Evidence for dominant suppression of repeat-induced point mutation (RIP) in crosses with the wild-isolated *Neurospora crassa* strains Sugartown and Adiopodoume-7

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Abstract

A convenient assay to score repeat-induced point mutation (RIP) in *Neurospora* employs the *erg-3* locus as a mutagenesis target. Using this assay we screened 132 wild-isolated *Neurospora crassa* strains for ability to dominantly suppress RIP. RIP was exceptionally inefficient in crosses with the wild isolates Sugartown (P0854) and Adiopodoume-7 (P4305), thereby suggesting the presence of dominant RIP suppressors in these strains. In other experiments, we found no evidence for dominant RIP suppression by the *Spore killer* haplotypes *Sk-2* and *Sk-3*.

[Noubissi F. K., Aparna K., McCluskey K. and Kasbekar D. P. 2001 Evidence for dominant suppression of repeat-induced point mutation (RIP) in crosses with the wild-isolated *Neurospora crassa* strains Sugartown and Adiopodoume-7. *J. Genet.* **80**, 55–61]

Introduction

Repeat-induced point mutation (RIP) is a mutational process that was discovered in *Neurospora*, and more recently found also in the related genus *Podospora* (Graia *et al.* 2001). RIP occurs during the sexual cycle in the dikaryotic stage between fertilization and karyogamy and targets duplicated DNA sequences for multiple G : C to A : T mutations and methylation of cytosine residues (for reviews see Selker 1990; Irelan and Selker 1996). Karyogamy produces a diploid zygote nucleus, in a cell called the ascus, which immediately undergoes meiosis and a postmeiotic mitosis whereby the RIP-induced mutations are segregated into progeny ascospores. In *N. crassa*, RIP occurs in > 90% asci bearing a linked duplication and in 5–90% asci with an unlinked duplication. We have developed a convenient assay to score RIP in crosses with strains bearing a tagged duplication of a fragment of the distal linkage group (LG) III locus *erg-3* (Prakash *et al.* 1999; Noubissi *et al.* 2000; Bhat and Kasbekar 2001). Presence of this duplication in crosses can generate RIP-induced *erg-3* mutant progeny. Ascospores mutant for *erg-3* produce colonies with a characteristic morphology on Vogel's 'sorbose' agar medium which allows them to be easily distinguished from sibling wild-type colonies (see Noubissi *et al.* 2000 for a picture). Thus by counting the number of mutant and wild-type colonies one can estimate RIP efficiency in the parental cross. Additionally, *erg-3* mutants have altered sensitivities to isoflavonoids and to the steroidal glycoside *a*-tomatine (Sengupta *et al.* 1995), which makes it possible to subsequently verify the genotype assignment made on the basis of the ascospore phenotype.

Previously this assay was used to screen 71 wild-isolated *N. crassa matA* strains and it revealed that RIP was unusually inefficient in crosses with a strain from Adiopodoume, Ivory Coast (Noubissi *et al.* 2000).

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Keywords. *Spore killer*; meiotic drive; *Neurospora crassa*; RIP.

Interestingly, this strain was reported by Kinsey (1989) to be the only *Neurospora* strain that contains active copies of the transposable element *Tad*. All the other (> 400) *Neurospora* strains that had been examined by Kinsey (1989) contained only RIP-inactivated relics of *Tad*. These findings provided experimental support to the idea that RIP may protect the genome against the proliferation of transposons and other repeated DNA elements. The convenience of the RIP assay and the success of our first screen prompted us to continue searching for novel dominant RIP suppressors among more recent accessions of wild strains with the Fungal Genetics Stock Center (FGSC) (Turner *et al*. 2001). We report here the screening of a second tranche of 132 *N. crassa matA* wild strains which resulted in the identification of two more dominant RIP suppressor strains, Sugartown (P0854) and Adiopodoume-7 (P4305).

We report also our examination of *Spore killer* meiotic drive elements for dominant suppression of RIP. *Spore killer-2* (*Sk-2*, also referred to as $Sk\text{-}2^K$) and *Spore killer-3* (*Sk-3*, also referred to as $Sk\text{-}3^K$) are two gene complexes (haplotypes) that were identified in a subset of strains of the sibling species *N. intermedia* that were isolated from Borneo, Java and Papua New Guinea based on the observation that in heterozygous $Sk^K \times Sk^S$ crosses, progeny ascospores that did not inherit the complex are killed (reviews: Raju 1994, 1996). No killing occurs in homozygous *Sk-2* × *Sk-2* and *Sk-3* × *Sk-3* crosses, and all ascospores are killed in *Sk-2* × *Sk-3* intercrosses. Although no killers have yet been found in *N. crassa*, the *N. intermedia Sk* factors are fully functional when introgressed into *N. crassa*. Resistant strains that neither kill nor are killed have been identified in both *N. intermedia* and *N. crassa* but most wild-isolated strains of both species are sensitive (i.e. Sk^S) (Turner 2001). *Sk-1* was identified in *N. sitophila*, but it could not be introgressed into *N. crassa*. *Sk-2* and *Sk-3* map to a 30-unit interval that spans the centromere of LG III. Recombination is blocked in this interval, presumably because of numerous small rearrangements. It was conceivable that repeated sequences might be involved in killing. Alternatively, transposable elements might have simply hitchhiked onto these meiotic drive elements. We reasoned that if repeated sequences were involved in killing, then the Sk^K haplotypes might also include dominant suppressors of RIP.

Materials and methods

***Strains*:** The 132 *N. crassa matA* wild-isolated strains screened for dominant RIP suppressors are designated by 'P' numbers (see table 2) and were obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas Medical Center, Kansas City, KS 66103, USA. The standard laboratory wild-type strains *74-OR23-1 matA* (FGSC #987) and *OR8-1 mata* (FGSC #988), and Spore killer strains *fl; Sk-2 matA* (FGSC #3297 bearing the Brunei (Borneo) allele) and *fl; Sk-3 matA* (FGSC #3579 bearing the Papua New Guinea allele) were also obtained from the FGSC. The $Dp\ 1.3^{ec}$ *hph mata* and $Dp\ 1.3^{ec}$ *hph matA* strains have been described by Prakash *et al*. (1999).

***Growth conditions*:** Crossing and maintenance of the *Neurospora* strains was essentially as described by Davis and De Serres (1970). Antibiotic resistance was scored by streaking conidia onto 1.5% agar plates containing Vogel's N medium plus sorbose (0.05% fructose, 0.05% glucose and 2% sorbose) and supplemented with the antibiotic. The antibiotics tested were *a*-tomatine (Sigma) at 90 μg/ml made from a 25 mg/ml stock solution in dimethylformamide and hygromycin B (Sigma) 200 μg/ml made from a 100 mg/ml aqueous stock solution. After an overnight incubation at 30°C on tomatine-supplemented medium, growth can be observed of only the *erg-3* mutant strains (Sengupta *et al*. 1995). Only strains expressing the *hph* gene could grow on hygromycin medium.

***Crosses, ascospore collections and RIP efficiency determinations*:** Crosses were performed by confrontation between mycelia inoculated as plugs on synthetic crossing medium in petri dishes. Generally ascospores began to be shot within 14–16 days. Ascospores were harvested by washing the lids with ~ 1 ml water. Unless indicated otherwise, a first harvest was made 31 days after the crosses were set up. Then the petri dish lids were replaced, and a second harvest was made after an additional 14 days.

Ascospores bearing RIP-induced *erg-3* mutations exhibit a characteristic growth morphology on Vogel's 'sorbose' agar medium that enables them to be scored merely by inspection (see Noubissi *et al*. 2000 for a picture). Reliability of identifying the *erg-3* mutant phenotype in this way was established by confirming the ability of the conidia to germinate and grow on tomatine medium.

***Double-blind test for dominant RIP suppression*:** Double-blind experiments to test whether dominant suppression of RIP is a defining phenotype of the Sugartown (P0854) and the Adiopodoume-7 (P4305) strains were performed essentially as described by Noubissi *et al*. (2000). F. K. N. handed over to Mr A. Bhat (A. B.) four cultures each of the Sugartown and Adiopodoume strains, as well as 18 other wild-isolated strains. For each experiment, A. B. relabelled three of the test cultures (e.g. P0854 or P4305) and seven other wild isolates with the numerals 1, 2, . . ., 10, and handed them over to Ms Meenal Vyas (M. V.) who rerelabelled the cultures with the letters A, B, . . ., J. The rerelabelled cultures were returned to F. K. N. who made crosses with $Dp\ 1.3^{ec}$ *hph mata*. The experiments to test Sugartown and Adiopodoume were completely

independent of each other. There were thus two keys for each experiment, one with A. B. indicating the cultures labelled 1, 2, . . ., 10 and the other with M. V. indicating rerelabelling with A, B, . . ., J. F. K. N. knew only that there was at least one test culture (i.e. Sugartown or Adiopodoume) in each experiment. The challenge was to predict which strains were Sugartown (or Adiopodoume) and which were non-Sugartown (or non-Adiopodoume) based on only the RIP efficiencies and to compare the predictions by reference to the keys.

In the 'Sugartown' experiment, the cultures labelled B, C and F were correctly identified as Sugartown and the rest were correctly identified as non-Sugartown. The non-Sugartown strains were A, Colonia Paraiso (P1306); D, Agudas Rd-1 (P3978); E, Colonia Paraiso (P1303); G, Roanoke (P0519); H, Welsh (P0504); I, Friendship Village (P4774); and J, Dagguluru (P1129). In the 'Adiopodoume' experiment, the cultures labelled D, E and J were correctly identified as Adiopodoume and the rest were correctly identified as non-Adiopodoume. The non-Adiopodoume strains were A, Rondon (P4033); B, Esterillo Este (P4006); C, Mallilinatham (P4333); F, Issia (P3618); G, Franklin (P4467); H, Saratoga (P0822); and I, Tanjong Tokong (P2673).

Results and discussion

Sk-2 and Sk-3 do not dominantly suppress RIP in erg-3

The strains *fl; Sk-2 matA* (FGSC #3297) and *fl; Sk-3 matA* (FGSC #3579) were each crossed with *Dp 1.3^{ec} hph mata* and ascospores were harvested after 27 and 53 days. Although the parental strains are *erg-3^{+}*, *erg-3* mutants can be generated in these crosses by RIP in the *Dp 1.3^{ec} hph* nucleus. Among segregants examined at 27 days from *fl; Sk-2 matA* × *Dp 1.3^{ec} hph mata*, 14/1169 (1.2%) were mutant in *erg-3*. The corresponding frequency for *fl; Sk-3 matA* × *Dp 1.3^{ec} hph mata* was 9/1147 (0.8%). At 53 days the frequencies were, respectively, 13/312 (4.16%) and 8/175 (4.57%). The differences between the early and late harvests are consistent with earlier observations that the RIP frequency increases with the age of the cross (Singer *et al*. 1995). These results suggested that *Sk-2* and *Sk-3* do not suppress RIP in *trans*.

However, it was conceivable that suppression might be limited to the *Sk^{K}*-bearing nucleus (i.e. in *cis*). Progeny from the above crosses all contain *Sk-2* or *Sk-3*. Additionally, those with a hygromycin-resistant phenotype would have also inherited the *Dp 1.3^{ec}* transgene. Twenty each of *Sk-2 Dp 1.3^{ec} hph* and *Sk-3 Dp 1.3^{ec} hph* segregants were crossed with the wild-type strains *74-OR23-1 matA* or *OR8-1 mata* and *erg-3* mutation frequencies were determined in ascospores harvested after 31 days (table 1). In the majority of crosses the mutation frequencies were > 1%, which suggested that *Sk-2* and *Sk-3* also do not suppress RIP in *cis*.

Suppression of RIP in crosses with Sugartown and Adiopodoume-7

Each of the 132 wild-isolated strains was crossed with a strain bearing the *Dp 1.3^{ec} hph* transgene and the frequency of RIP-induced *erg-3* mutants was determined among ascospores harvested after 31 days (table 2). Three strains, Lankala Koderu-1 (P1108), Madurai (P4359) and Rameshwaram (P4361), behaved in our hands as *mata*, but the remaining 129 strains were *matA*, as expected. For eight crosses that gave low *erg-3* mutation frequencies

Table 1. *erg-3* mutation frequencies in progeny of *Sk Dp 1.3^{ec} hph* × wild type.

Strain	Number of progeny examined	Frequency of *erg-3* mutants (%)
Set 1		
1	445	0.9
2	198	1.5
3	617	1.8
4	599	1.6
5	284	2.8
6	406	2.0
7	407	0.7
8	591	2.0
9	242	3.7
10	426	1.4
11	613	1.8
12	232	1.7
13	1406	0.9
14	327	0.3
15	1328	2.2
16	552	0.4
17	338	1.5
18	289	1.7
19	215	2.3
20	301	1.3
Set 2		
1	405	0.5
2	290	1.7
3	151	0.7
4	212	0.9
5	393	2.0
6	243	1.2
7	255	1.2
8	437	2.3
9	472	2.1
10	94	1.6
11	321	0.6
12	706	1.7
13	359	1.4
14	407	3.4
15	319	2.2
16	261	2.7
17	146	0.7
18	290	1.7
19	365	1.1
20	303	1.3

Set 1: *Sk-2 Dp 1.3^{ec} hph* × wild type.
Set 2: *Sk-3 Dp 1.3^{ec} hph* × wild type.

Table 2. *erg-3* mutation frequencies in crosses of wild-isolated strains with *Dp 1.3*^{ec} *mata*.

Strain			Number of segregants examined		Frequency of *erg-3* mutants (%)	
P0352	Pakistan	Lahore	279		1.5	
P0354	Pakistan	Lahore	377		3.5	
P0497	USA	Houma	686		1.1	
P0502	USA	Houma	342		8.4	
P0504	USA	Welsh	171		10.0	
P0515	USA	Welsh	153		5.9	
P0518	USA	Roanoke	865		3.8	
P0519	USA	Roanoke	480		15.0	
P0521	USA	Roanoke	313		1.3	
P0523	USA	Roanoke	433		1.4	
P0666	India	Vehar-1 (Bombay)	475		3.4	
P0670	India	Vehar-1 (Bombay)	474		1.1	
P0671	India	Vehar-1 (Bombay)	593		1.7	
P0673	India	Vehar-1 (Bombay)	469		1.9	
P0683	India	Aarey-1 (Bombay)	808		2.1	
P0684	India	Aarey-1 (Bombay)	282		7.8	
P0729	India	Mughalsarai-1	262		1.9	
P0732	India	Mughalsarai-2	342		0.6	
P0736	India	Mughalsarai-2	406		3.2	
P0741	India	Mughalsarai-2	531		1.5	
P0752	India	Bichpuri-1	426		1.1	
P0753	India	Bichpuri-1	184		4.9	
P0822	USA	Saratoga	247		17.4	
P0836	USA	Spurger	348		5.5	
P0839	USA	Spurger	462		5.0	
P0841	USA	Spurger	855		10.0	
P0844	USA	Spurger	775		8.0	
P0845	USA	Spurger	827		12.5	
P0853	USA	Sugartown	733		11.5	
P0854	USA	Sugartown	428	(322)	0.5	(0.3)
P0861	USA	Elizabeth	194		5.2	
P0866	USA	Elizabeth	880		2.5	
P0869	USA	Elizabeth	457		5.3	
P0872	USA	Bayou Chicot	287		3.5	
P0884	USA	Coon	396		1.5	
P0886	USA	Coon	960		6.6	
P1108*	India	Lankala Koderu-1	445		7.4	
P1109	India	Lankala Koderu-1	193		3.1	
P1125	India	Dagguluru	828		1.5	
P1128	India	Dagguluru	690		1.8	
P1129	India	Dagguluru	922		1.9	
P1135	USA	Saratoga	349		6.6	
P1139	USA	Elizabeth	280		4.3	
P1297	Puerto Rico	Colonia Paraiso	278		7.6	
P1299	Puerto Rico	Colonia Paraiso	555		2.7	
P1300	Puerto Rico	Colonia Paraiso	137		3.7	
P1303	Puerto Rico	Colonia Paraiso	277		2.9	
P1306	Puerto Rico	Colonia Paraiso	619		4.7	
P1307	Puerto Rico	Colonia Paraiso	417		8.7	
P1443	USA	Everglades	305		7.6	
P1445	USA	Everglades	331		2.4	
P1463	USA	Homestead-3	306		4.9	
P1465	USA	Homestead-3	261		4.2	
P1466	USA	Homestead-3	638		2.1	
P2539	India	Madurai	171		3.0	
P2556	India	Rameshwaram	165		3.1	
P2561	India	Vallancheri	132		2.3	
P2591	Malaya (Penang)	Georgetown-2	598	(122)	0.9	(2.45)
P2609	Malaya (Penang)	Georgetown-4	756		4.4	
P2612	Malaya (Penang)	Georgetown-5	373		2.4	
P2613	Malaya (Penang)	Georgetown-5	172		1.8	
P2625	Malaya (Penang)	Georgetown-7	709		1.1	

Table 2 (*continued*)

Strain			Number of segregants examined		Frequency of *erg-3* mutants (%)	
P2633	Malaya (Penang)	Georgetown-8	465		1.8	
P2635	Malaya (Penang)	Georgetown-8	877		2.0	
P2673	Malaya (Penang)	Tanjong Tokong	748		1.6	
P2676	Malaya (Penang)	Tanjong Tokong	303		5.0	
P2677	Malaya (Penang)	Tanjong Tokong	257		3.1	
P2690	Malaya (Penang)	Batu Ferringi-2	398		1.5	
P2691	Malaya (Penang)	Sunshine Beach	546	(385)	0.7	(1.03)
P2724	Malaya (Penang)	Telok Kumbar	479		1.9	
P2900	Thailand	Klong Rangsit No. 5	229		2.2	
P2903	Thailand	Klong Rangsit No. 5	254		2.8	
P3387	Brazil	Caracarai	270		4.1	
P3395	Brazil	Rondon	357		3.4	
P3397	Brazil	Rondon	555	(318)	0.7	(0.94)
P3398	Brazil	Rondon	324		1.9	
P3428	Haiti	Carrefour Dufort	378		3.2	
P3429	Haiti	Carrefour Dufort	442		3.9	
P3482	Haiti	Berard	125		2.4	
P3616	Ivory Coast	Balayo	253		1.2	
P3618	Ivory Coast	Issia	186		2.7	
P3807	Congo	Lebanda	222		5.9	
P3842	Congo	Madingo	164		7.4	
P3971	Costa Rica	Jaco-1	153		2.6	
P3975	Costa Rica	Agudas Rd-1	566		3.9	
P3977	Costa Rica	Agudas Rd-1	942		1.9	
P3978	Costa Rica	Agudas Rd-1	300		1.7	
P3984	Costa Rica	Esterillo Este Rd-1	1021		0.9	
P3988	Costa Rica	Esterillo Este Rd-2	493		2.7	
P3991	Costa Rica	Esterillo Este Rd-2	244		3.3	
P3997	Costa Rica	Esterillo Este Rd-3	750		1.4	
P3999	Costa Rica	Esterillo Este Rd-3	253		3.2	
P4000	Costa Rica	Esterillo Este Rd-3	403		5.7	
P4001	Costa Rica	Esterillo Este Rd-3	270		6.7	
P4006	Costa Rica	Esterillo Este	183		7.1	
P4010	Costa Rica	Esterillo Este	207		3.0	
P4016	Costa Rica	Jaco-2	742		12.7	
P4030	Venezuela	Puerto Ayachucho	164		4.9	
P4033	Brazil	Rondon	220		7.3	
P4128	Mexico	Kabah	370		1.4	
P4129	Mexico	Kabah	295		2.4	
P4155	Mexico	Uman	231		3.5	
P4208	Puerto Rico	Colonia Paraiso	501		1.2	
P4210	Puerto Rico	Colonia Paraiso	548		1.3	
P4212	Puerto Rico	Colonia Paraiso	413		6.3	
P4214	Brazil	Rondon	433		3.3	
P4216	Thailand	Klong Rangsit No. 7	257		2.3	
P4247	Thailand	Khao Eto	379	(600)	0.5	(1.66)
P4292	Ivory Coast	Brabadougou	17		11.2	
P4295	Ivory Coast	Brabadougou	135		5.9	
P4305	Ivory Coast	Adiopodoume-7	584	(356)	0.3	(0.28)
P4306	Ivory Coast	Adiopodoume-7	537		1.1	
P4330	India	Konappatti	854		3.6	
P4333	India	Mallilinatham	482		2.5	
P4334	India	Mallilinatham	473		1.1	
P4359*	India	Madurai	500		1.0	
P4361*	India	Rameshwaram	263		14.1	
P4453	USA	Franklin	413		2.7	
P4464	USA	Franklin	355		2.0	
P4467	USA	Franklin	295		9.2	
P4480	USA	Franklin	447		2.2	
P4484	USA	Franklin	355		6.2	
P4485	USA	Franklin	215		6.5	
P4486	USA	Franklin	377		3.2	

Table 2 (*continued*)

Strain			Number of segregants examined	Frequency of *erg-3* mutants (%)
P4487	USA	Franklin	318	9.1
P4490	USA	Franklin	243	16.1
P4494	USA	Franklin	339	2.4
P4510	USA	Georgia Plantation	368	1.4
P4515	USA	Northside Planting	272	3.0
P4723	Grand Cayman	Old Man Bay-2	698	2.6
P4762	India	Lankala Koderu-2	377 (354)	0.8 (0.8)
P4769	Swaziland	Enzulini	806 (208)	0.8 (4.3)
P4774	Guyana	Friendship Village	320	2.5

∗These strains behave as *mata*.
Numbers in parenthesis refer to the ascospores harvested at 45 days. All other values refer to ascospores harvested at 31 days.

Table 3. *erg-3* mutation frequencies in second set of crosses between wild-isolated strains and *Dp 1.3*^{ec} *hph mata.*

Strain	Number of segregants examined	Frequency of *erg-3* mutants (%)
P0732	342	0.9
P0854	1007 (728)	0.2 (0.1)
P2591	182	1.1
P2691	876	0.7
P3397	555	0.9
P3984	491	1.2
P4305	935 (1096)	0.3 (0.1)
P4762	789	2.0

Numbers in parenthesis refer to the ascospores harvested at 45 days. All other values refer to ascospores harvested at 31 days.

(< 1%), we also determined the RIP frequencies among ascospores harvested at 45 days. Crosses with the Sugartown (P0854) and Adiopodoume-7 (P4305) strains continued to show low RIP frequencies even at 45 days (table 2). To verify that these results were not a sampling artifact we repeated the crosses with Sugartown, Adiopodoume-7, and a few other wild-isolated strains. Again the *erg-3* mutation frequencies were very low only in the crosses with Sugartown and Adiopodoume-7 (table 3). These results suggest that Sugartown and Adiopodoume-7 exert a dominant suppression of RIP in *erg-3*. We also noticed that crosses with the Sugartown strain consistently produced approximately 100× fewer ascospores.

Dominant RIP suppression identifies Sugartown and Adiopodoume-7 in 'double-blind' experiments

We asked whether suppression of RIP in *erg-3* could be used as a defining character to identify Sugartown and Adiopodoume-7 in a double-blind experiment. Crosses were made between *Dp 1.3*^{ec} *hph mata* and 10 coded wild strains, and the frequencies of RIP-induced *erg-3* mutants were determined in ascospores harvested after 31 days (the experimental design is detailed in Materials and methods).

In the experiment to test Sugartown the mutation frequencies (%) were: A, 1.54; B, < 0.49; C, < 0.26; D, 1.87; E, 1.04; F, < 0.82; G, 1.66; H, 2.01; I, 3.42; and J, 0.97. From these results B, C and F were correctly identified as Sugartown and A, D, E, G, H, I and J as non-Sugartown strains. In the experiment to test Adiopodoume-7 the frequencies were: A, 2.11; B, 3.22; C, 1.25; D, < 0.13; E, < 0.08; F, 0.9; G, 2.9; H, 10.0; I, 2.22; and J, 0.13. On the basis of these results D, E and J were correctly identified as Adiopodoume-7 and A, B, C, F, G, H and I as non-Adiopodoume-7 strains. Thus Sugartown and Adiopodoume-7 can indeed be distinguished from other wild isolates solely on the basis of their ability to suppress RIP in *erg-3*.

Conclusions

Our results allow us to conclude that *Sk-2* and *Sk-3* do not exert a dominant suppression of RIP. This suggests that repeated sequences may not be required for meiotic drive by the *Sk* haplotypes. Alternatively, if repeated sequences are involved, then such sequences might evade RIP by a mechanism that is limited to the *Sk* complex in some unknown way.

The results reported here bring the number of wild strains examined for dominant RIP suppressors to 203. Of these, dominant suppression was exhibited by three strains (~ 1.5%). It is remarkable that two suppressor strains, Adiopodoume (FGSC 430) and Adiopodoume-7 (P4305), were isolated from the same geographical location in Ivory Coast, West Africa. We are examining whether these strains have the same or different genetic and molecular basis for RIP suppression. Unlike Adiopodoume (FGSC 430), neither Adiopodoume-7 (P4305) nor Sugartown (P0854) possesses sequences that hybridize with *Tad* (data not shown). But Sugartown and Adiopodoume-7

might turn out to host active copies of other *Neurospora* transposons.

Acknowledgements

We thank Ashwin Bhat and Meenal Vyas for assisting in the 'double-blind' experiments. F. K. N. was supported by a fellowship from the Third World Organization of Women in Science.

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Received 7 August 2001