Fungal Genetics and Biology **35,** 171–182 (2002)
doi:10.1006/fgbi.2001.1323, available online at http://www.idealibrary.com on IDEAL®

Ds-like *Restless* Deletion Derivatives Occur in *Tolypocladium inflatum* and Two Foreign Hosts, *Neurospora crassa* and *Penicillium chrysogenum*[1]

Frank Windhofer,* Katarzyna Hauck,* David E. A. Catcheside,† Ulrich Kück,* and Frank Kempken*,[2]

**Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität Bochum, Bochum, Germany; and †School of Biological Sciences, Flinders University, Adelaide, South Australia, Australia*

Accepted for publication November 28, 2001

Windhofer, F., Hauck, K., Catcheside, D. E. A., Kück, U., and Kempken, F. 2002. *Ds*-like *Restless* deletion derivatives occur in *Tolypocladium inflatum* and two foreign hosts, *Neurospora crassa* and *Penicillium chrysogenum. Fungal Genetics and Biology* **35,** 171–182. Single copies of the transposon *Restless* from *Tolypocladium inflatum* were introduced into *Neurospora crassa* and *Penicillium chrysogenum.* Excision of *Restless* from its donor site was investigated in *N. crassa* and in *P. chrysogenum* using direct selective conditions. In *N. crassa,* forward selection was also analyzed. Deleted *Restless* elements were frequently obtained in addition to the expected complete removal of *Restless* from its donor site. Similar deleted elements were also identified in *T. inflatum* employing a PCR amplification strategy. These deleted *Restless* copies strongly resemble maize *Ds* elements of various types, and direct repeated sequences of 3 to 16 bp were found to flank the truncated regions. In addition *Ds1*-like *Restless* elements were identified that carried foreign sequences between the inverted repeats. We discuss how *Ds*-like *Restless* elements might be generated by inaccurate excision from an active transposon copy. © 2002 Elsevier Science (USA)

Index Descriptors: transposon *Restless; Neurospora crassa; Penicillium chrysogenum; Tolypocladium inflatum; Ds*-like elements; excision.

Mobile genetic elements and particularly transposons are known from all branches of the tree of life (Finnegan, 1989; Kempken and Kück, 1998b; McDonald, 1993; Saedler and Gierl, 1996). While often disregarded as selfish DNA (Doolittle *et al.,* 1984; Doolittle and Sapienza, 1980; Orgel and Crick, 1980), growing evidence now supports the view that transposable elements have an important impact on the structure and organization of the genomes they inhabit and consequently influence their host's evolution (Federoff, 2000; Kempken and Windhofer, 2001; Kidwell and Lisch, 1997; Nordborg and Walbot, 1995). Transposons often resemble element families which consist of a few active and a large number of inactive transposable elements. The latter may be activated *in trans* by one of the active copies. This is exemplified by the classic *Activator/Dissociation* elements from maize (Kunze, 1996), first described by Barbara McClintock in the late 1940s (McClintock, 1947, 1951). Little however is known about how *Ds* elements are derived from *Ac* elements.

Transposable elements similar to *Ac/Ds* have been identified in many organisms and resemble the *hAT* family of eukaryotic transposons, present in humans, animals,

[1] Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AJ311539–AJ311546 and AY062266–AY062268.

[2] To whom correspondence should be addressed at present address: AG Botanik mit Schwerpunkt Genetik und Molekularbiologie, Botanisches Institut, Christian-Albrechts-Universität Bochum, Olshausenstrasse 40, 24098 Kiel, Germany. Fax: +49-431-880-4248. E-mail: fkempken@bot.uni-kiel.de.

1087-1845/02 $35.00
© 2002 Elsevier Science (USA)
All rights reserved.

plants, and fungi (Kempken and Windhofer, 2001). Filamentous fungi are excellent experimental systems, due to their short life cycles, small genomes, and the feasibility of genetic manipulation. The discovery of *hAT* transposons in fungi (Gómez-Gómez *et al.,* 1999; Hua-Van *et al.,* 2000; Kempken and Kück, 1996; Okuda *et al.,* 1998) enables some of the unsolved problems regarding these genetic traits to be addressed more easily. The transposon *Restless* from the cyclosporin-producing fungus *Tolypocladium inflatum* is a well-characterized fungal *hAT* transposon (Kempken and Kück, 1996). Its occurrence in different fungal strains (Kempken *et al.,* 1998), alternative splicing of its primary transcript (Kempken and Kück, 1996), and extrachromosomal transposon copies (Kempken and Kück, 1998a) have been described in detail. In addition, the element has already successfully been employed for gene tagging (Kempken and Kück, 2000).

Originally, using expand PCR and Southern hybridization, only full-length *Restless* copies were observed, leading to the conclusion that, in contrast to the *Ac/Ds* elements and many other transposons, *Restless* consists only of full-length copies (Kempken and Kück, 1996). More recently, we were able to transform *Restless* into the filamentous fungus *Neurospora crassa* without triggering vegetative methylation, thereby retaining the element's activity in its new host (Windhofer *et al.,* 2000). The *Restless* element was also introduced into the asexual and commercially valuable fungus *Penicillium chrysogenum.* Here we report the detection of autonomous excision of *Restless* in both *N. crassa* and *P. chrysogenum.* Besides the expected complete removal of *Restless* from its donor site, we frequently found deleted *Restless*-like elements. Similar deleted elements were identified in *T. inflatum* employing a PCR strategy. These deleted *Restless* copies strongly resemble *Ds* elements of various types. We discuss the hypothesis that *Ds*-like elements are being generated by inaccurate excision of an active copy.

MATERIALS AND METHODS

Strains and Culture Conditions

The following fungal strains were used in this study: *N. crassa* F10292-1 (*arg-1* K166, *his-3* K480, *cot-1* C102T, *rec-2,* A), *P. chrysogenum* strain CRB47 (niaD$^-$ phenotype, E. Friedlin and H. Kürnsteiner, personal communication), and *T. inflatum* ATCC34921. Culture conditions were as described (Davis and DeSerres, 1970; Stimberg *et al.,* 1992). *Escherichia coli* strain XL-Blue1 (Stratagene, La Jolla, CA) was used for propagation of vector constructs under standard culture conditions.

DNA Isolation

A published protocol (Lecellier and Silar, 1994) was used with two modifications: fungal mycelium was ground in a mortar under liquid nitrogen and the volume of buffers, etc., was increased fivefold for better DNA yield. Bacterial plasmid DNA was isolated using NucleoSpin or NucleoBond reagent sets (Macherey-Nagel, Düren, Germany).

Gel Electrophoresis, Blotting, and Hybridization Conditions

Agarose gels, Southern blotting, and DNA–DNA hybridization were performed according to Sambrook *et al.* (1989). A decaprime kit (Ambion, Austin, TX) was used to label 20 to 30 ng of template DNA with [α-^{32}P]dCTP.

PCR Amplification

PCR was carried out as described previously (Howad and Kempken, 1997).

Oligonucleotides

These were synthesized by MWG-Biotech (Ebersberg, Germany). Sequences and locations are shown in Table 1.

E. coli Transformation

Cloning and *E. coli* transformation were done according to standard procedures (Sambrook *et al.,* 1989). Vector construction is described below. All cloning and transformation experiments were done according to the requirements of the German Gene Technology Law (Gentechnikgesetz).

N. crassa Transformation and Analysis

Spheroplasts were prepared from *N. crassa* and used for DNA-mediated transformation as described by Yuan and Marzluf (1992). Primary transformants were obtained by site-specific recombination at the *his-3* locus and selection for growth on minimal medium (Bowring and Catcheside, 1993). Microconidia were isolated from primary transfor-

© 2002 Elsevier Science (USA)
All rights reserved.

TABLE 1
Oligonucleotides Used

Name	Sequence (5′-3′)	Location	Used for
FK47	TCAGTGGCCATCCGACAG	*Restless* 3752–3735	Generating modified splice site in vector pFW182
FK57	ACGAGAATCCCACGCCTA	*Restless* 1617–1634	Generating modified splice site in vector pFW182
FK90	GACTCTTTACCCGCAGACA	*Restless* 2683–2701	Generating target site duplication in vector pFW193
FK96	CAGAGTGCGTAATCAACCAA	*Restless* 1–20 and 4097–4078	Amplification of *Restless* deletion derivatives in *T. inflatum*
FK97	CTACTTCCAACCGTTGAAGCC	*Restless* 21–41	Amplification of *Restless* deletion derivatives in *T. inflatum*
FK163	CGTGATCTTGAGTCATCGTTCGCGAATCGC	pUT737 2091–2120	Obtaining amplicons after transposition of *Restless*
FK164	GTCGTCCCGGACCACACCGGCGAAGTCGTC	pUT737 2400–2381	Obtaining amplicons after transposition of *Restless* and generating target site duplication in vector pFW193
FK219	GCGCATCTCTTCGATTAAGCC	pUT737 2127–2147	Obtaining amplicons after transposition of *Restless*
FK220	AGTCGTCCTCCACGAAGTCCC	pUT737 2387–2368	Obtaining amplicons after transposition of *Restless*
FK240	AATACCAGTTGAGTTGAATTGGTTGATTACGCACTCTGGAGCAAGGCATCCCGACTACTC	pFW128 6301–6360	Generating target site duplication in vector pFW193
FK245	TTGGCCATGGTGATATCTAGTTAACTAGTTCGAGTAGTCGGGATGCCTTGCTCCAGAGTGC	pFW128 6391–6330	Generating target site duplication in vector pFW193
FK251	TCCATGGGATCAAGGTAAGTAATTGATTCCTGCC	*Restless* 1878–1911	Generating modified splice site in vector pFW182
FK252	GGCAGGAATCAATTACTTACCTTGATCCCATGGA	*Restless* 1911–1878	Generating modified splice site in vector pFW182
FK278	GCCACGCCCAACGCTTGATGAGTACGAACG	*Restless* 3615–3644	Reverting point mutation in vector pFW224
FK279	CGTTCGTACTCATCAAGCGTTGGGCGTGGC	*Restless* 3644–3615	Generating target site duplication in vector pFW193

mants to obtain homokaryons as described (Ebbole and Sachs, 1990). Selection for *mtr* mutants was done according to published procedures (Stadler *et al.,* 1991). Phleomycin selection used as an assay for transposition of transposon *Restless* was done according to a described method (Austin *et al.,* 1990). A maximum of 5×10^7 conidia were spread on one 8.5-cm plate to avoid inhibition of germination.

P. chrysogenum Transformation and Analysis

The generation of protoplasts was done as described (Bull *et al.,* 1988) with some modifications. Before protoplast formation, the strain was grown for 36 to 48 h on complete medium followed by transfer to minimal medium for 24 h. The mycelium was incubated for 2 h (27°C)

© 2002 Elsevier Science (USA)
All rights reserved.

with Novozyme (Novo Industrie AIS) (5 mg/ml of 0.9 M NaCl) to obtain sufficient protoplasts. Transformed protoplasts were selected on minimal medium containing 35 mM $NaNO_3$. Transformants with an excised *Restless* transposon were selected on complete medium containing 50 μg phleomycin per milliliter.

Vector Construction

Vector constructs used here are based on vector pUT737, which carries a phleomycin-resistance gene with a fungal promoter and terminator (Jain *et al.,* 1992).

(1) Vector pFW128. Plasmid pUT737 was digested with *Sal*I and sticky ends were removed with S1 nuclease. A PCR-amplified *Restless* copy was inserted between the promoter and the phleomycin open reading frame (see Fig. 1).

(2) Vector pFW182. A modified 5′ splice site (5′-GTAAGT-3′) was inserted into vector pFW128. This was intended to increase splicing of the *Restless* mRNA, as RT-PCR had indicated that mRNA carrying the native splice site (5′-GCAAGC-5′) was partially spliced only (Kempken and Windhofer, unpublished data). The modification of the splice site was done using *in vitro* mutagenesis as described (Urban *et al.,* 1997). Two overlapping PCR amplicons were amplified using vector pFW128 as template. One amplicon obtained from PCR with oligonucleotides FK57–FK252 was 294 bp in size. The second fragment obtained with oligonucleotides FK251–FK47 was about 1.9 kb in size. Aliquots of 6% of the volume of these PCRs were used for an additional amplification using oligonucleotides FK57–FK47. The resulting 2.1-kb PCR product was digested with *Sac*I. This *Sac*I fragment was then inserted instead of the native fragment and ligated to a 7.98-kb *Sac*I vector fragment of pFW128 purified from an agarose gel, in order to remove the unmodified *Sac*I fragment.

(3) Vector pFW186. To allow for site-specific integration in *N. crassa,* a 1.6-kb *Not*I/*Cla*I fragment from pFJB1 (Bowring and Catcheside, 1993) carrying *his-3* sequences truncated at the 5′ end was inserted into the *Not*I/*Cla*I-digested vector pFW182.

(4) Vector pFW193. An intact 8-bp target site duplication was inserted into vector pFW128 using *in vitro* mutagenesis as described by Urban *et al.* (1997). A 1.5-kb *Hpa*I fragment carrying the right-hand *Restless* terminus from plasmid pFW128 was replaced with a *Hpa*I-digested 1.5-kb PCR amplicon containing an engineered 8-bp target site, to generate a vector with 8-bp target site duplications flanking the transposon. Two overlapping PCR amplicons were amplified using vector pFW128 as template. The first fragment was about 200 bp in size using oligonucleotides FK240–FK164. The second fragment was a product of about 1.5 kb using oligonucleotides FK90–FK245. Aliquots of 6% of the volume of these PCRs were used for a further PCR using oligonucleotides FK90–FK164. The 1.5-kb PCR product was digested with *Hpa*I, purified from a gel, and ligated with an 8.5-kb *Hpa*I vector fragment of pFW128 purified from a agarose gel. To allow for site-specific integration, a 1.6-kb *Not*I/*Cla*I fragment from pFJB1 (Bowring and Catcheside, 1993) carrying *his-3* sequences truncated at the 5′ end was inserted into the *Not*I/*Cla*I-digested vector.

(5) Vector pFW224. pFW193 was modified by using the "QuikChange" Site-Directed Mutagenesis Kit (Stratagene). A point mutation in exon 2 of *Restless* at position 5870 was modified using oligonucleotides FK278 and FK279, restoring to wild-type a mutation from the original sequence that led to a substitution of proline for leucine, in all other vector constructs compared to the originally published sequence (Kempken and Kück, 1996), the rationale being that an amino acid substitution in the open reading frame might affect transposase activity.

(6) Vector pFW249. This vector carries a deleted transposase gene and was generated by digestion of vector pFW224 with *Sal*I. This resulted in three fragments of 0.77, 2.67, and 7.72 kb. The two larger fragments were eluted from a gel and religated. The resulting vector (pFW249) carries a 0.77-kb deletion in exon 2 of the transposase.

(7) Vector pKC2. The transposase coding region including the intron was PCR amplified using vector pFW128 as a template and oligonucleotides FK114 and FK115. The PCR fragment was cloned into the *Sma*I site of vector pPLKS-, carrying the repressible *phoA* promoter (Graessle *et al.,* 1997). Downstream of the transposase gene a *trpC* terminator was added.

Vectors pFW128, pFW182, pFW186, pFW193, pFW224, pFW249, and pKC2 are shown in Fig. 1.

Sequence Analysis

All sequence analysis was done by MWG-Biotech and SEQlab (Göttingen, Germany).

RESULTS

In a previous study we introduced the transposon *Restless* from the cyclosporin-producing fungus *T. inflatum*

© 2002 Elsevier Science (USA)
All rights reserved.

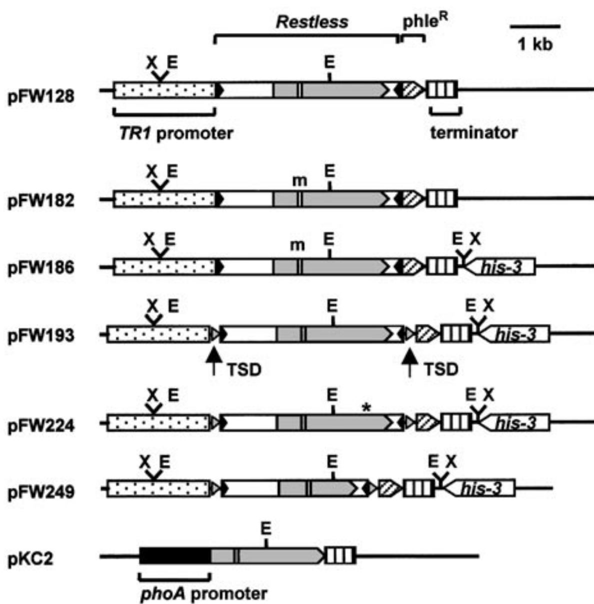

FIG. 1. Vectors used for transformation of *N. crassa*. Details of vector construction are given under Materials and Methods. Components of the *Restless* element are as follows: target site duplication (light gray triangle), terminal inverted repeat (black triangle), and transposase gene (gray pointed rectangle; intron white). A point mutation introduced into the intron 5′ splice site (see Materials and Methods) is indicated by "m" and the site of reversion of a point mutation leading to an amino acid substitution in the transposase is indicated by an asterisk. Dotted box, *TR1* promoter (Jain *et al.*, 1992); black box, *phoA* promoter (Graessle *et al.*, 1997); pointed box with inclined stripes, phleomycin-resistance gene; box with vertical stripes, *trpC* promoter; E, *Eco*RI; X, *Xho*I.

into *N. crassa*, in order to exploit the abilities of transposons to tag genes. We found that although in multiple-copy transformants, *Restless* was methylated and therefore inactive in *N. crassa*, single-copy transformants integrated at the *N. crassa his-3* locus were not (Windhofer *et al.*, 2000), enabling further analysis of the activity of *Restless* in this foreign host. To this end, two different experimental approaches were employed, using vectors carrying full-length copies of *Restless* and others with a truncated transposase (see Fig. 1) to enable us to distinguish host-specific effects from transposase activity. In addition to using *N. crassa*, we also analyzed the activity of *Restless* in the asexual fungus *P. chrysogenum*.

Since transposition is a rare event, even in its natural host where transposition of *Restless* occurs at a frequency of less than 10^{-5} (Kempken, unpublished results), we employed selection strategies to detect transposition: (i) forward selection in *N. crassa* using the *mtr* gene (Stadler *et al.*, 1991) and (ii) direct selection in *N. crassa* and *P. chrysogenum* for excision of a *Restless* element placed between the promoter and the coding sequence of the dominant phleomycin-resistance gene (*Sh ble*) (Austin *et al.*, 1990).

Analysis of Excision Events upon Forward Selection in *N. crassa*

The *N. crassa mtr* gene encodes a transporter specific for neutral amino acids (Koo and Stuart, 1991; Stadler *et al.*, 1991). Mutations that inactivate *mtr* can be selected by their ability to grow on medium containing the amino acid analogue *p*-fluorophenylalanine. We sought to use this rather simple system to forward select for transposition of the *Restless* element into *mtr*. Indeed, *Restless* transformants made with vectors pFW193 or pFW224 (which are identical except for one point mutation in exon 2; see Fig. 1) yielded up to 30-fold higher frequency of spontaneous *mtr* mutations than were obtained from untransformed *N. crassa*. DNA isolated from spontaneous *mtr*$^-$ mutants derived from monokaryotic strains transformed with pFW193 were assayed for excision of *Restless* from its donor site using the PCR primers shown in Fig. 2A. Amplicons of approximately 260, 350, and 450 bp were obtained (Fig. 2B). The smallest of these amplicons is the size of an empty donor site after excision of *Restless*. This was confirmed by DNA sequencing (Fig. 3A; sequence data were deposited in the EMBL database under Accession Nos. AJ311545 and AJ311546). Most interestingly,

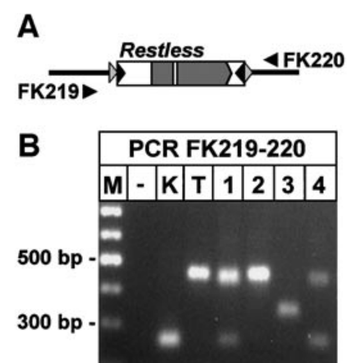

FIG. 2. Amplicons obtained from the donor site after forward selection for transposition of *Restless* in *N. crassa* by selection of *mtr* mutants. (A) *Restless* element with target site duplication (light gray triangle), terminal inverted repeat (black triangle), and transposase gene (dark gray pointed rectangle; intron white). (B) Amplicons obtained upon PCR with oligonucleotides FK219 and FK220. Lanes M, marker; −, without DNA; K, vector pUT737 (without *Restless*); T, original transformant prior to selection; 1–4, four different mutants; for details see main text.

© 2002 Elsevier Science (USA)
All rights reserved.

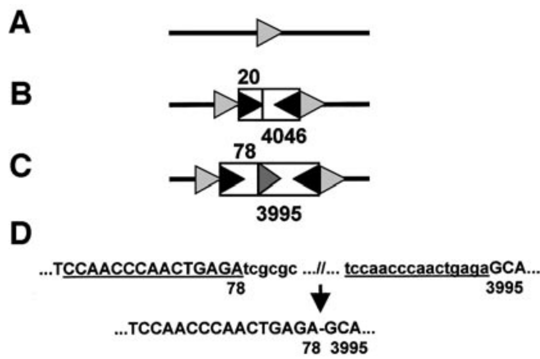

FIG. 3. Sequence analysis of amplicons from lanes 1–4 of Fig. 2. Schematic representation of sequencing data. (A) The 260-bp amplicon has lost the entire *Restless* element including one copy of the direct repeat. One copy of the direct repeat remains (light gray rectangle). (B) The 350-bp amplicon carries a much truncated copy of the *Restless* element. All sequence between position 20 and 4046 (see Kempken and Kück (1996)) has been removed and only the right- and left-hand terminal sequences have been retained. The truncated copy still possesses a target site duplication and terminal inverted repeats (black triangles). (C) Similar to (B), the 450-bp amplicon exhibits a large truncation of the *Restless* element, between nucleotides 78 and 3995. A small portion of the 3′ end of the transposase gene (dark gray triangle) is still present in addition to the terminal inverted repeats and target site duplication. (D) Sequences upstream and downstream of the deleted area of the 450-bp amplicon are shown. Sequences retained in the truncated copy are in capital letters. A 16-bp direct repeat flanking the deleted region is underlined. Dots and slashes indicate additional sequences not shown in the figure.

the sequence of the two 260-bp amplicons (Fig. 2B) shows that the *Restless* element and one of the direct repeats had been removed, restoring the sequence to that which would have been present prior to transposon insertion. However, the target site duplications were generated in a vector carrying *Restless* by *in vitro* mutagenesis, not by transposition; this finding may be an indication that *Restless* excision proceeds by a mechanism dissimilar to that of transposons in higher plants where small deletions are usually generated at the excision site (Kunze, 1996).

Sequencing of the 350-bp amplicon (see Fig. 3B) revealed the presence of a 71-bp *Restless* fragment at the donor site which had lost most of its internal DNA, except for 20 bp resembling one of the inverted repeats at the left terminus and 51 bp at the right terminus (position 4046–4097 (Kempken and Kück, 1996)).

All but one *mtr* mutant, as well as the transformant from which the *mtr* mutants were obtained, also gave rise to a 450-bp amplicon. Southern hybridization revealed that a full-length *Restless* copy was present in the transformant prior to the experiment, indicating that the deleted version present in the 450-bp amplicon may have been present in a low percentage of the nuclei of the primary transformant. The 450-bp amplicon contained 181 bp of *Restless* (Fig. 3C) DNA, consisting of bases 1–78 and 3995–4097, flanked by vector sequences (Kempken and Kück, 1996). The 181-bp deleted copy of *Restless* may have been generated by recombination between 16-bp direct repeats of 5′-TCCAACCCAACTGAGA-3′, present in the subterminal regions at both ends of the *Restless* element and flanking the truncated region in the 450-bp amplicon (Fig. 3D). The smaller 71-bp truncated *Restless* element found in the 350-bp amplicon may have been generated by recombination at a 3-bp direct repeat sequence, 5′-CAA-3′.

Analysis of Excision Events Selected by Phleomycin Resistance in N. crassa

The vectors pFW193 and pFW224 used for transformation of *N. crassa* (see Fig. 1) included the *Sh ble* gene and its promoter, separated by the *Restless* transposon. This arrangement allows direct selection for excision of the *Restless* element by adding phleomycin to the growth medium. Conidia are able to germinate and grow on phleomycin only after excision of *Restless* allows expression of the phleomycin-resistance gene. Although wild-type conidia of *Neurospora* are resistant to 50 μg/ml phleomycin they were found to be completely inhibited by 5 μg/ml in the presence of 0.5 mg/ml caffeine, a strategy for enhancing sensitivity to phleomycin previously reported (Austin *et al.,* 1990). A total of 26 colonies resistant to 35 μg/ml phleomycin were obtained using conidia of strains transformed with either vector pFW193 or pFW224. Phleomycin-resistant colonies occurred at a frequency of $1–5 \times 10^{-7}$. No phleomycin-resistant colonies were obtained when 10^8 wild-type conidia were plated on phleomycin. Phleomycin-resistant colonies were also not obtained from transformants made with vectors pFW186 and pFW249. In pFW186, the intron in the *Restless* transposase gene was modified to prevent alternative splicing, a process believed to be essential for the function of *Restless,* and in pFW249, the *Restless* element has a truncated transposase gene. Since both pFW186 and pFW249 contain the subterminal repeats involved in generation of deletion derivatives, we can exclude involvement of endogenous recombination enzymes from the fungal host in the generation of *Ds*-like elements.

DNA of the 26 phleomycin-resistant colonies from *N. crassa* was isolated from microspore progeny to select for homokaryotic mycelia and subjected to PCR analysis and

© 2002 Elsevier Science (USA)
All rights reserved.

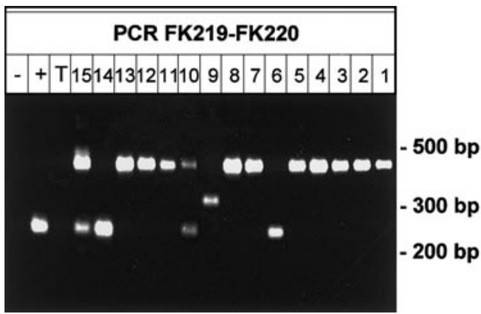

FIG. 4. Donor site amplicons obtained after phleomycin selection for transposition of *Restless* in *N. crassa.* Amplicons were obtained by PCR with oligonucleotides FK219 and FK220. Lanes −, without DNA; +, vector pUT737 (without *Restless*); T, original transformant prior to selection; 1–15, different phleomycin-resistant mutants; for details see main text.

gel electrophoresis. Examples are shown in Fig. 4. It should be noted that the pFW193 transformant used here was different from that shown in Fig. 2, as it did not give rise to an additional 450-bp fragment. Three different sized amplicons were obtained from phleomycin-resistant mycelia. (i) In nine colonies amplicons of 260 bp were observed; (ii) in one colony an amplicon of 350 bp was observed; and (iii) in 22 colonies a 450-bp amplicon was observed. Some of the transformants carried both a 260- and a 450-bp amplicon, indicating that these are heterokaryotic mycelia despite passage through microconidia, which are expected to be uninucleate. This may have resulted from colony overlap when microspores were plated.

Sequence analysis of these amplicons confirmed the results found with amplicons obtained by forward selection as shown in Fig. 3. Both types of experiments, forward selection using *mtr* and direct selection by phleomycin resistance, thus give similar results. Most frequently observed is the 450-bp amplicon, which may be due to homologous recombination in the 16-bp direct repeats flanking the truncated region. Indeed, the 350-bp amplicon, which has only a 3-bp repeat flanking the truncated region, was found only twice. In the case of phleomycin selection, partial excision of *Restless* apparently still allowed expression of the phleomycin gene. This may be due to the promoter immediately in front of the open reading frame being tolerant of small insertions.

All 260-bp amplicons resemble a complete excision of *Restless* including one of the direct repeats, as shown by sequence analysis.

Excision Events in P. chrysogenum

Phleomycin-resistant colonies from *P. chrysogenum* transformants carrying vector pFW128 were observed at frequencies of 3×10^{-2} to 5×10^{-4}, about 10^3 times higher than observed in *N. crassa.* Cotransformants which also carried vector pKC2 (see Fig. 1) allowing for expression of the transposase gene under control of the repressible acid phosphatase gene (*phoA*) promoter (Graessle *et al.,* 1997) led to an even higher frequency of 5×10^{-1}. Phleomycin-resistant colonies were not obtained from transformants carrying vector pFW182, which has a mutated intron splice site and is known to be deficient in alternative splicing, which is believed to be necessary for the activity of the element (Windhofer and Kempken, in preparation).

PCR amplicons were obtained from phleomycin-resistant colonies (data not shown) and sequenced. It should be noted that vector pFW128 does not have target site duplications and, due to the cloning procedure, has short inverted repeats flanking the transposon instead. Four different footprints were observed: (i) complete excision of *Restless,* associated with a deletion of one nucleotide of vector DNA (Fig. 5A);

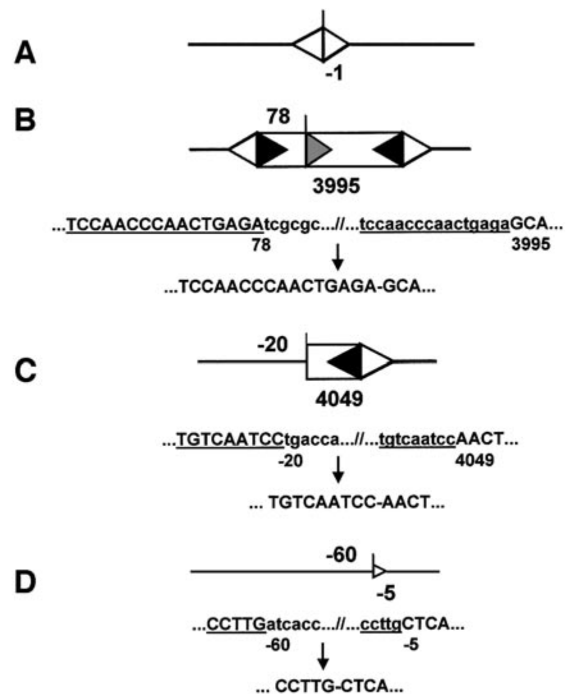

FIG. 5. Schematic representation of sequence data from donor site amplicons following phleomycin selection for transposition of *Restless* in *Penicillium chrysogenum.* (A–D) Different types of excision events; Type (A) and (B) were identified twice, (C) was identified three times, and (D) was identified once only. For more details see main text.

© 2002 Elsevier Science (USA)
All rights reserved.

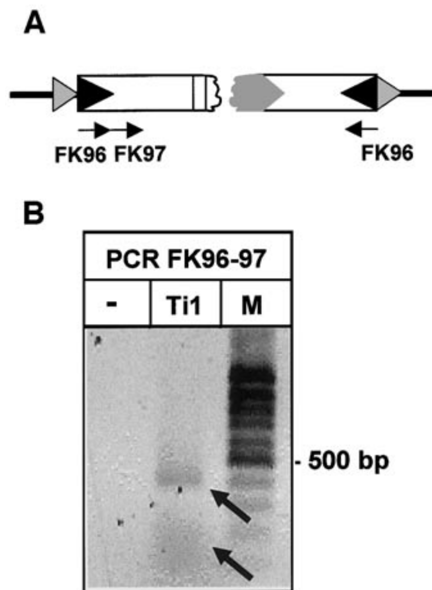

FIG. 6. PCR amplification of *Restless* deletion derivatives from *T. inflatum.* (A) Left and right ends of the *Restless* transposon are shown (for description see Fig. 3) as well as the localization of oligonucleotides FK96 and FK97. (B) PCR was performed using oligonucleotides FK96 and FK97 using high-molecular-weight DNA from *T. inflatum* ATCC34921. A short elongation time was used for PCR to preferentially amplify small molecules. The arrows indicate an amplicon of about 400 bp and a smear of different sized molecules at about 100 to 200 bp. Lanes −, control without DNA; Ti1, *T. inflatum.* This figure is shown with dark bands on a light background to increase reproducibility.

(ii) a deletion giving rise to the same 450-bp amplicon found in *N. crassa* (Fig. 5B); and (iii) excisions extending 20 and 60 bp into the vector DNA (Figs. 5C and 6D). These are reminiscent of some of the excision sites described for the *Ascot* transposon in *Ascobolus immersus* (Colot *et al.,* 1998). Each of the excision events shown in Figs. 5B to 5D can be accounted for by recombination at direct repeats of 5 to 16 bp.

Deleted Restless Copies in Tolypocladium inflatum

Restless transposase activity in *N. crassa* and *P. chrysogenum* was frequently associated with incomplete excision and generation of deleted *Restless* elements. We therefore were interested in whether similar events occurred in *T. inflatum* ATCC34921, the natural host of *Restless.* To this end a nested PCR amplification was performed using oligonucleotides FK96 and FK97 located at or close to the transposon termini. The PCR products were subjected to gel electrophoresis (Fig. 6). The low-molecular-weight amplicons obtained were shotgun cloned into the *Xcm*I site of vector pMON (Borovkov and Rivkin, 1997). DNA was prepared from individual clones, digested with *Eco*RI and *Xba*I to release the cloned DNA, and subjected to gel electrophoresis. A variety of different sized amplicons of approximately 100 to 450 bp were found (Fig. 7) and subsequently, 17 amplicons were sequenced (EMBL Accession Nos. AJ311539–AJ311544).

Three types of truncated *Restless* elements were identified from *T. inflatum* (Fig. 8): (i) a highly truncated version (Δ*Rst1*) retaining only 41 bp at the left-hand end and 20 bp at the right-hand end (control experiments in which no fungal DNA was used suggest that these are not artifacts generated by ligation of the primer oligonucleotides); (ii) elements carrying unrelated sequences, as exemplified by Δ*Rst2* and Δ*Rst3,* which possess 24 and 131 bp of DNA of unknown origin; and (iii) truncated *Restless* elements (Δ*Rst4–6*) which retain internal sequences of varying size at one or both ends of the element. It should be noted that (Δ*Rst5*) is identical to the *Restless* truncation found in the 450-bp amplicon obtained from *N. crassa,* the difference in size being due to the different amplification strategies (compare Fig. 2A and Fig. 6). Both Δ*Rst5* and Δ*Rst6* were obtained four times (Fig. 7). At the borders of the Δ*Rst6* truncation, a 4-bp direct repeated sequence, 5′-CTGG-3′, is present. Another 4-bp repeat, 5′-TTGG-3′, flanks the deleted sequences of the Δ*Rst1* element. However, no obvious repeated sequence was found that could account for the 192-bp amplicon Δ*Rst4,* which was found only once.

DISCUSSION

In 1996, Kempken and Kück reported finding *Restless* in *T. inflatum,* the first *hAT*-like transposon to be identi-

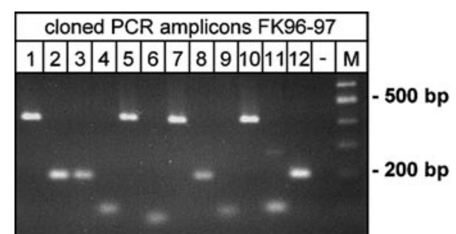

FIG. 7. *Restless* deletion derivatives cloned from donor sites following transposition of *Restless* in *T. inflatum.* The amplicons obtained from PCR were shotgun cloned into vector pMON. Twelve clones were digested with *Xba*I and *Eco*RI to release the cloned fragments and analyzed by gel electrophoresis. The lower part of the gel below the vector band is shown. Inserts vary in size from about 100 to 400 bp.

© 2002 Elsevier Science (USA)
All rights reserved.

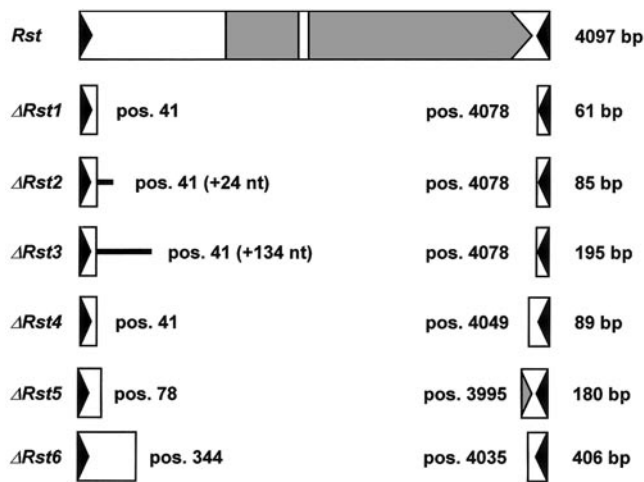

FIG. 8. Sequence analysis of the donor site deletions cloned from *T. inflatum* (lanes 1–15 of Fig. 4). These amplicons, obtained with oligonucleotides FK96 and FK97, all represent truncated *Restless* elements (Δ*Rst*). For comparison, a complete copy of *Restless* (*Rst*) is also shown. The primers yield 41 bp left and 20 bp right of the *Restless* sequences, not including the direct repeats. Total sizes of all fragments are shown on the right-hand side of the figure. The symbols used are defined in the captions of earlier figures. The lines shown for Δ*Rst2* and Δ*Rst3* indicate sequences of unknown origin, 24 and 131 nucleotides in length, respectively. In Δ*Rst6,* deletions may have occurred at two different sites. The deleted copy Δ*Rst5* is identical to the truncated *Restless* copy present in the 450-bp amplicons found in *N. crassa.*

fied in a filamentous fungus. Since then, similar elements have also been identified in *Fusarium oxysporum* (Gómez-Gómez *et al.,* 1999; Hua-Van *et al.,* 2000; Okuda *et al.,* 1998). However, a much larger number of *hAT* transposons are known from a variety of plant and animal species including human (Kempken and Windhofer, 2001). Quite often, full-length and truncated copies of the same element are found in the same species. This is well known for the *Ac/Ds* family from maize (Kunze, 1996), where several types of *Dissociation* or *Ds* elements are known: *Ds2d1, Ds2d2, Ds6,* and *Ds9* (Fedoroff, 1989), for instance, that have deletions of various sizes compared to the full-length *Activator* or *Ac* element. In remarkable contrast, *Ds1* (Gerlach *et al.,* 1987; Sutton *et al.,* 1984), *Ds5933* (Döring *et al.,* 1984), or *wxB4-Ds* (Varagona and Wessler, 1990) elements consist of short terminal stretches of *Ac*-like sequences, necessary for recognition *in trans* by the *Ac* transposase (Kunze, 1996), with a central DNA sequence derived from unknown genomic sources. Truncated copies were also shown for the *F. oxysporum Folyt1* element (Gómez-Gómez *et al.,* 1999). In contrast, in previous studies, truncated copies of *Restless* were not detected in *T. inflatum* strain ATCC34921 (Kempken and Kück, 1996). However, in a different strain, ATCC42437, a single truncated *Restless* copy was observed, but no full-length copies were found (Kempken *et al.,* 1998).

In this study we have provided direct evidence for the presence of *Ds*-like deletion derivatives of *Restless* in its natural host *T. inflatum,* as well as in two foreign hosts, *N. crassa* and *P. chrysogenum.* Due to the small size of these deletion derivatives, Δ*Rst* elements had not been detected in earlier work using Southern blot analysis.

When compared to *Ds* elements, the Δ*Rst* elements, albeit much smaller, bear a striking similarity. Δ*Rst2* and Δ*Rst3* are most similar to *Ds1* (Gerlach *et al.,* 1987; Sutton *et al.,* 1984), *Ds5933* (Döring *et al.,* 1984), and *wxB4 Ds* (Varagona and Wessler, 1990) elements, which also carry unknown sequences between their terminal inverted repeats. Δ*Rst4–6,* however, resemble counterparts of the *Ds6* type of *Ds* elements (Fedoroff, 1989) in having large internal deletions. Therefore, we conclude that the Δ*Rst* elements in fungi are similar to *Ds* elements identified in maize.

Little is known of how truncated elements like Δ*Rst* or *Ds* are generated. In the case of Δ*Rst* elements, direct repeats of 3 to 16 bp are present in most cases immediately upstream and downstream of the truncated region. This may suggest an involvement of these repeats in the generation of the Δ*Rst* elements. This may be achieved by the activity of the *Restless* transposase or else by homologous recombination mediated by host enzymes. Homologous recombination is a rather rare event in vegetative cells of filamentous fungi (Fierro and Martin, 1999), but nevertheless may occur (Casqueiro *et al.,* 1999), even at very short sequences of 6 bp (Fierro *et al.,* 1995). Therefore, the possibility cannot be excluded that deleted *Restless* copies are generated by the recombination promoting enzymes of the host. However, we obtained no phleomycin-resistant colonies from untransformed *N. crassa* or *P. chrysogenum* conidia, even when using significantly larger numbers of conidia than with *Restless* transformants. The failure to obtain phleomycin-resistant colonies with cells transformed with vector pFW249, which carries a truncated transposase gene, provided an important control. If generation of *Ds*-like elements is caused by endogenous host-encoded enzymes, phleomycin-resistant colonies should occur with vector pFW249 as well, since it contains all of *Restless* with the exception of an internal piece of the transposase gene. The absence of phleomycin-resistant colonies in transformants carrying that vector strongly argues for an active role of the *Restless* transposase in the excision process. In addition, we also did not obtain phleo-

© 2002 Elsevier Science (USA)
All rights reserved.

mycin-resistant colonies from transformants carrying vector pFW186, which has a mutated *Restless* 5′ intron splice site (Windhofer and Kempken, in preparation), preventing alternative splicing (Kempken and Kück, 1996) and thus proper transposase gene expression. Similarly, no phleomycin-resistant colonies were obtained when a comparable vector (pFW182, see Fig. 1) was employed in *P. chrysogenum.* In sum, phleomycin-resistant colonies were not obtained in *N. crassa* or *P. chrysogenum,* without expression of a *Restless* transposase. Cumulatively, these data are inconsistent with deleted *Restless* elements being generated as a result of nonspecific host enzyme activity and suggest that they arise due to activity of the *Restless* transposase.

It is known that the expression of a transposase may generally induce recombination at direct repeats. Expression of the *Ac* transposase in plants led to a 1000-fold increase in the spontaneous recombination frequency in *Arabidopsis,* when a *Ds* element was inserted between two repeated sequences (Xiao and Peterson, 2000) or at the *p1* locus (Xiao *et al.,* 2000). Likewise, the *Mutator* transposon also promotes recombination between direct repeat sequences of about 17 kb in the maize *knotted* locus (Lowe *et al.,* 1992). However, these direct repeats were much larger than the 16-bp direct repeat implicated in the generation of the Δ*Rst5* element obtained in both *N. crassa* and *T. inflatum;* for example, the 618-bp direct repeat found in an *Arabidopsis* study (Xiao *et al.,* 2000), is almost 40 times larger than those described here.

So far, no information is available about the DNA binding properties of the *Restless* transposase, mainly due to the lack of a suitable expression system. However, DNA binding studies have been performed with the *Ac* transposase from maize. It was shown that the transposase enzyme binds to a short subterminal hexamer motif, 5′-AAACGG-3′, which is repeated several times in the *Ac* sequence (Kunze and Starlinger, 1989). In an additional study, a more sensitive approach was possible and led to the conclusion that the transposase also bound to the inverted repeat. Moreover, it was shown that two trinucleotide sequences, 5′-ACG-3′ and 5′-TCG-3′, are the minimal binding sites for the *Ac* transposase (Becker and Kunze, 1997). The right- and left-hand subterminal repeats of *Restless* contain six direct repeats of the sequence 5′-CCAAC-3′. This sequence is part of the 16-bp direct repeat mentioned previously (Kempken and Kück, 1996). It is possible that these repeated sequences are the target for specific DNA binding of the *Restless* transposase. Inaccurate excision of the *Restless* element may occur when the transposase cuts the DNA sequence close to its binding site instead of outside the terminal inverted repeat sequence. Further experimental studies are necessary to elucidate the enzymatic properties of the *Restless* transposase.

Another unusual observation stems from analysis of *Restless* excision sites, which in *N. crassa* all lacked the typical footprints known from other *hAT* transposons, e.g., *Ac/Ds* elements from maize (Kunze, 1996) or the *Ascot* transposon from *A. immersus* (Colot *et al.,* 1998). Yet, we observed footprints in *P. chrysogenum.* Another example monitored for excision, the *hAT* transposon *Folyt1* from *F. oxysporum,* also did not leave footprints (Gómez-Gómez *et al.,* 1999). Thus it appears that *hAT* transposons do not generate footprints at the donor site when they transpose in some fungal hosts. Further characterization of the *Restless–N. crassa/P. chrysogenum* experimental systems and particularly the characterization of reintegration events should help to elucidate the functional properties of the *Restless* transposase.

ACKNOWLEDGMENTS

The authors thank Mrs. G. Isowitz for excellent technical assistance and Drs. E. Friedlin (Kundl), H. Kürnsteiner (Kundl), and H. Haas (Innsbruck) for providing fungal strains and expression vectors. This work was funded by a grant from the "Deutsche Forschungsgemeinschaft, Bonn" to F.K. (Ke409/5-3). F.W. and K.H. received a grant from the "Graduiertenförderung des Landes Nordrhein-Westfalen." D.E.A.C. received a travel grant from the German–Australian Cooperative Science Program of the Department of Industry, Science and Resources, Canberra, Australia. U.K. and K.H. are grateful to Biochemie AG (Kundl, Austria) for financial support.

REFERENCES

Austin, B., Hall, R. M., and Tyler, B. M. 1990. Optimized vectors and selection for transformation of *Neurospora crassa* and *Aspergillus nidulans* to bleomycin and phleomycin resistance. *Gene* **93:** 157–162.

Becker, H. A., and Kunze, R. 1997. Maize *Activator* transposase has a bipartite DNA binding domain that recognizes subterminal sequences and the inverted repeats. *Mol. Gen. Genet.* **254:** 219–230.

Borovkov, A. Y., and Rivkin, M. I. 1997. *Xcm*I-containing vector for direct cloning of PCR products. *BioTechniques* **22:** 812–814.

Bowring, F. J., and Catcheside, D. E. A. 1993. The effect of *rec-2* on repeat-induced point mutation (RIP) and recombination events that excise DNA sequence duplications at the *his-3* locus of *Neurospora crassa. Curr. Genet.* **23:** 496–500.

© 2002 Elsevier Science (USA)
All rights reserved.

Bull, J. H., Smith, D. J., and Turner, G. 1988. Transformation of *Penicillium chrysogenum* with a dominant selectible marker. *Curr. Genet.* **13:** 377–382.

Casqueiro, J., Banuelos, O., Gutierrez, S., Hijarrubia, M. J., and Martin, J. F. 1999. Intrachromosomal recombination between direct repeats in *Penicillium chrysogenum:* Gene conversion and deletion events. *Mol. Gen. Genet.* **261:** 994–1000.

Colot, V., Haedens, V., and Rossignol, J. L. 1998. Extensive, nonrandom diversity of excision footprints generated by *Ds*-like transposon *Ascot-1* suggests new parallels with V(D)J recombination. *Mol. Cell. Biol.* **18:** 4337–4346.

Davis, R. H., and DeSerres, E. F. 1970. Genetic and microbiological research techniques for *Neurospora crassa. Methods Enzymol.* **17:** 79–143.

Doolittle, W. F., Kirkwood, T. B. L., and Dempster, M. A. H. 1984. Selfish DNAs with self-restraint. *Nature* **307:** 501–502.

Doolittle, W. F., and Sapienza, C. 1980. Selfish genes, the phenotype paradigm and genome evolution. *Nature* **284:** 601–603.

Döring, H. P., Tillmann, E., and Starlinger, P. 1984. DNA sequence of the maize transposable element *Dissociation. Nature* **307:** 127–131.

Ebbole, D., and Sachs, M. S. 1990. A rapid and simple method for isolation of *Neurospora crassa* homokaryons using microconidia. *Fung. Genet. Newslett.* **37:** 17–18.

Federoff, N. 2000. Transposons and genome evolution in plants. *Proc. Natl. Acad. Sci. USA* **97:** 7002–7007.

Fedoroff, N. 1989. About maize transposable elements and development. *Cell* **56:** 181–191.

Fierro, F., Barredo, J. L., Diez, B., Gutierrez, S., Fernandez, F. J., and Martin, J. F. 1995. The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. *Proc. Natl. Acad. Sci. USA* **92:** 6200–6204.

Fierro, F., and Martin, J. F. 1999. Molecular mechanisms of chromosomal rearrangement in fungi. *Crit. Rev. Microbiol.* **25:** 1–17.

Finnegan, D. J. 1989. Eukaryotic transposable elements and genome evolution. *Trends Genet.* **5:** 103–107.

Gerlach, W. L., Dennis, E. S., Peacock, W. J., and Clegg, M. T. 1987. The *Ds1* controlling element family in maize and *Tripsacum. J. Mol. Evol.* **26:** 329–334.

Gómez-Gómez, E., Anaya, N., Roncero, M. I. G., and Hera, C. 1999. *Folyt1,* a new member of the *hAT* family, is active in the genome of the plant pathogen *Fusarium oxysporum. Fung. Genet. Biol.* **27:** 67–76.

Graessle, S., Haas, H., Friedlin, E., Kürnsteiner, H., Stöffler, G., and Redl, B. 1997. Regulated system for heterologous gene expression in *Penicillium chrysogenum. Appl. Environ. Microbiol.* **63:** 753–756.

Howad, W., and Kempken, F. 1997. Cell-type specific loss of *atp6* RNA editing in cytoplasmic male sterile *Sorghum bicolor. Proc. Natl. Acad. Sci. USA* **94:** 11090–11095.

Hua-Van, A., Daviere, J. M., Kaper, F., Langin, T., and Daboussi, M. J. 2000. Genome organization in *Fusarium oxysporum:* Clusters of class II transposons. *Curr. Genet.* **37:** 339–347.

Jain, S., Durand, H., and Tiraby, G. 1992. Development of a transformation system for the thermophilic fungus *Talaromyces sp.* CL240 based on the use of phleomycin resistance as a dominant selectable marker. *Mol. Gen. Genet.* **234:** 489–493.

Kempken, F., Jacobsen, S., and Kück, U. 1998. Distribution of the fungal transposon *Restless:* Full-length and truncated copies in closely related strains. *Fung. Genet. Biol.* **25:** 110–118.

Kempken, F., and Kück, U. 1996. *Restless,* an active *Ac*-like transposon from the fungus *Tolypocladium inflatum:* Structure, expression, and alternative RNA splicing. *Mol. Cell. Biol.* **16:** 6563–6572.

Kempken, F., and Kück, U. 1998a. Evidence for circular transposition derivatives from the fungal *hAT*-transposon *Restless. Curr. Genet.* **34:** 200–203.

Kempken, F., and Kück, U. 1998b. Transposons in filamentous fungi—Facts and perspectives. *BioEssays* **20:** 652–659.

Kempken, F., and Kück, U. 2000. Tagging of a nitrogen pathway-specific regulator gene in *Tolypocladium inflatum* by the transposon *Restless. Mol. Gen. Genet.* **263:** 302–308.

Kempken, F., and Windhofer, F. 2001. The *hAT* family: A versatile transposon group common to plants, fungi, animals, and man. *Chromosoma* **110:** 1–9.

Kidwell, M. G., and Lisch, D. 1997. Transposable elements as sources of variation in animals and plants. *Proc. Natl. Acad. Sci. USA* **94:** 7704–7711.

Koo, K., and Stuart, W. D. 1991. Sequence and structure of *mtr,* an amino acid transport gene of *Neurospora crassa. Genome* **34:** 644–651.

Kunze, R. 1996. The maize transposable element *Activator* (*Ac*). *Curr. Top. Microbiol. Immunol.* **204:** 161–194.

Kunze, R., and Starlinger, P. 1989. The putative transposase of transposable element *Ac* from *Zea mays* L. interacts with subterminal sequences of *Ac. EMBO J.* **8:** 3177–3185.

Lecellier, G., and Silar, P. 1994. Rapid methods for nucleic acids extraction from petri dish grown mycelia. *Curr. Genet.* **25:** 122–123.

Lowe, B., Mathern, J., and Hake, S. 1992. Active *Mutator* suppress the knotted phenotype and increase recombination at the *Kn1-0* tandem duplication. *Genetics* **132:** 813–822.

McClintock, B. 1947. Cytogenetic studies of maize and *Neurospora* mutable loci. *Carnegie Inst. Washington Year Book* **46:** 146–152.

McClintock, B. 1951. Chromosome organization and genic expression. *Cold Spring Harbor Symp. Quant. Biol.* **16:** 13–47.

McDonald, J. F. 1993. Evolution and consequences of transposable elements. *Curr. Opin. Genes Dev.* **3:** 855–864.

Nordborg, M., and Walbot, V. 1995. Estimating allelic diversity generated by excision of different transposon types. *Theor. Appl. Genet.* **90:** 771–775.

Okuda, M., Ikeda, K., Namiki, F., Nishi, K., and Tsuge, T. 1998. *Tfo1,* an *Ac*-like transposon from the plant pathogenic fungus *Fusarium oxysporum. Mol. Gen. Genet.* **258:** 599–607.

Orgel, L., and Crick, F. H. C. 1980. Selfish DNA—The ultimate parasite. *Nature* **284:** 604–607.

Saedler, H., and Gierl, A. 1996. *Transposable Elements.* Springer Verlag, Berlin/Heidelberg/New York.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stadler, D., Macleod, H., and Dillon, D. 1991. Spontaneous mutation at the *mtr* locus of *Neurospora:* The spectrum of mutant types. *Genetics* **129:** 39–45.

© 2002 Elsevier Science (USA)
All rights reserved.

Stimberg, N., Walz, M., Schörgendorfer, K., and Kück, U. 1992. Electrophoretic karyotyping from *Tolypocladium inflatum* and six related strains allows differentiation of morphologically similar species. *Appl. Microbiol. Biotechnol.* **37:** 485–489.

Sutton, W. D., Gerlach, W. L., Schwartz, D., and Peacock, W. J. 1984. Molecular analysis of *Ds* controlling element mutations at the *Adh1* locus of maize. *Science* **223:** 1265–1268.

Urban, A., Neukirchen, S., and Jaeger, K. E. 1997. A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR. *Nucleic Acids Res.* **25:** 2227–2228.

Varagona, M., and Wessler, S. R. 1990. Implications for the *cis*-requirements for *Ds* transposition based on the sequence of the *wxB4 Ds* element. *Mol. Gen. Genet.* **220:** 414–418.

Windhofer, F., Catcheside, D. E. A., and Kempken, F. 2000. Methylation of the foreign transposon *Restless* in vegetative mycelia of *Neurospora crassa. Curr. Genet.* **37:** 194–199.

Xiao, Y.-L., Li, X., and Peterson, T. 2000. Ac insertions site affects the frequency of transposon-induced homologous recombination at the maize *p1* locus. *Genetics* **156:** 2007–2017.

Xiao, Y.-L., and Peterson, T. 2000. Intrachromosomal homologous recombination in *Arabidopsis* induced by a maize transposon. *Mol. Gen. Genet.* **263:** 22–29.

Yuan, G. F., and Marzluf, G. A. 1992. Molecular characterization of mutations of *nit4,* the pathway-specific regulatory gene which controls nitrate assimilation in *Neurospora crassa. Mol. Microbiol.* **6:** 67–73.

© 2002 Elsevier Science (USA)
All rights reserved.