mating loci. The proportion of selfed and crossed cleistothecia would depend on the ability of the different pairs of nuclei to switch to opposite mating types, which in turn might depend on the genetic markers segregating and/or subtle and uncontrolled environmental cues. In somatic diploids both mating types would be in the same state (possibly off). When somatic diploid nuclei find themselves in fruiting bodies, different combinations of mating gene switching would be possible. Only the switching patterns that mimic exactly that of germinal diploids would allow the completion of meiosis (Figure 2b), the others leading to aberrant meioses. The availability of the genome sequences, rapid gene replacement techniques and the fluorescent tagging of proteins involved in karyogamy and meiosis should enable the verification or falsification of this hypothesis.

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Shall I compare thee to a GM potato?

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A fundamental issue in the safety assessment of genetically modified crops is the question of whether unintentional changes have occurred in the crop plant as a consequence of the genetic modification. This question was addressed recently by using a powerful metabolite fingerprinting and metabolite profiling method to assess whether genetically modified potatoes are substantially similar to their corresponding conventional cultivars.

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Introduction

One of the stages in the safety assessment of genetically modified (GM) crop plants is the compositional comparison of the GM line with its corresponding traditionally bred cultivar. This is to identify any unintended changes resulting from the genetic modification (such as insertion of the transgene into another gene, or the production of new metabolites), a process formalized as the Principle of Substantial Equivalence [1,2]. Any changes detected in the GM line are assessed in the context of the range of values for a given variable found within different conventionally bred