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Hiroshi Shiotani · Takashi Tsuge

Efficient gene targeting in the filamentous fungus *Alternaria alternata*

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Abstract To characterize homologous recombination of transforming DNA in the filamentous fungus *Alternaria alternata*, we have compared the frequencies of gene targeting by circular and linear DNA fragments in the fungus. The *A. alternata BRM1* gene, which is an essential gene for melanin biosynthesis, was selected as a target locus. *BRM1* targeting events are easily identified because loss of function leads to a change in mycelial color from black to light brown. We constructed targeting vectors by inserting 0.6 to 3.1 kb internal *BRM1* segments into a plasmid containing the hygromycin B phosphotransferase gene. When circular plasmids were used, melanin-deficient (Mel$^-$) transformants accounted for 30 to 80% of hygromycin B-resistant (Hy^R) transformants, correlating closely with the size of the *BRM1* segment in the transforming DNA. Restriction enzyme digestion within the *BRM1* region greatly enhanced the frequency of gene targeting: integration of the linear plasmids was almost completely attributable to homologous recombination, regardless of the size of the *BRM1* segments. Plasmids carrying both *BRM1* segments and rDNA segments were transformed into the fungus to examine the effect of the number of target copies on homologous recombination. Using the circular plasmids, Mel$^-$ transformants accounted for only 5% of Hy^R transformants. In contrast, when the linear plasmid produced by restriction enzyme digestion within the *BRM1* segment was used, almost all transformants were Mel$^-$. These results indicate that homologous integration of circular molecules in *A. alternata* is sensitive to the length of homology and the number of targets, and that double-strand breaks in transforming DNA greatly enhance homologous recombination.

Key words *Alternaria alternata* · Gene targeting · Melanin biosynthesis gene

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H. Shiotani[1] · T. Tsuge (✉)
School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01, Japan

Present address:
[1]Fruit Tree Research Station, Kuchinotsu Branch, Otsu 870, Kuchinotsu-Cho, Minamitakagi-Gun, Nagasaki 859-25, Japan

Introduction

Gene transfer by transformation is crucial to the development of gene cloning systems and has been described in a number of fungi (Fincham 1989; Hynes 1986). Most transformation schemes in filamentous fungi are based on integration of donor DNA into a chromosomal locus. In *Saccharomyces cerevisiae* cells, integration appears to be completely attributable to homologous recombination (Hinnen et al. 1978; Kingsman et al. 1981). In contrast, ectopic integration of transforming DNA is more common in other eukaryotes, including filamentous fungi (Capecchi 1989; Fincham 1989; Hynes 1986; Zimmer 1992). Homologous recombination of exogenous DNA permits integrative gene disruption and the replacement of genomic segments by sequences that have been altered in vitro. Since such techniques provide a powerful tool for evaluating gene functions and constructing well defined mutations, they were originally developed in *S. cerevisiae* (Orr-Weaver et al. 1983; Rothstein 1991; Winston et al. 1983) and expanded to several filamentous fungi, such as *Aspergillus nidulans* (Miller et al. 1985), *Neurospora crassa* (Paietta and Marzluf 1985) and *Ustilago maydis* (Fotheringham and Holloman 1989; Kronstad et al. 1989). However, integrative gene disruption and replacement do not seem as easily attainable in filamentous fungi as in *S. cerevisiae* (Fincham 1989; Hynes 1986).

We previously observed that the filamentous fungus *Alternaria alternata* possesses a homologous

recombination system similar to that of *S. cerevisiae* (Tsuge et al. 1990). The deuteromycotine fungus *A. alternata* is one of the most cosmopolitan of fungal species. It contains seven phytopathogenic variants, designated pathotypes (Kusaba and Tsuge 1994; Nishimura and Kohmoto 1983). These seven pathotypes cause severe diseases on different plants by producing host-specific toxins effective only against the host plant (Nishimura and Kohmoto 1983). When we used integrative transformation vectors carrying *A. alternata* rDNA segments, the plasmids integrated into the target rDNA locus of the fungal chromosome by a single-crossover event, which dramatically improved the transformation frequency (Tsuge et al. 1990). This feature of *A. alternata* is expected to be useful and critical for analysis of fungal genes of interest, such as pathogenicity-related genes.

Here, we investigate gene targeting in *A. alternata* using a phenotypic marker. To facilitate detection of the homologous recombination event, we targeted the *A. alternata BRM1* gene, which is essential for melanin biosynthesis in the fungus (Kimura and Tsuge 1993). The fungus produces melanin via polyketide biosynthesis, the last step being the polymerization of 1,8-dihydroxynaphthalene (Kimura and Tsuge 1993; Tanabe et al. 1988). The *BRM1* product is involved in conversion of scytalone to 1,3,8-trihydroxynaphthalene, which is an essential step in the melanin biosynthetic pathway (Kimura and Tsuge 1993). The $brm1^-$ mutation is not lethal, but causes a distinct phenotypic alteration in mycelial color: mycelial color of the $brm1^-$ strains cultured on an agar medium is light brown, while the wild-type strains produce dark green to black mycelia (Kimura and Tsuge 1993; Tanabe et al. 1988).

Homologous recombination of exogenous DNA has been well characterized in *S. cerevisiae* (Capecchi 1989; Orr-Weaver et al. 1983; Rothstein 1991; Shortle et al. 1982; Szostak and Wu 1980; Wilson et al. 1994; Winston et al. 1983). It has been reported that the presence of double-strand breaks in donor DNA promotes homologous recombination (Orr-Weaver and Szostak 1983; Orr-Weaver et al. 1981); such promotion has also been observed in other eukaryotes (Capecchi 1989; Fincham 1989; Hynes 1986; Szostak et al. 1983; Zimmer 1992). Furthermore, the frequency of gene targeting depends on the number of target copies in the yeast genome (Szostak and Wu 1980; Wilson et al. 1994), although targeted integration is insensitive to the number of targets in mammalian cells (Zheng and Wilson 1990). While homologous recombination of transforming DNA has been reported in several filamentous fungi, it has not been studied systematically. This communication reports on homologous recombination of exogenous DNA in the filamentous fungus *A. alternata*, investigating the effects of double-strand breaks in transforming DNA, and the size and copy number of target DNA.

Materials and methods

Fungal and bacterial strains

The strains 15A and AKT88-2 of the Japanese pear pathotype of *A. alternata* were used in this experiment. The pathotype causes black spot on certain Japanese pear cultivars by producing a host-specific toxin, called AK-toxin (Nakashima et al. 1985; Otani et al. 1985; Tanaka 1933). Strain 15A is a wild type, and strain AKT88-2 is a light-brown mutant carrying the $brm1^-$ mutation. The mutant was isolated from strain 15A by N-methyl-N′-nitro-N-nitrosoguanidine treatment of conidia (Tanabe et al. 1988). The *BRM1* gene is involved in conversion of scytalone to 1,3,8-trihydroxynaphthalene in the melanin biosynthetic pathway, the $brm1^-$ mutation leads to abnormal pigmentation (light brown) of fungal tissues (Kimura and Tsuge 1993; Tanabe et al. 1988). The strains were routinely maintained on potato sucrose agar.

Escherichia coli strain HB101 was used for cloning and propagation of plasmids.

Construction of plasmids

Plasmid vectors used are shown in Fig. 1. All manipulations for construction of plasmids were performed by standard recombinant DNA techniques (Sambrook et al. 1989). The pGDB1 series plasmids used for the *BRM1* gene targeting were constructed by inserting internal segments of this gene into the fungal integrative transformation vector pSH75 (Kimura and Tsuge 1993) (Fig. 1A). The plasmid pSH75 carries the *hph* (hygromycin B phosphotransferase) gene (Gritz and Davies 1983), which is regulated by the *Aspergillus nidulans trpC* promoter and terminator sequences (Mullaney et al. 1985), as a selectable marker. Four fragments (0.6, 1.4, 2.2 and 3.1 kb in length, respectively) from within the *BRM1* transcription unit were isolated and blunted with T4 DNA polymerase (Fig. 1B). Each of these fragments was introduced into the *Eco*RV site of pSH75 to produce pGDB1 series plasmids.

The pDH25 series plasmids were made by inserting the 2.2-kb *BRM1* setment (*Eco*RI-*Eco*RV fragment in Fig. 1B) and/or the 2.7-kb rDNA segment (Tsuge et al. 1989) into plasmid pDH25 containing the *hph* gene as a selectable marker (Cullen et al. 1987) (Fig. 1C). The *BRM1* and rDNA segments were introduced into the *Pvu*II and *Xba*I sites, respectively, in pDH25.

Fungal transformation

Protoplasts of *A. alternata* were prepared and transformed with plasmid DNA according to the method described previously (Tsuge et al. 1990). Transformation was conducted using about 2×10^7 protoplasts and 5 μg plasmid DNA. Following transformation, the protoplasts were plated directly onto a selective regeneration medium (0.1% yeast extract, 0.1% casein enzymatic hydrolysate, 1 M sucrose, 2% agar, 100 μg hygromycin B per ml). To evaluate melanin production by transformants, colonies that appeared on the regeneration medium were transferred to PSA plates containing 100 μg hygromycin B per ml. Mycelial color was observed after incubation at 25° C for 5 days.

DNA extraction

Fungi were grown in 50 ml of potato sucrose broth in 100-ml Erlenmeyer flasks at 25° C for 3 days on an orbital shaker (120 rpm). Total cellular DNA was prepared as described previously (Adachi et al. 1993).

Plasmid DNA was isolated from *E. coli* cells by the alkaline lysis method (Sambrook et al. 1989).

Southern blot analysis

Restriction enzyme digestion of DNA and fractionation in agarose gels were performed by standard methods (Sambrook et al. 1989). Fractionated DNA was transferred to Hybond N^+ nylon membrane (Amersham) by the alkaline transfer method (Reed and Mann 1985), and subjected to hybridization according to the manufacturer's recommendations. Hybridized blots were washed at 65° C; the final wash was done in 0.1 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, pH7.7, and 1 mM EDTA) plus 0.1% sodium dodecyl sulphate.

DNA probes were produced by labeling with [α-^{32}P] dCTP by the random-primer method (Feinberg and Vogelstein 1983).

Quantification of scytalone in fungal culture

The accumulation of scytalone, an intermediate in the direct pathway to melanin, was analyzed by high performance liquid chromatography (HPLC). Fungi were cultured on 20 ml of potato sucrose agar in petri dishes (9 cm diameter) at 25° C for 10 days. Scytalone was extracted from the fungal culture as described by Bell et al. (1976). The extract was dissolved in methanol (500 µl/plate), and an aliquot (1 to 20 µl) was subjected to HPLC. The HPLC system used was a Model BIP-1 instrument (Japan Spectroscopic). The samples were fractionated on reverse-phase Develosil ODS_7 column (4.6 × 250 mm, Nomura Chemicals) with methanol/acetic acid/water (30:1:69, v/v/v) at 1.0 ml/min. Detection was by monitoring absorbance at 275 nm. Authentic scytalone was purifed from the culture extract of strain AKT88-2 by the method of Bell et al. (1976).

Results

Effect of the size of homologous targets on recombination

The pGDB1 series plasmids were constructed by ligating internal fragments of the *A. alternata BRM1* transcription unit into pSH75 containing the *hph* gene (Fig. 1). Four plasmids with inserted *BRM1* fragments of different sizes were used to observe the size dependency of target DNA for homologous recombination.

pGDB1 series plasmids were used as circular molecules for transformation of *A. alternata* strain 15A, and transformants were selected for hygromycin B resistance (Hy^R). Hy^R transformants were assayed for melanin production. The circular pGDB1 series vectors produced melanin-deficient (Mel^-) transformants with mycelial color similar to that of strain AKT88-2 carrying the *brm1*$^-$ mutation; the vector plasmid pSH75 induced no Mel^- transformants (Table 1). The frequency of appearance of Mel^- transformants differed among the transforming plasmids. The difference correlated closely with the size of the *BRM1* segments introduced into the donor plasmids: the 3.1-kb, 2.2-kb, 1.4-kb and 0.6-kb internal *BRM1* segments generated Mel^-

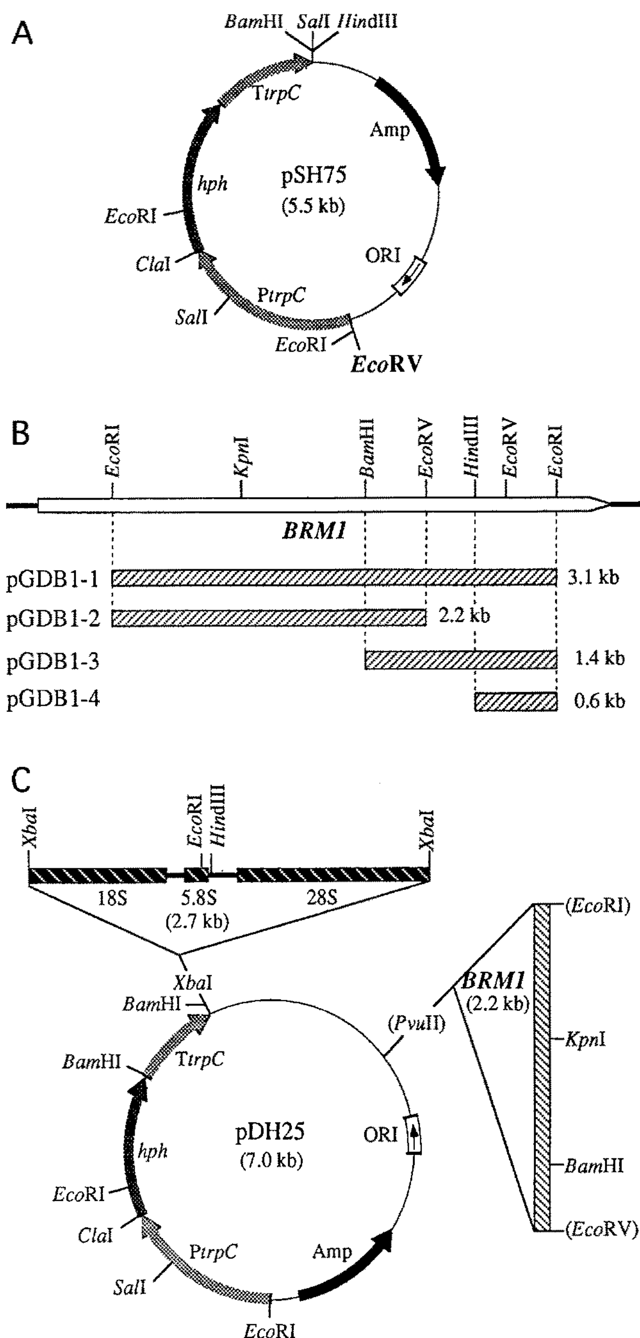

Fig. 1A Plasmid pSH75 used for construction of the pGDB1 series vectors containing the *A. alternata BRM1* gene. **B** Physical map of the *BRM1* locus. The *BRM1* transcription unit and its transcription direction are shown by the *arrow*. Four restriction fragments within the transcription unit were blunted with T4 DNA polymerase and inserted into the *Eco*RV site of pSH75 to produce pGDB1 series plasmids. **C** Transformation vectors containing internal sequences of the *A. alternata BRM1* gene and rDNA. Plasmids pDH25B1 and pDH25r1a possess the 2.2-kb *BRM1* (*Eco*RI-*Eco*RV fragment) and the 2.8-kb rDNA fragments, respectively, in pDH25. Plasmid pDH25B1r1a contains both fragments. Abbreviations: P*trpC* and T*trpC*, the *Aspergillus nidulans trpC* promoter and terminator sequences, respectively; *hph*, hygromycin B phosphotransferase gene; ORI, origin of replication; Amp, gene conferring resistance to ampicillin

Table 1 Frequency of melanin-deficient transformants following transformation of *Alternaria alternata* with circular or linear molecules of pGDB1 series plasmids

Plasmid[a]	*BRM1* insert (kb)	Transformants /µg DNA[b]	No. of transformants[c]		Mel$^-$ transformants (%)
			Mel$^+$	Mel$^-$	
Circular					
pSH75	None	2.0 ± 0.8	125	0	0.0
pGDB1-1	3.1	3.3 ± 1.8	34	158	82.3
pGDB1-2	2.2	4.1 ± 1.9	59	155	72.4
pGDB1-3	1.4	3.6 ± 1.8	60	104	62.4
pGDB1-4	0.6	2.1 ± 1.0	68	30	30.6
Linear					
pSH75	None	1.7 ± 0.3	83	0	0.0
pGDB1-1	3.1	31.1 ± 8.4	1	168	99.4
pGDB1-2	2.2	30.4 ± 7.7	2	178	98.9
pGDB1-4	0.6	10.7 ± 3.1	2	166	98.8

[a] Transformation vectors pSH75, pGDB1-1, pGDB1-2 and pGDB1-4 were linearized by cutting with *Eco*RV, *Kpn*I, *Kpn*I, and *Eco*RV, respectively (Fig. 1)
[b] Each value is the average with S.D. of five and three experiments using circular plasmids and linear plasmids, respectively. Each experiment, done with three to four replications, was conducted using protoplasts prepared separatively
[c] Mel$^+$, melanin-producing; Mel$^-$, melanin-deficient

transformants at frequencies of 82.3, 72.4, 62.4 and 30.6%, respectively (Table 1).

To confirm that the Mel$^-$ transformants were attributable to integrative disruption of the *BRM1* locus via homologous recombination of donor plasmids, we analyzed the integration mode of pGDB1-2 in the Mel$^-$ transformant chromosomes. Five each of Mel$^-$ and Mel$^+$ transformants obtained with pGDB1-2 DNA were subjected to Southern blot analysis. Total DNA of the transformants and the wild-type strain was digested with *Sal*I, which has two recognition sites in pGDB1-2, and the blot was probed with the 2.2-kb *BRM1* sequence present in pGDB1-2 (Fig. 2A). The probe hybridized only to an expected 15.5-kb band in the wild-type strain. In contrast, DNA of Mel$^-$ transformants lacked this fragment, indicating that pGDB1-2 DNA has inserted in the *BRM1* locus of the Mel$^-$ transformants (Fig. 2A). DNA from the Mel$^-$ transformants shared common 5.5-kb, 8.6-kb and 12.8-kb fragments (Fig. 2A). The 8.6-kb and 12.8-kb fragments corresponded to the junctions between the recipient *BRM1* region and integrated pGDB1-2 (Fig. 2C). An additional 5.5-kb band was derived from tandem repeats of integrated vector DNA (Fig. 2C). Two Mel$^-$ transformants (lanes 1 and 2 in Fig. 2A) possessed several additional fragments. These fragments seem to have resulted from the recombination of plasmids integrated at the *BRM1* locus or from the nonhomologous integration of the plasmid. DNA from five Mel$^+$ transformants carried the 15.5-kb fragment which was detected in the wild-type strain (Fig. 2A), showing that these transformants resulted from ectopic integration of pGDB1-2 DNA (Fig. 2C). The 5.5-kb fragment derived from tandem integrations of pGDB1-2 was also detected in all Mel$^+$ transformants.

The *brm1*$^-$ mutation is known to induce the accumulation of scytalone, an intermediate of melanin biosynthesis, in culture (Bell et al. 1976; Bell and Wheeler 1986; Kimura and Tsuge 1993; Tanabe et al. 1988). Five Mel$^-$ transformants obtained with pGDB1-2 were quantitatively analyzed for scytalone accumulation in culture, and the amounts were found to be similar to that seen for strain AKT88-2 carrying the *brm1*$^-$ mutation. The amount of scytalone detected ranged from 45 to 71 µg per ml of culture for the Mel$^-$ transformants and strain AKT88-2. Scytalone was not detected in the culture of wild-type strain or Mel$^+$ transformants. These results confirm that the Mel$^-$ transformants carry a *brm1*$^-$ mutation brought about by targeting of the *BRM1* gene.

Effect of double-strand breaks in transforming DNA on recombination

We also used pGDB1 series plasmids as linear molecules; these were produced by cleavage at a unique restriction site present in the *BRM1* insert, giving rise to a DNA segment that has homology with the target site at each end (Fig. 1B). Plasmids pGDB1-1 and pGDB1-2 were linearized by digestion with *Kpn*I. Linearized pGDB1-4 was produced by *Eco*RV digestion. Since no available enzyme was found for pGDB1-3, it could not be used in this experiment. The linearized pGDB1-1, pGDB1-2 and pGDB1-4 significantly enhanced transformation frequency of the fungus, as compared with the circular molecules (Table 1). The frequency of *BRM1* targeting was increased dramatically by using the linear molecules (Table 1). Size dependency of targeting DNA for homologous integration was not

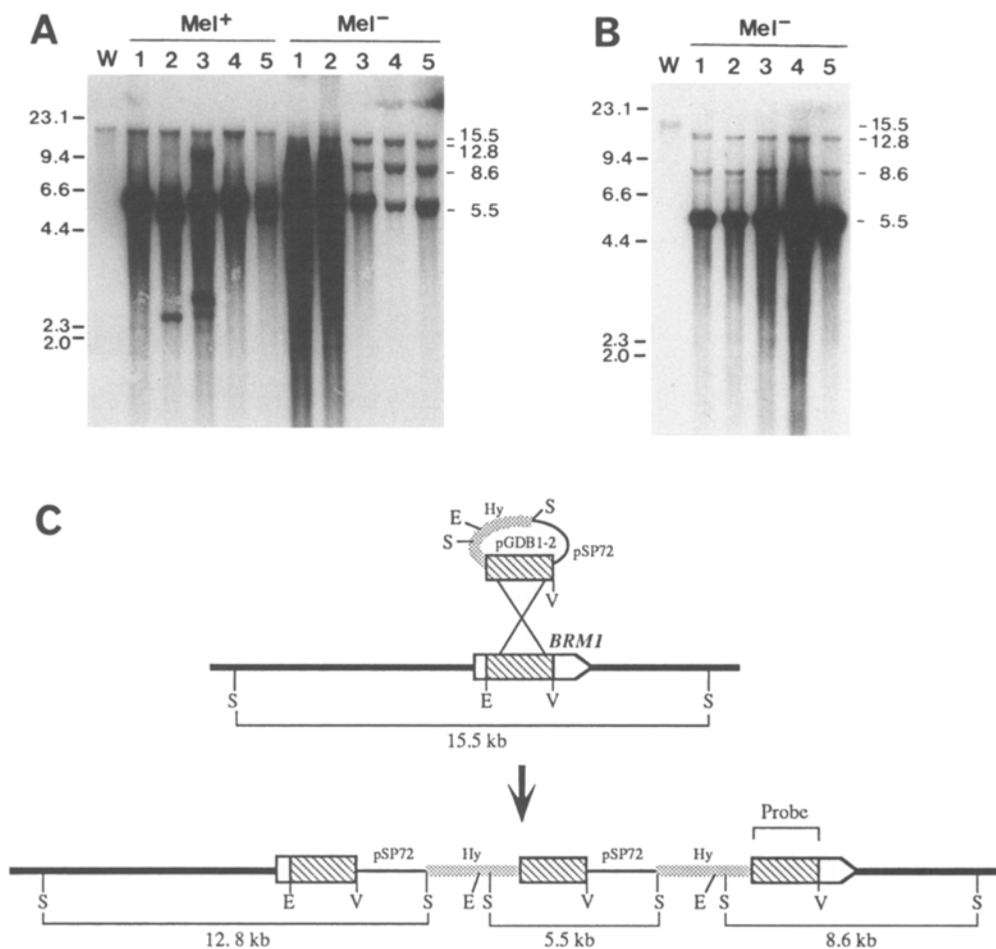

Fig. 2 DNA blot analysis of melanin-deficient strains produced by transformation of strain 15A with pGDB1-2. Total DNA digested with *Sal*I was electrophoresed in 0.8% agarose gel, and the blot was probed with ^{32}P-labeled *BRM1* sequence (the 2.2-kb *BRM1* fragment from pGDB1-2). The sizes (in kb) of marker DNA fragments (*Hin*dIII-digested λ DNA) are indicated on the left. **A** Strain 15A (lane W) and melanin-producing (Mel$^+$) and melanin-deficient (Mel$^-$) transformants produced with the circular molecules of pGDB1-2. **B** Strain 15A (lane W) and Mel$^-$ transformants produced with the linear molecules of pGDB1-2. **C** Structure of the *A. alternata BRM*1 locus before and after recombination with gene disruption vector pGDB1-2. The 5.5-kb fragment, which resulted from the integration of pGDB1-2 as a tandem array, was detected in all transformants. The integration of two copies of pGDB1–2 into the *BRM1* locus by homologous recombination through a single crossover event is shown as an example. Restriction site abbreviations: E, *Eco*RI; S, *Sal*I; V, *Eco*RV. Hy, hygromycin resistance cassette; the *thin line* represents sequences derived from pSP72 (Promega), which form part of the pSH75 vector (see Fig. 1)

observed when the linear molecules were used: almost all colonies transformed with the linearized pGDB1-1, pGDB1-2 and pGDB1-4 were Mel$^-$.

Southern blot analysis of the Mel$^-$ transformants showed that the transforming DNA was targeted to the *BRM1* site of the recipient chromosome (Fig. 2B).

Effect of the number of homologous targets on recombination

To observe the effect of the number of homologous targets on recombination of donor DNA we used pDH25 series plasmids (Fig. 1C). In these plasmids *BRM1* and/or rDNA segments are inserted in pDH25. The *A. alternata* genome contains a single copy of the *BRM1* gene and about 200 copies of the rDNA units in tandem array (Kimura and Tsuge 1993; Tsuge et al. 1989).

The pDH25 series plasmids were used as circular molecules for transformation of strain 15A. Plasmids pDH25rla and pDH25B1r1a containing rDNA segments produced many more transformants than the original vector pDH25 (Table 2). In contrast, pDH25B1, carrying the *BRM1* segment, did not significantly enhance transformation frequency (Table 2). Although all transformants obtained with pDH25 and pDH25r1a produced melanin, pDH25B1 and pDH25B1r1a yielded Mel$^-$ transformants whose mycelial color was identical to that of the *brm1*$^-$ strain AKT88-2 (Table 2). The frequency of Mel$^-$ transformants, however, differed markedly between pDH25B1 and pDH25B1r1a. Mel$^-$ transformants accounted for 73.3% of pDH25B1 transformants but only 5.1% of pDH25B1r1a transformants, indicating that the circular pDH25B1r1a DNA was integrated predominantly into the rDNA locus.

Since the pDH25B1r1a DNA contained a unique *Kpn*I site within the *BRM1* segment and a unique *Hin*dIII site within the rDNA segment, the plasmid DNA was linearized by cutting with *Kpn*I or *Hin*dIII and used for transformation of strain 15A. Use of the *Kpn*I-digested molecules markedly increased the frequency of *BRM1* targeting: Mel$^-$ transformants accounted for 97% of the transformants (Table 2). This frequency was similar to that observed in the transformation with the *Kpn*I-digested pDH25B1. The *Hin*dIII-digested pDH25B1r1a enhanced the transformation efficiency

Table 2 Frequency of melanin-deficient transformants of *Alternaria alternata* obtained using pDH25 series plasmids

Plasmid[a]	Insert (kb)		Transformants /μg DNA[b]	No. of transformants[c]		Mel^- transformants (%)
	BRM1	rDNA		Mel^+	Mel^-	
Circular						
pDH25	None	None	1.8 ± 1.0	41	0	0.0
pDH25B1	2.2	None	3.2 ± 1.4	20	55	73.3
pDH25r1a	None	2.7	48.1 ± 19.4	346	0	0.0
pDH25B1r1a	2.2	2.7	58.0 ± 22.4	426	23	5.1
Linear						
pDH25B1 (*Kpn*I digest)	2.2	None	32.2 ± 10.3	10	193	95.1
pDH25B1r1a (*Kpn*I digest)	2.2	2.7	33.7 ± 13.3	5	195	97.5
pDH25B1r1a (*Hin*dIII digest)	2.2	2.7	121.5 ± 27.7	572	0	0.0

[a] See Fig. 1
[b] Each value is the average with S.D. of four experiments which were conducted using protoplasts prepared separately
[c] Mel^+, melanin-producing; Mel^-, melanin-deficient

relative to circular pDH25B1r1a, and produced only Mel^+ transformants (Table 2).

Discussion

We studied homologous recombination of exogenous DNA in *A. alternata* to gain a better understanding of its transformation mechanism and to expand the range of genetic manipulations possible with this fungus. The *BRM1* gene, which is one of the essential genes for melanin biosynthesis of *A. alternata*, was selected as a target locus because disruption leads to a change in mycelial color easily distinguishable from the parental phenotype. An integrative disruption system (Shortle et al. 1982) was employed to observe the frequency of gene targeting.

The first experimental approach was to observe the effects of the size and double-strand breaks of substrate DNA on homologous recombination using vector plasmids containing different-sized segments of the *BRM1* gene. Although homologous integration of circular molecules is rather rare in transformation of filamentous fungi (Fincham 1989; Hynes 1986), both linear and circular molecules produced *brm1*$^-$ transformants in *A. alternata*. When we used circular plasmids, the frequency of gene targeting was proportional to the length of homologous sequence. This indicates that successful homologous integration of the circular molecules is rate-limited by the length of homology. However, there were no significant differences in transformation efficiency among plasmids, irrespective of the presence or absence of the *BRM1* segments or the segment length.

Integration of the linearized molecules of the pGDB1 series was completely attributable to homologous recombination, regardless of the size of homology. When pGDB1-1, pGDB1-2 and pGDB1-4 were cut at single restriction sites within the *BRM1* segments and transformed into *A. alternata*, almost all transformants contained a *brm1*$^-$ mutation due to *BRM1* targeting. This indicates that double-strand breaks could play a more important role in homologous recombination than the length of homology over the range of length we examined. Double-strand breaks in transforming plasmids also enhanced the transformation efficiency of pGDB1 series plasmids, although the effect was not as marked as in *S. cerevisiae*. Orr-Weaver et al. (1983, 1981) reported that the introduction of a double-strand break into a region of the yeast DNA on a transforming plasmid leads to as much as 1000-fold stimulation of the transformation frequency. Promotion of homologous integration and of transformation frequency by double-strand breaks of transforming DNA has also been observed in other eukaryotes, including filamentous fungi (Capecchi 1989; Fincham 1989; Hynes 1986; Szostak et al. 1983: Zimmer 1992). The impact observed on transformation of *A. alternata* seems to be greater than seen for the filamentous fungi investigated to date. Linearization of pSH75 DNA, which carries no sequence homologous to the *A. alternata* genome, had no impact on transformation frequency, suggesting that ectopic integration of transforming DNA in *A. alternata* is not rate-limited by double-stranded breaks.

The role of the length of homology on the frequency of transformation has not been studied systematically. Rothstein (1991) reported experimental data concerning the minimum length of homology necessary for homologous integration of plasmid vectors in *S. cerevisiae*. In one case, cleaved sequences with 125 necleotides of homology (37 nucleotides on one side and 88 on the other) did not target the homologous site, whereas targeted integration was obtained with a sequence containing 250 nucleotides of homology (40 and

210 nucleotides) (Rothstein 1991). A low frequency of transformation (less than 10 transformants per 85 μg DNA) at the *CYC1* locus has been observed in yeast with a 20-bp oligonucleotide, although no transformants were detected using 10- or 15-bp oligonucleotides (Moerschell et al. 1988). Schiestl and Petes (1991) reported that four basepairs of homology are sufficient for some types of homologous recombination in *S. cerevisiae*. They employed DNA fragments with no homology to the *S. cerevisiae* genome; when these fragments were cut with *Bam*HI and transformed into *S. cerevisiae*, they were integrated into genomic GATC sites. Since the GATC sequence is present at the ends of *Bam*HI fragments, they proposed the conclusion mentioned above (Schiestl and Petes 1991). Although these studies did not provide a definite minimum length, it is evident that for successful homologous recombination short stretches of nucleotide homology to the *S. cerevisiae* genome are frequently sufficient. The linearized molecules of pGDB1-4, which contains the shortest (0.6 kb in length) *BRM1* segment among the plasmids used, carried about 0.2 kb *BRM1* sequence on one side and about 0.4 kb on the other side. Thus, these lengths of homology could be enough for homologous recombination of exogenous DNA in *A. alternata*.

The second experimental approach was to investigate the effect of the number of target copies on the frequency of gene targeting, using plasmid vectors containing a *BRM1* segment and an rDNA segment. The *A. alternata* genome possesses a single copy of the *BRM1* gene and about 200 copies of the rDNA units in tandem array (Kimura and Tsuge 1993; Tsuge et al. 1989). When we used circular DNA molecules for transformation of *A. alternata*, the transformation frequency was dependent on the number of target copies: it was markedly higher in plasmids carrying the rDNA segment than in plasmids containing only the *BRM1* segment. The frequency of homologous integration of the circular molecules also depended on the number of target copies. Plasmid pDH25B1r1a, containing both rDNA and *BRM1* segments, integrated predominantly into the rDNA locus. Furthermore, linearized pDH25B1r1a DNA cut within the rDNA segment produced many more transformants than that cut within the *BRM1* segment. These results are in agreement with those of previous studies of transformation in *S. cerevisiae* (Szostak and Wu 1980; Wilson et al. 1994), but not to those in mammalian cells (Zheng and Wilson 1990). We conclude that the search for a homologous partner is the rate-limiting step in the successful integration of the circular and linear DNA molecules in *A. alternata*.

There were no significant differences in transformation or *BRM1* targeting frequency between pDH25B1 and pDH25B1r1a digested with *Kpn*I. This enzyme cuts at the boundaries of the DNA segments that share homology with the target *BRM1* locus. Thus, the rDNA segment within pDH25B1r1a could not disturb the *BRM1* targeting by *Kpn*I-digested pDH25B1r1a.

One more characteristic of *A. alternata* transformation is high-copy-number integration of transforming DNA in tandem arrays. Estimation of the approximate number of integrated plasmid copies showed that some transformants carry more than 100 copies of the plasmid per haploid genome. Such high-copy-number integration was observed in transformation of *A. alternata* with both circular and linear plasmids that contain the rDNA segment or the *BRM1* segment or lack any homologous segment. Lopes et al. (1991) reported high-copy-number integration of a transformation vector carrying rDNA segments in *S. cerevisiae*. They demonstrated that such integration is achieved by amplification of a small number of copies initially integrated in the rDNA locus. We also observed amplification of transforming DNA under strong selection pressure, i.e., application of a high concentration of hygromycin B during culture of transformants (H. Shiotani and T. Tsuge, unpublished results). These features in transformation of *A. alternata* are useful for making mutants that express genes of interest at high level in the fungal cells. Mechanisms of homologous recombination, high-copy-number integration and amplification of settled plasmid in *A. alternata* remain to be identified.

Several genes possibly involved in pathogenicity of phytopathogenic fungi have recently been cloned, and one-step gene replacement and integrative gene disruption were applied to characterize the crucial roles of cloned genes in pathogenicity (Desjardins et al. 1992; Marmeisse et al. 1993; Panaccione et al. 1992; Rogers et al. 1994; Scott-Craig et al. 1990; Stahl and Shäfer 1992; Sweigard et al. 1992; Talbot et al. 1993). However, many transformants needed to be screened for preparation of gene-targeted strains, because homologous integration of exogenous DNA was rather rare in most fungi, especially using circular DNA molecules. As far as we know, homologous transformation similar to that in *A. alternata* has been reported only in *Cochliobolus heterostrophus* (Turgeon et al. 1987). When circular plasmids containing a *C. heterostrophus* chromosomal segment were transformed into the fungus, most, but not all, plasmid integration events were at homologous chromosomal sites. The gene targeting technique described here is simple and can be used more easily in *A. alternata* than in other filamentous fungi. This will certainly contribute to our long-term goal – the elucidation of the genetic regulation of the pathogenicity of *A. alternata* pathogens that depend on host-specific toxins.

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