Multiple cotransformations in *Coprinus cinereus*

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Plasmids usually integrate ectopically into the genome of the homobasidiomycete *Coprinus cinereus* in transformations. Often, integration occurs at multiple sites indicating that more than one plasmid copy was incorporated. This feature prompted us to study transformation with mixtures of several different plasmids in several genetic backgrounds. We found multiple cotransformation to be efficient even with four different plasmids. Simultaneous uptake of a second plasmid was between 5-55%, of two additional plasmids between 3-18% and 3% for three additional plasmids. These high frequencies make possible the analysis of interactions between different heterologous genes introduced into the same nucleus.

Transformation in *C. cinereus* is an efficient method to introduce foreign DNA into the fungal host. Several hundreds of transformants can be obtained per µg DNA. Due to uptake of several DNA molecules, a high percentage of these transformants contains multiple plasmid copies integrated at different places and/or in tandem arrangement (Binninger *et al.* 1987 EMBO J. **6**:835-840; Granado *et al.* 1997 Mol. Gen. Genet. **256:**28-36). Use of this feature has been made in cotransformations where one plasmid with a selectable marker serves first to identify transformants and integration of another plasmid with a gene of interest is secondarily scored (Kües *et al.* 1992 Genes Dev. **6**:568-577; O'Shea *et al.* 1998 Genetics **148**:1081-1090). Isolated mating type genes have been introduced into *C. cinereus* monokaryons by cotransformation. Expression of heterologous *A* mating type genes in transformants resulted in the formation of clamp cells at the hyphal septa due to an interaction between compatible products of heterologous and endogenous *A* genes (Kües *et al.* 1992). Transfer of heterologous *B* genes was identified in matings with semi-compatible monokaryons differing in the *A* but not in the *B* specificity from the strain used in transformation. Transformants expressing heterologous *B* genes in addition to their endogenous *B* genes form dikaryons with fused clamp cells with the mating partner (O'Shea *et al.* 1998). In this study, we took advantage of these phenotypes to analyse the efficiency of multiple cotransformation. As far as we are aware, such multiple cotransformation experiments have not been performed with any filamentous fungus before.

Multiple cotransformation of monokaryons. In a first set of experiments, we used the tryptophan auxotrophic monokaryons AT8 (*A43*, *B43*, *trp-3*, *ade-8*), FA2222 (*A5 B6 acu-1 trp-1.1,1.6*), LT2 (*A6 B6 trp-1.1,1.6*) and 218 (*A3 B1 trp-.1.1,1.6*) (Kertesz-Chaloupková *et al.* 1998 Fungal Genet. Biol. **23**:95-109) as hosts and either plasmid pDB3 with the *C. cinereus trp-3*$^+$ wild type gene (Burrows 1991 PhD Thesis, London University, London UK) or plasmid pCc1001 with the *C. cinereus trp-1*$^+$ wild type gene (Binninger *et al.* 1989) for first selection of transformants. Plasmids with isolated mating type genes were pE12P5-3 carrying the *A43* mating type gene *a1-2* (Kües *et al.* 1994 Mol. Gen. Genet. **245**:45-52), pUK2 carrying the compatible *A42* mating type gene *a2-1* (Kües *et al.* 1992) and cosmid cJH8 carrying the complete DNA sequence of *B42* mating type locus (Halsall *et al.* 2000 Genetics **154**:1115-1123). Trp$^+$-transformants expressing heterologous *A* genes or heterologous *A* and *B* genes were identified microscopically due to presence of unfused and fused clamp cells, respectively. AT8, FA2222, LT2 and 218 transformants expressing heterologous *B42* genes were identified in crosses with monokaryon MK-50 (*A3 B43 trp-1*), monokaryon LT2, monokaryon FA2222 or monokaryons MK-45 (*A43 B1 ade-8*) and MK-70 (*A43 B1 trp-3 ade-8*), respectively. MK strains originated from a cross 210 x AT8 (this study).

Simultaneous transfer of two plasmids into the four different monokaryons was frequent in all experiments. Cotransformation frequencies for the *trp-3*$^+$ or *trp-1*$^+$ selection marker and a heterologous *A* gene were between 29-48% and for the respective selection marker and the heterologous *B* genes between 11-55% (Table 1 and 2). Moreover, expression of the three different genes originating from three different plasmids (*trp* selection marker, heterologous *A* gene and heterologous *B* genes) per Trp$^+$-transformant was also high and varied between 7-16% in the different experiments (Table 1 and 2). Cotransformation rates of the same plasmid(s) into different monokaryons were often but not always comparable (Table 1). Explicitly, transfer of the *B42* cosmid cJH8 in monokaryon 218 was considerably less often detected than in the other monokaryons (Table 1 and 2). When doubling the concentration of cosmid cJH8 (2 µg) in cotransformation of monokaryon 218, we did not obtain a significant increase in transformants expressing the *B42* genes (only 18%). Likewise, when repeating the set of transformations with monokaryon 218 using 700 ng pUK2 and 1 µg *B42* DNA cotransformation rates were similar (31% for plasmid pUK2 and 15% for the *B42* cosmid). These results might indicate that the system reached saturation. Work in *Neurospora crassa* suggested that appropriate chromosomal sites with double-strand breaks cause competence of nuclei for incorporation of incoming plasmid DNA. Only a few nuclei in a transformation experiment are competent for DNA uptake and it appears that these few nuclei have often more than one double-strand break, offering several sites for plasmid integration (Grotelueschen and Metzenberg 1995 Genetics **139**:1545-1551). A complete exploitation of the available plasmid integration sites within a nucleus offers an attractive explanation for the saturation levels possibly observed for *B42* DNA in *C. cinereus* monokaryon 218. However, since we scored transfer only by gene expression it is also possible that interaction of the *B42* gene products with those of the endogenous *B1* genes is generally poor in monokaryon 218 and, in consequence, actual DNA integration events might have been overlooked. Large variations in cotransformation efficiencies of mating type genes, as measured by phenotypic expression, are not uncommon with different monokaryons, even with DNA of the same amount from the same sample (Kües *et al.* 2001 Curr. Genet. 39:16-24), arguing for a variability in interactions between products of mating type genes. To have an overview of whether there are indeed insertions

undiscovered by phenotypic expression, Southern blots might be performed. However, for studying the interactions between products of heterologous genes in transformants, such an analysis is not very relevant but rather the frequency of phenotypic expression as determined here.

Multiple cotransformation of an A*-null strain.* In another set of experiments, using the tryptophan auxotrophic *C. cinereus* strain NA2 (*A B6 ade-8 trp-1.1,1.6*) and plasmid pCc1001 for selection, we studied uptake of up to four different plasmids in transformation. Strain NA2 is an *A*-null mutant whose *A* genes have been inactivated through gene replacement (E.H. Pardo 1995, PhD thesis, University of Oxford, Oxford UK). Thus, the strain only produces clamp cells when two compatible *A* genes such as *a1-2* and *a2-1* were newly introduced into the strain on separate plasmids. Fused clamp cells appeared when in addition to the compatible *A* genes heterologous *B42* genes have been transformed. Expression of only the *a1-2* gene from the *A43* locus was detected through mating with monokaryons 26 (*A5 B5 met-1*) or LN118 (*A42 B42 ade-2 trp-1.1,1.6*) (Kertesz-Chaloupková et al. 1998), expression of only the *a2-1* gene from the *A42* locus through mating with either the *A5 B5* monokaryon 26 or the *A43 B43* monokaryon AT8. To identify *a1-2* + *B42* and *a2-1* + *B42* transformants by dikaryon formation, crosses were performed with the *A5 B6* monokaryon FA2222.

Generally, the transformation efficiencies for mating type genes in these sets of experiments were lower as compared to data obtained with the monokaryons. Cotransformation frequencies of a second plasmid was detected at frequencies between 5-35%, of two additional plasmids at frequencies between 3-18% and of three additional plasmids at a frequency of 3% (Table 2). Thus, cotransformation frequencies of multiple plasmids do not decrease in relative relations to the frequencies of transfer of individual plasmids. In fact, the results indicate that plasmid uptake into a cell and/or integration into a nucleus is not random. We conclude, that in case a nucleus integrated a second plasmid, it will integrate with high probability a third or even a fourth plasmid. In consequence, multiple cotransformation in *C. cinereus* is an efficient way to introduce several different plasmids simultaneously into the same nucleus.

General conclusions. When wanting to study interactions between different heterologous genes in *C. cinereus*, one might choose to sequentially transform a strain with the genes of interest. However, every single transformation procedure does need its own selection marker and there are only a few available, mostly auxotrophy markers that will have to be combined in one background. Moreover, performance of a number of subsequent transformations is time-consuming. In this paper, we show that it is possible to simultaneously introduce mixtures of several plasmids into a cell by a single transformation step, needing only one selection marker. Even in the least optimal case (strain NA2), cotransformation efficiencies were high enough to detect positive clones in a reasonable sample number of tested transformants. We successfully have transformed five different *Coprinus* strains showing that the technique of multiple cotransformation will be widely applicable in *C. cinereus.*

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Table 1. Cotransformation of the *trp-1*$^{+}$ plasmid pCc1001 or the *trp-3*$^{+}$ plasmid pDB3 and plasmids with mating type genes into *C. cinereus* monokaryons[1]

Mating type gene(s) used in cotransformation	Mating type gene(s) expressed in Trp^{+} transformants	Cotransformation frequency (number of transformants expressing mating type gene(s)/ number of tested Trp^{+} transformants)		
		Monokaryon AT8	**Monokaryon FA2222**	**Monokaryon 218**
a2-1	*a2-1*	29% (24/83)	39% (25/64)	32 % (24/76)
B42	*B42*	55% (34/62)	54% (45/84)	11 % (3/28)
a2-1 + *B42*	*a2-1*	21% (23/110)	48% (29/60)	43% (30/70)
	a2-1 + *B42*	13% (14/110)	13% (8/60)	16% (11/70)

[1] 400 ng pBD3 (for strain AT8), 400 ng pCc1001 (for strains FA2222 and 218), 700 ng pUK2 with gene *a2-1* and 1 µg cJH8 with the *B42* mating type genes were applied in transformations, following the protocol of Granado *et al*. (1997).

Table 2. Cotransformation of the *trp-1*$^{+}$ plasmid pCc1001 and plasmids with mating type genes into *C. cinereus* monokaryon LT2[1]

Mating type gene(s) used in cotransformation	Mating type gene(s) expressed in Trp^{+} transformants	Cotransformation frequency (number of transformants expressing mating type gene(s)/number of tested Trp^{+} transformants)
a1-2	*a1-2*	48% (10/21)
B42	*B42*	31% (12/39)
a1-2 + B42	*a1-2*	44% (48/108)
	a1-2 + B42	7% (8/108)

[1] 400 ng pCc1001, 700 ng pE12P5-3 and 1 µg cJH8 were applied in transformations, following the protocol of Granado *et al.* (1997).

Table 3. Cotransformation of the *trp-1*$^{+}$ plasmid pCc1001 and plasmids with mating type genes into the *A*-null strain NA2[1]

Mating type gene(s) used in cotransformation	Mating type gene(s) expressed in Trp^{+} transformants	Cotransformation frequency	Number of transformants expressing mating type gene(s)/number of tested Trp^{+} transformants
a1-2	*a1-2*	10%	4/39
a2-1	*a2-1*	22%	8/36
B42	*B42*	20%	11/54
a1-2 + a2-1	*a1-2*	18%	3/17
	a2-1	35%	6/17
	a1-2 + a2-1	18%	3/17
a1-2 +B42	*a1-2*	13%	14/111
	B42	5%	6/111
	a1-2 + B42	3%	3/111
a2-1 + B42	*a2-1*	26%	18/69
	B42	16%	11/69
	a2-1 + B42	9%	6/69
a1-2 + a2-1 + B42	*a1-2*	12%	13/112
	a2-1	21%	24/112
	B42	5%	6/112
	a1-2 + a2-1	9%	10/112
	a1-2 + B42	4%	4/112
	a2-1 + B42	4%	4/112
	a1-2 + a2-1 + B42	3%	3/112

[1] 1µg DNA per plasmid (pCc1001, pE12P5-3, pUK2, cJH8) and per transformation was used in the transformation protocol of Granado *et al.* (1997).