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Genome quality control: RIP (repeat-induced point mutation) comes to *Podospora*

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Summary

RIP (repeat-induced point mutation) is a silencing process discovered in *Neurospora crassa* and so far clearly established only in this species as a currently occurring process. RIP acts premeiotically on duplicated sequences, resulting in C-G to T-A mutations, with a striking preference for CpA/TpG dinucleotides. In *Podospora anserina*, an RIP-like event was observed after several rounds of sexual reproduction in a strain with a 40 kb tandem duplication resulting from homologous integration of a cosmid in the mating-type region. The 9 kb sequenced show 106 C-G to T-A transitions, with 80% of the replaced cytosines located in CpA dinucleotides. This led to the alteration of at least six genes, two of which were unidentified. This RIP-like event extended to single-copy genes between the two members of the repeat. The overall data show that the silencing process is strikingly similar to a light form of RIP, unaccompanied by C-methylation. Interestingly, the *N. crassa* zeta–eta sequence, which acts as a potent *de novo* C-methylation RIP signal in this species, is weakly methylated when introduced into *P. anserina.* These results demonstrate that RIP, at least in light forms, can occur beyond *N. crassa*.

Introduction

Transgene-induced gene silencing (TIGS) was discovered in filamentous fungi about a dozen years ago (Selker *et al*., 1987a; Cambareri *et al*., 1989; Goyon and Faugeron, 1989; Rhounim *et al*., 1992). This phenomenon (which covers mechanistically distinct processes) is not limited to fungi: it was also described in plants (reviewed by Vaucheret *et al*., 1998) and, more recently, reported in animals (Pal-Bhadra *et al*., 1997; Garrick *et al*., 1998) and protists (Ruiz *et al*., 1998). In plants, TIGS has been divided into two classes, according to the level at which silencing occurs: transcriptionally (TGS) or post-transcriptionally (PTGS) (Stam *et al*., 1997). The few cases of TIGS so far reported in animals and protists belong to the PTGS class, at least in terms of mechanisms. In filamentous fungi, three modes of TIGS have been described: quelling, RIP (repeat-induced point mutation) and MIP (methylation-induced premeiotically). Quelling, which occurs vegetatively in *Neurospora crassa*, is related to PTGS (Romano and Macino, 1992; Cogoni and Macino, 1999 and references therein). RIP in *N. crassa* and MIP in *Ascobolus immersus* are developmentally linked processes: both act at a very precise stage of the sexual cycle, i.e. between fertilization and meiosis (Selker *et al*., 1987a; Goyon and Faugeron, 1989). However, RIP is distinguishable from other TIGS processes, in which silencing is reversible. RIP is irreversible, leading to mutations: C-G to T-A transitions with a significant bias for 5′-CpA-3′ contexts (Cambareri *et al*., 1989; Grayburn and Selker, 1989). Sequences submitted to RIP are methylated when the mutation level is over 1% (Singer *et al*., 1995a; see *Discussion* for further details and references on RIP).

After the discovery of RIP and MIP, similar premeiotic silencing processes were sought in other filamentous fungi. In *Sordaria macrospora*, which is very closely related to *N. crassa*, tandem duplications created by transformation experiments seem very stable through several serial rounds of sexual reproduction and do not exhibit methylation (Le Chevanton *et al*., 1989). In *Aspergillus nidulans*, tandemly repeated sequences arising from plasmid integration are unstable over the sexual cycle. Sequence losses are caused by either unequal cross-overs or intrachromosomal recombination (Timberlake, 1991 and references therein). Such recombination/excision (pop-out) events have also been reported for large tandem duplications resulting from homologous integration of cosmids in *Podospora anserina.* In this case, it was shown that the events mostly occur premeiotically (Picard *et al*., 1987; Coppin-Raynal *et al*., 1989), as for RIP and MIP. It is noteworthy that recombination/excision processes leading to the loss of one copy of the tandem duplications, along with the vector sequence, also occur premeiotically in *A. immersus*

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(Goyon and Faugeron, 1989) and *N. crassa* (Selker *et al*., 1987a; Cambareri *et al*., 1991). Thus, RIP (MIP) can co-exist with excision processes in the same organism. However, no cases of RIP (MIP) have so far been reported in filamentous ascomycetes other than the two species in which they were discovered, with the exception of a putative RIP-like process mentioned briefly in *P. anserina* (Hamann *et al*., 2000a; see *Discussion*). However, a MIP-like phenomenon has been described in the basidiomycete *Coprinus cinereus* (Freedman and Pukkila, 1993). In *A. immersus* and *C. cinereus*, MIPs occur premeiotically but, in the latter case, the process is less efficient and scarcely results in gene silencing. These data suggest that MIP might be more widespread than is currently assumed. However, its mild forms (causing no phenotypic changes) require identification by systematic studies at the molecular level. This also applies to RIP. Although so far described only in *N. crassa*, RIP (or RIP-like processes) are suspected to play a role in the inactivation of transposable elements, even in species evolutionarily distant from *N. crassa* (for references, see *Discussion*). A RIP-like process has even been proposed to act in the evolution of mammalian genomes (Kricker *et al*., 1992). However, no bona fide RIP event, other than those seen in *N. crassa*, has been observed yet.

In this paper, we describe an event akin to RIP in *P. anserina*, occurring after serial rounds of sexual reproduction in a transformant with a large tandem duplication, which led to 106 C-G to T-A transitions (in 9 kb sequenced) with 80% of the replaced cytosines located in a CpA context. The lack of C-methylation is in agreement with a mild RIP event. The overall data suggest that RIP can be a general silencing process, at least in filamentous fungi.

Results

Historical background: fate of a large tandem duplication

The N9 cosmid (Picard *et al*., 1991) contains, at one end of the genomic insert, the three *mat*– genes, *FMR1*, *SMR1* and *SMR2* (Debuchy and Coppin, 1992; Debuchy *et al*., 1993) and, at the other end, the *su12* gene encoding a protein of the cytosolic ribosomes (Silar *et al*., 1997). The vector carries the *su8-1* gene acting as a UGA tRNA suppressor (Debuchy and Brygoo, 1985). Transformation of a *mat*+ *leu1-1* recipient strain with this cosmid leads to (leu$^+$) transformants, owing to suppression of the UGA *leu1-1* mutation. Crossing the transformants against a *193* strain (Picard, 1971) aids in tracking segregation of the vector marker; *193 su8-1* ascospores are green, whereas *193 su8*$^+$ ascospores are white (wild-type ascospores are black). Transgenic strains expressing both *mat*+ and *mat*– functions were recovered (Picard *et al*., 1991). Those carrying the cosmidic information integrated through homologous recombination have a tandem duplication (Fig. 1) and are highly unstable during sexual reproduction: when crossed to a *mat*– strain, a large fraction of their progeny no longer express the *mat*– and *su8-1* information (Fig. 2, Picard *et al*., 1991). Molecular analyses showed that the relevant strains have lost one copy of the duplicated sequence, along with the vector, through an intramolecular recombination event (pop-out). These events mostly occur between fertilization and premeiotic replication (Picard *et al*., 1987), and the number of asci issued from a pop-out event increases strikingly with the age of the fruiting body (Rossignol and Picard, 1991).

A puzzling phenotype

During the systematic analysis of one of these transformants (called N9B2; Picard *et al*., 1991), we found an ascospore (called 1e, Fig. 2) in the late progeny of the fourth serial cross with an unexpected phenotype: it no longer expressed the *mat*– information although still expressed the *su8-1* marker and displayed a male sterile phenotype. Southern analyses demonstrated that the tandem duplication was maintained and that the *mat*– sequence was still physically present (data not shown). Crosses of this strain revealed that its peculiar phenotype could be transmitted over meiosis (Fig. 2). In addition, pop-out events (ascertained by hybridization experiments; data not shown) led to offspring that have lost one copy of the duplicated sequence, along with the *su8-1* marker. These strains still displayed the male sterile phenotype of the parental strain but also exhibited an unexpected property: female sterility. Analyses of two such strains (4d and 1e-ex, Fig. 2) showed that one (4d) grew very poorly. Initially, we were unable to explain the male and female sterility of these strains but thought that the poor growth of 4d could result from an alteration in the *su12* gene carried by the N9 cosmid and thus originally present in the duplication. This idea was confirmed in the following way: normal growth of 4d was restored by transformation with a 3 kb DNA fragment encompassing the wild-type *su12* gene (Silar *et al*., 1997). Similarly, complementation assays with N9 subclones revealed that the male and female sterile phenotypes resulted from an alteration in two genes so far unidentified. One of them, *ami1*, is the functional orthologue of the *A. nidulans apsA* gene: male sterility is explained by the fact that, in the mutant context, microconidia (which act as male gametes in *P. anserina*) are anucleate (Graïa *et al*., 2000). The gene responsible for female sterility (called *mim1*) will be described elsewhere (F. Graïa *et al*., in preparation).

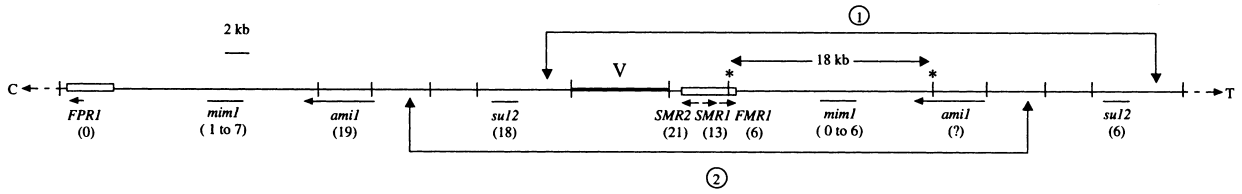

Fig. 1. Schematic map of the duplicated sequence resulting from integration of the N9 cosmid. The length of the genomic insert carried by the cosmid is 43 kb. The open rectangles represent the mating type-specific sequences with the *FPR1* (*mat*+) and *FMR1*, *SMR1*, *SMR2* (*mat*–) genes. The thick line corresponds to the pHSU8 vector sequence (Debuchy *et al*., 1988) that contains the *su8-1* gene as the selective marker (V). Positions and sizes of the genes are roughly indicated along with their transcription orientation (when available). Vertical bars indicate the positions of the *Eco*RI sites. The 18 kb fragment used as a probe in some hybridization experiments is bordered by stars. T and C mean telomere and centromere, respectively. Arrows numbered 1 indicate a possible recombination/excision event leading to the 4d strain, which differs from the 1e-ex strain (Fig. 2) by the status of the *su12* gene. Correspondingly, arrows numbered 2 indicate a possible pop-out event leading to the 1e-ex strain. Numbers in brackets below the genes give the number of C-G to T-A mutations for ≈1000 bp sequenced. The ambiguity with *mim1* results from its sequencing from the 3a strain, which contains the two copies of the gene (Fig. 2). In any case, the *mim1* allele borne by the excised strains (4d and 1e-ex, Fig. 2) contains at least one stringent mutation, as the two strains display a female sterile phenotype. The *ami1* copy lost after the pop-out events should have at least one stringent mutation, as the 3a strain, which contains the two copies, exhibits a male sterile phenotype (Fig. 2).

The altered sequences do not exhibit methylation but contain C-G to T-A mutations

To determine whether the duplication sequence was the target of a MIP-like process, Southern blot analyses were performed with DNA from the 3a strain, which still contained the duplication, from the 4d (excised) strain and from a wild-type *mat*+ strain after digestion with several pairs of restriction enzymes currently used to reveal C-methylated sites (see *Experimental procedures*). Thus, at least 49 restriction sites were tested: the patterns were the same in the two relevant strains and their control (data not shown), suggesting that no methylation occurred.

Sequencing was then performed. The three *mat*– genes (*FMR1*, *SMR1* and *SMR2*) and the *mim1* gene were sequenced from the 3a strain, which still carried the duplication (with respect to the *mim1* gene, the sequence is therefore derived from a mixture of the two alleles). The *su12* gene and the 5′ portion of the *ami1* gene were sequenced from the two excised strains, 4d and 1e-ex (Fig. 2). The data are summarized in Tables 1 and 2 and Fig. 1. The 9 kb sequence showed 106 point mutations, all C-G to T-A transitions. Furthermore, there is a striking bias concerning the nucleotide context of the altered C-G pairs: >80% of the cytosines replaced are located 5′ to adenines, and nearly 50% are located 3′ of thymines (Table 1). In the coding sequences, the types of mutant codons agree well with the degree of phenotypic alterations. The *ami1* mutant contains nonsense mutations, as do the three *mat*– genes. The *su12* allele, carried by the strain that grows normally (1e-ex), contains silent mutations, whereas the allele of the slow-growing strain (4d) shows missense mutations (Table 2). These data also clarify the phenotypic properties of the original 1e strain (Fig. 2), which still carried the duplication but no longer expressed the *mat*– mating ability. The nonsense mutation in *FMR1* leads to a truncated protein without the domain required for *mat*– fertilization functions (Debuchy and Coppin, 1992). The 1e strain grew normally because one copy of the *su12* gene was functional. One can also

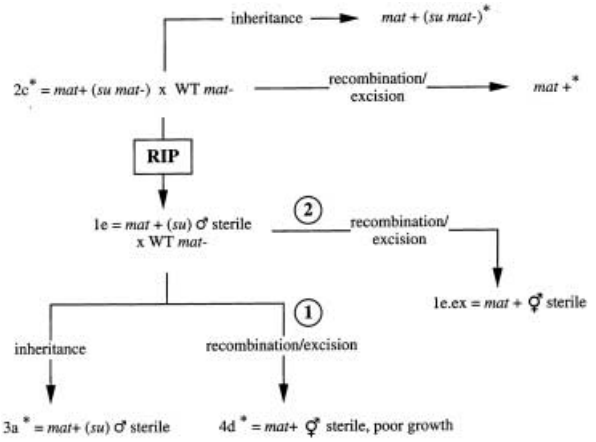

Fig. 2. Pedigree and phenotypic features of strains used. The 2c strain is a secondary (purified) transformant with a tandem duplication arising from the integration of a cosmid by homologous recombination in the mating type region (see Fig. 1). It arises from the N9B2 primary transformant (Picard *et al*., 1991) after three serial crosses to *mat*– reference strains. It expresses the resident *mat*+ and the transgenic *mat*– information along with the *su8-1* (indicated here as *su*) marker carried by the vector. Crosses of 2c with a *mat*– strain give rise to a mixed progeny: some ascospores exhibit the parental phenotype (inheritance), whereas others no longer express the transgenic information. In this case, the strains were shown to have lost one copy of the duplicated sequence along with the vector through a recombination/excision event (pop-out). One ascospore (1e) displayed an unexpected phenotype: it no longer expressed the *mat*– information (although retaining the *su8-1* marker) and showed a male sterile phenotype. The different types of progeny issued from 1e are indicated. Transgenic information is in brackets. The strains submitted to Southern tests are marked with a star. Numbers 1 and 2 refer to two different pop-out events (see Fig. 1). Sequencing was performed on strains 3a, 1e-ex and 4d.

Table 1. Site specificity of C-G to T-A mutations.

X	XpCpA	XpCpG	XpCpT	XpCpC	Total
A	15	1	1	0	17 (16)
G	15	0	1	3	19 (18)
T	40	4	4	1	49 (46)
C	18	1	1	1	21 (20)
Total	88	6	7	5	106
	(83)	(5.7)	(6.6)	(4.7)	

The sequence context of the C-G to T-A mutations was examined on the strand showing the C to T change. X represents the nucleotide located 5′ to the C. Values in brackets give the percentage of each dinucleotide with respect to the total number of mutations.

infer, from the male sterile and female fertile phenotype of this strain, that the two copies of *ami1 and* a sole copy of *mim1* were non-functional. The fact that the two excised strains bear the same *ami1* allele (see legend to Table 2) suggests that both carry the same non-functional *mim1* allele (Fig. 1), which fits with their common female sterile phenotype.

Methylation and RIP: the zeta–eta test

The fact that the mutated region was not methylated could be explained in two non-exclusive ways. First, the RIP event described above is sparse, and the mutation level is in the range of 1%: in *N. crassa*, RIP is accompanied by methylation only when the level of mutations is over 1% (Singer *et al*., 1995a). Secondly, *P. anserina* might be insensitive to the RIP signal and thus unable to methylate the relevant region. We therefore used the *N. crassa* zeta–eta (ζ–η) sequence (Selker and Stevens, 1985), which is an efficient target for *de novo* methylation not only in *N. crassa* (Selker *et al*., 1987b) but also in *A. immersus*, when introduced into this foreign context (Malagnac *et al*., 1999). The ζ–η sequence was introduced into *P. anserina* by transformation. DNA from five primary and four secondary transformants (issued from two primary transformants through one sexual cycle) were tested by Southern analysis after digestion with either *Mbo*I or *Sau*3A. Both enzymes recognize the sequence GATC, but *Sau*3A does not cleave if the cytosine is methylated. The restriction pattern, observed in all cases, is shown in Fig. 3. The *Mbo*I digests reveal the two bands expected from complete digestion of the ζ–η region, i.e. a–b (535 bp) and c–d (702 bp). Interestingly, the most prominent *Sau*3A bands are those observed after digestion with *Mbo*I, demonstrating that the b, c and d sites are not completely blocked. Furthermore, lack of the a–c fragment (627 bp) suggests that the b site is very rarely (if ever) methylated. The band at 794 bp may correspond to fragments b–d and/or c–e, revealing that the c or d sites are blocked in a fraction of the nuclei. The highest band probably represents the b–x fragment, showing that, in a few nuclei, the c, d and e sites are methylated. To ensure that this pattern was not the result of partial digestion, the membrane was probed with the *AS1* gene (Dequard-Chablat and Sellem, 1994), which contains eight *Mbo*I–*Sau*3A sites: the same bands were observed after digestion with both enzymes. Thus, *P. anserina* is sensitive (albeit very weakly) to the ζ–η signal. Furthermore, the pattern is very reproducible and does not depend on the integration site of the sequence: it was observed in all transformants analysed.

Discussion

The RIP paradigm

RIP was discovered in *N. crassa* (Selker *et al*., 1987a; Cambareri *et al*., 1989), in which it has been submitted to extensive analyses. The main features of this process are as follows. First, RIP acts premeiotically (Selker *et al*., 1987a; Selker, 1990), i.e. between fertilization and

Table 2. Types of mutations observed in the genes altered by RIP.

				Coding regions			
Strains	Genes	Length (bp)	Non-coding regions	Silent	Missense	Nonsense	Total
3a	*SMR2*	1176	2	10	7	2	21 (0)
3a	*SMR1*	1054	6 (4)	3	3	1	13 (4)
3a	*FMR1*	1450	3	2	2 (1)	1	8 (1)
3a	*mim1*	1000 × 2	1 (1)	3 (3)	3 (3)	0	7 (7)
4d	*ami1*	1725	19	9	2	3	33 (0)
4d	*su12* (1)	1078	12 (9)	2 (2)	4 (4)	0	18 (15)
1e.ex	*su12* (2)	1078	2	4	0	0	6 (0)

The origin and phenotype of each strain are given in Fig. 2. Note that strain 3a still contains the duplication: sequencing of the *mim1* gene was thus performed on a mixture of the two copies. *su12* (1) and *su12* (2) correspond, respectively, to the allele present after excision events (1) and (2) as shown in Fig. 1. Sequencing of the *ami1* allele borne by the 1e-ex strain was also done on 600 bp of the coding region and showed the same mutations as those observed in the 4d strain. Sequencing of the *mat*+ gene (*FPR1*), which lies on the centromeric border of the duplication (Fig. 1), did not reveal any mutation over 1367 bp sequenced. Numbers in brackets give the numbers of C-to-T mutations observed on the transcribed strand.

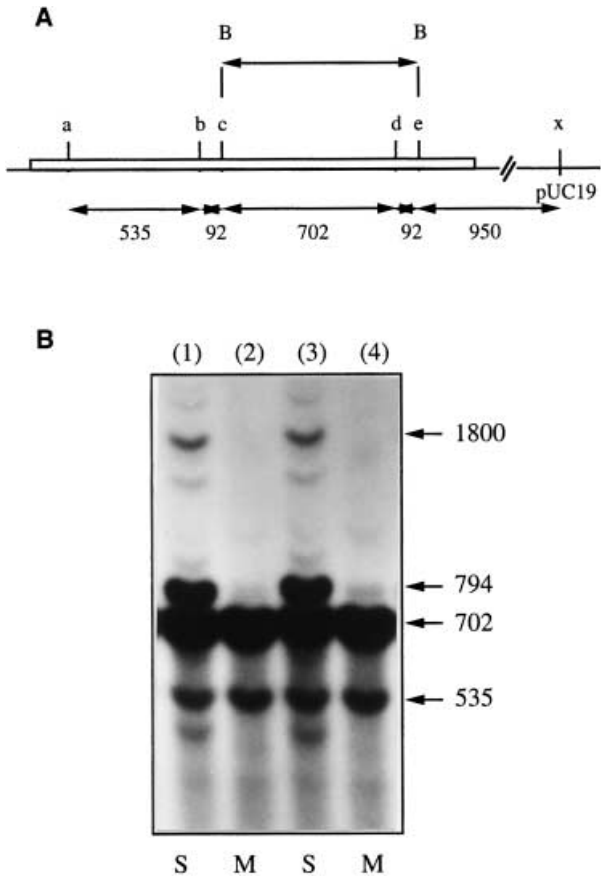

Fig. 3. C-methylation of the ζ–η sequence integrated into the *P. anserina* genome.
A. Restriction map of the ζ–η sequence (open rectangle) with respect to the *Mbo*I–*Sau*3A (a to e) and *Bam*HI (B) sites. The x site is the nearest *Mbo*I–*Sau*3A site downstream of the ζ–η sequence in pUC19. The *Bam*HI fragment used as a probe is shown over the map. Note that, owing to duplication inside the ζ–η sequence, this probe is able to hybridize the a–b fragment.
B. Southern blot of one purified transformant. DNAs from a *mat*+ (lanes 1 and 2) and a *mat*– (lanes 3 and 4) strain were digested by either *Sau*3A (S) or *Mbo*I (M). The sizes of the fragments are given in basepairs.

meiosis, probably during the sexual dikaryotic stage. At this stage, the two parental nuclei divide synchronously in a common cytoplasm before undergoing karyogamy (nuclear fusion) in specialized cells, the ascus mother cells. These cells are unable to divide: their nuclei enter meiosis, and the cells differentiate into asci, which contain the sexual progeny, the ascospores (for reviews, see Raju, 1992; Coppin *et al*., 1997). Secondly, RIP is characterized by mutations that are exclusively C-G to T-A transitions with a striking preference for CpA:TpG contexts (Cambareri *et al*., 1989; Grayburn and Selker, 1989). Thirdly, RIP is triggered by repeats that can occur spontaneously by chromosomal rearrangements (Perkins *et al*., 1997) or by transformation experiments (Selker *et al*., 1987a; Selker and Garrett, 1988), if the repeat length is over 400 bp (Watters *et al*., 1999). Fourthly, although it is less efficient on unlinked than on linked duplications, RIP systematically affects both members of a duplication (Selker *et al*., 1987a; Selker and Garrett, 1988; Fincham *et al*., 1989). Fifthly, when the mutation level is $>1\%$ (Singer *et al*., 1995a), the sequences altered by RIP are methylated in almost all cytosine residues (Selker *et al*., 1993). Sixthly, RIP can extend beyond the duplicated sequences to unique sequences at least up to 4 kb (Irelan *et al*., 1994). Lastly, frequencies of RIP increase with the age of the fruiting bodies (Singer *et al*., 1995b).

RIP-like processes as possible transposon-inactivating mechanisms in fungi

It has been shown that RIP acts not only on *N. crassa* sequences but also on foreign sequences, once they are duplicated in the fungal genome (Foss *et al*., 1991). Thus, RIP seems to be a potent mechanism acting against its own duplications, which could affect viability through either gene imbalance or chromosome rearrangements caused by recombination between repeats. But it can also detect and inactivate foreign duplications caused, for instance, by transposons and thus block their propagation. Transposons that have probably been inactivated by RIP have been reported in *N. crassa* (Schechtman, 1990; Kinsey *et al*., 1994; Cambareri *et al*., 1998; Margolin *et al*., 1998). In one case, the data have been extended to several *Neurospora* species including homothallics, suggesting that RIP is (was) not limited to *N. crassa* (Kinsey *et al*., 1994). Other reports have extended this idea to fungal species evolutionarily very distant from the *Neurospora* genus, such as *Aspergillus fumigatus* (Neuveglise *et al*., 1996), *Fusarium oxysporum* (Hua-Van *et al*., 1998), *Pyricularia grisea* (Nakayashiki *et al*., 1999) and *P. anserina* (Hamann *et al*., 2000a, b). In each case, the inactivated transposons show numerous C-G to T-A mutations. The contexts in which the replaced cytosines are located do not differ clearly from those characteristic of the canonical RIP. Although the transitions occurred at Cp(A/G) dinucleotides in *F. oxysporum* and at Cp(A/T) dinucleotides in *P. grisea* and *P. anserina*, CpA represents half the relevant dinucleotides in the three cases (Hua-Van *et al*., 1998; Nakayashiki *et al*., 1999; Hamann *et al*., 2000a). With respect to the *A. fumigatus* transposon, the authors (Neuveglise *et al*., 1996) claimed that mutations occurred preferentially in the CpG context (owing to the deficit of CpG in the sequence). However, inspection of the two studied copies of the transposon reveals that they differ by C to T transitions mostly located in the CpA context: among 53 single (non-adjacent) mutations (nucleotide changes that are directly adjacent cannot be classified), we counted 39 transitions at CpA dinucleotides. *A. fumigatus and F. oxysporum* have no

known sexual stage, whereas the sexual stage of *P. grisea* has been reported only under laboratory conditions. As suggested by the authors, the observed mutations might be relics of an RIP-like process that occurred at a time when these fungi possessed a functional sexual cycle (Neuveglise *et al*., 1996; Hua-Van *et al*., 1998; Nakayashiki *et al*., 1999).

RIP in P. anserina*: sparse but conspicuous*

The event described in this study exhibits the main properties characteristic of a canonical sparse RIP. It was observed in a tandem duplication after sexual reproduction in one of the last ascospores produced by the cross. All mutations are C-G to T-A transitions with a strong preference for the CpA dinucleotide. The sequence concerned shows 1% mutations and does not exhibit C-methylation, although *P. anserina* is able (albeit weakly) to methylate *de novo* a *N. crassa* sequence acting as a portable signal for this type of methylation. The mutational process extended beyond the repeat into single-copy genes, which stand between the two members of the repeat (i.e. the *mat*– genes), but the *mat*+ gene, which lies outside, was unaffected. In fact, the slight differences between the *P. anserina and N. crassa* processes reside in the nucleotide contexts of the replaced cytosines outside the CpA dinucleotide, which is highly conserved between the two species. In *N. crassa*, the other dinucleotides submitted to RIP exhibit a clear-cut order (CpT > CpG > CpC) (Selker, 1990), whereas in *P. anserina*, there are no preferences between these three contexts. Otherwise, mutations occur more frequently on cytosines located downstream of adenines in *N. crassa* (Grayburn and Selker, 1989) and downstream of thymines in *P. anserina.* It is important to emphasize that the preferential context for RIP cannot be explained by a bias in dinucleotide frequencies in either *N. crassa* (Cambareri *et al*., 1989; Watters *et al*., 1999) or *P. anserina* (data not shown).

As shown in Table 2, some genes show only C-to-T [*SMR2*, *ami1*, *su12*(2)] or G-to-A (*mim1*) transitions on a given strand, whereas others exhibit both patterns on the same strand. In *N. crassa*, sequences mutated by RIP frequently show only C-to-T or G-to-A changes (not both) on a given strand (Selker *et al*., 1993; Singer *et al*., 1995a; Watters and Stadler, 1995). In other cases, RIP sequences display a mix of C-to-T and G-to-A changes on the same strand (Cambareri *et al*., 1989; Grayburn and Selker, 1989). As discussed previously, several models may account for these situations (Watters and Stadler, 1995; Watters *et al*., 1999). A single round of RIP acting on a single strand and followed by heteroduplex resolution, by either repair or replication, leads to a meiotic product with a single pattern (C-to-T or G-to-A on a given strand). The result is the same if RIP acts on both strands during one cell cycle with resolution of the heteroduplex through replication. The dual pattern can also be explained in two ways. If there is a single round of RIP, the process should act on both strands, the heteroduplex being resolved by repair. But multiple rounds of RIP occurring over the few divisions between fertilization and meiosis could also be responsible. In addition, in the case of mild RIP, these multiple rounds could have occurred in serial crosses, with the upstream RIP events yielding no phenotypic changes. Unfortunately, this hypothesis can no longer be tested in the case reported here because the strains standing upstream in the relevant pedigree have been lost.

Sparse versus dense RIP: mechanistic aspects

The data reported in this paper clearly demonstrate that RIP is a process transcending *N. crassa*. However, in *P. anserina* and perhaps in other fungal species, it occurs in a light form. Thus, it could be detected only through a systematic sequencing of duplicated sequences in transgenic strains or through systematic phenotypic analyses, when the phenotypic screen detects a very low level of mutants. This was the case in the situation reported here: after four serial crosses, one ascospore (among 85 issued from different asci of the last cross) gave a strain with an unexpected phenotype. Furthermore, the first phenotypic alteration detected in this strain resulted from mutations in a single-copy gene (*FMR1*), whereas the second was caused by mutations in a gene (*ami1*) whose size (6kb) might have aided its inactivation on the two copies.

In any case, the reasons why RIP seems so inefficient in species other than *N. crassa* remain unclear. Assuming that the results reported by Hamann *et al*. (2000a) are extended to other cases, they rule out the hypothesis that RIP would be triggered only by large tandem duplications, at least in *P. anserina*: these authors analysed the sexual progeny of a strain in which a resident gene was duplicated by transformation, and they observed three C-to-T transitions in the ectopic copy. Another possibility is that RIP efficiency is lowered by repair mechanisms that faithfully resolve the heteroduplex generated by RIP (towards the wild-type sequence) before meiosis. In this regard, it would be interesting to look for RIP in mutant contexts more error-prone than the wild type. However, a simpler explanation resides in the low efficiencies of the enzymes (DNA-cytosine deaminase, DNA-(5-methylcytosine) deaminase, DNA-(5-cytosine) methyltransferase), which have been assumed to play a role in the RIP C-to-T transitions (Selker, 1990; Rossignol and Faugeron, 1994; Mautino and Rosa, 1998). Cloning of the relevant genes, coupled with their inactivation and overexpression in different systems, would help to understand how RIP has evolved in the fungal world.

In *N. crassa*, several studies (e.g. Selker *et al*., 1987a; Irelan *et al*., 1994; Watters *et al*., 1999) have emphasized a correlation between RIP and recombination, processes requiring pairing of homologous sequences. However, the data from Butler and Metzenberg (1990) suggest strict alternatives between premeiotic recombination and RIP for particular sequences: these authors show that rDNA repeats are prone to recombination/excision events (through sister chromatid exchanges) at the premeiotic stage but are not subject to RIP. The low level of RIP in *P. anserina* is not easily explained by a defect in the pairing process *per se*. In fact, recombination/excision (pop-out) events are highly efficient in this fungus when long tandem duplications are involved. These events mostly take place before premeiotic replication, and their frequencies increase with the age of the fruiting bodies (Picard *et al*., 1987; Coppin-Raynal *et al*., 1989; Rossignol and Picard, 1991), two parameters also characteristic of RIP in *N. crassa* (Selker *et al*., 1987a; Selker, 1990; Singer *et al*., 1995b). Thus, *P. anserina* performs pairing efficiently, followed by recombination, at the precise time when pairing followed by RIP occurs. An attractive model would be a competition between the two machines required for recombination and RIP with a strong advantage for the first in *P. anserina.* Once engaged in this mechanism, the relevant sequences would be immune to the second process even if the first aborts. This hypothesis could be tested by searching for RIP in mutant contexts that decrease the efficiency of premeiotic recombination between tandem repeats. In this regard, it is worth noting that the *rec-2* locus, which influences frequencies of meiotic recombination in *N. crassa*, also influences pop-out events but has no effect on RIP (Bowring and Catcheside, 1993).

Genome quality control and RIP

The role of RIP as a genome defence mechanism has been discussed extensively (Selker, 1990; Kricker *et al*., 1992; Rossignol and Faugeron, 1994). The strength of RIP (and RIP-like processes) in combating transposons is clearly established (see References above). However, RIP also modifies the host genes when duplicated. It has thus intrinsically beneficial and detrimental facets. Mutations resulting from RIP suppress recombination between the affected sequences (Selker, 1990; Cambareri *et al*., 1991), thus preventing chromosome rearrangements by ectopic recombination. But these mutations also prevent expression of the repeated sequences, thus precluding genomic evolution through gene duplication. As noted by Margolin *et al*. (1998), this detrimental facet of RIP might be the price to pay in fitness for an efficient genomic defence. This might explain why RIP may have disappeared from other fungal species. However, RIP could have been maintained in light forms, probably as a less efficient destroyer of transposons, but as a source of genetic diversity. Even in *N. crassa*, sparse RIP events have been observed (Fincham, 1990; Barbato *et al*., 1996). The discovery of a conspicuous sparse RIP event in *P. anserina* opens new insights in the investigation of the evolution of this type of genome quality control in fungi and in other species. In fact, an RIP-like process might exist in plants (quoted by Kumpatla *et al*., 1997) and may have played a key role in the evolution, organization and stability of the mammalian genomes (Kricker *et al*., 1992).

Experimental procedures

P. anserina *strains and media*

P. anserina is a filamentous ascomycete whose life cycle and general methods of genetic analysis have been described previously (Rizet and Engelmann, 1949; Esser, 1974). All strains are derived from the wild-type S strain. The origin and phenotypic properties of the strains used in this study are shown in Fig. 2. Tests currently used for male and female sterility have been detailed in Graïa *et al*. (2000). Transformation experiments were performed as described previously (Graïa *et al*., 2000). The culture and germination media have been reviewed recently (Berteaux-Lecellier *et al*., 1995).

Plasmids

The *N. crassa* zeta–eta (ζ–η) sequence (Selker and Stevens, 1985) is borne by the pVM57 plasmid, along with the bacterial *hph* gene (resistance to hygromycin) under the control of the *A. nidulans trp*C promoter (V. Miao and E. U. Selker, unpublished).

DNA procedures

Total DNA was extracted as described previously (Coppin-Raynal *et al*., 1989; Dequard-Chablat and Sellem, 1994; Lecellier and Silar, 1994). In genomic analyses, DNA was digested by *Eco*RI and probed with the entire N9 cosmid (Picard *et al*., 1991), the *Eco*RI 18 kb fragment (Fig. 1) or a *mat*– specific sequence. For analyses of the C-methylation pattern of genomic sequences, DNA was digested by the following pairs of restriction enzymes (of which the first member is insensitive and the second sensitive to the C-methylation status of their respective targets): *Mbo*I–*Sau*3A, *Bst*NI–*Eco*RII and by a pair of enzymes that are both sensitive to methylation, *Msp*I–*Hpa*II. The *Eco*RI 18 kb fragment was used as a probe in this set of experiments. For the investigation of methylation in the transgenic ζ–η fragment, DNA was digested by the *Mbo*I–*Sau*3A pair, and the probe was the *Bam*HI 0.8 kb fragment, containing one copy of the tandem duplication of the ζ–η sequence (Selker and Stevens, 1985). These probes were prepared with a random primer labelling kit (Roche Diagnostic). Standard procedures for Southern blotting on N+ membrane (Amersham) were used. Hybridizations were performed as described previously (Church and Gilbert, 1984).

Sequencing

Genes were obtained through amplification with polymerase chain reaction (PCR) on genomic DNA from the relevant strains (Fig. 2). Specific oligonucleotides were used to amplify each gene, and the amplified fragments were prepared for direct sequencing as described previously (Rosenthal *et al*., 1993). Sequencing was performed with an automatic sequencing machine (373A DNA sequencer; Applied Systems) using the Dye Terminator cycle sequencing kit (ABI-Prism; Perkin-Elmer).

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