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Technological Advancement

Cyclosporin A-resistance based gene placement system for *Neurospora crassa*

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Abstract

DNA introduced into *Neurospora crassa* are usually inserted at random ectopic sites of the genome, often in multiple copies. To facilitate the study of gene expression and function, transformation by a single-copy of a gene at a defined locus is desired. Although several targeted gene placement methods are available for *N. crassa*, they all require a specific genetic background in the recipient. We describe here the development of a new locus for targeted gene placement that does not require any pre-existing marker in the target strain. Our system takes advantage of the fact that disruption of the *csr-1* gene, which encodes the cyclosporin A-binding protein, leads to the resistance to cyclosporin A. By cloning a gene of interest into a *csr-1* knock-in vector and transforming a fungus with it, one can easily insert any gene, in single-copy, into a defined locus.

Keywords: *Neurospora crassa*; Targeted gene placement; Transformation; *Cyclosporin resistant-1*

1. Introduction

Blessed with its advanced genetics and ease of use, *Neurospora crassa* has become one of the most commonly studied eukaryotic systems. The recent release of the *Neurospora* genome sequence further cements its role as an ideal filamentous fungal model organism (Galagan et al., 2003). The functional genomic study of *Neurospora* holds great promise to provide insight into the many essential aspects of the biology of fungi as well as that of higher eukaryotes. However, the absence of certain molecular tools in *Neurospora* has hampered the ability of researchers to conduct full-scale genome-wide studies. One of these missing tools is an efficient gene knock-out system, which has been available in the best-characterized *Saccharomyces cerevisiae* for years. Fortunately, this monumental hurdle has been overcome recently by the creation of the *mus-51* and *mus-52* mutants, which allow 100% homologous recombination and thus provide an efficient gene disruption method (Ninomiya et al., 2004).

Another molecular tool that is essential to any model organism is a targeted gene placement system. The ability to insert a DNA sequence to a specific chromosomal location, as a single-copy, is extremely important for the characterization of a candidate gene, for example, the study of the promoter region and the protein motifs required for its activity. In *Neurospora*, integration next to the *his-3* gene is the most frequently used gene placement method (Aramayo and Metzenberg, 1996; Margolin et al., 1997). The method utilizes the replacement of a *his-3* mutation with the wild-type allele (as well as a cargo sequence) via a double crossover. This method is relatively easy and efficient, although the need of a specific genetic background (i.e. *his-3*$^-$) in the recipient strain may constitute a minor inconvenience.

Because of the aforementioned disadvantage of the *his-3* system and the need for a viable alternative, we examined other loci that can yield similar gene placement results without posing any genetic restrictions. One of the candidates we examined is *cyclosporin resistant-1* (*csr-1*). Cyclosporin A, a

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potent immunosuppressive drug under the trademark of Sandimmun® (Kunz and Hall, 1993), is produced by the fungus *Tolypocladium niveum* (formerly *Tolypocladium inflatum*; Cannon, 1986). A cyclic nonribosomal peptide of 11 amino acids, cyclosporin A was first discovered as an antifungal agent (Dreyfuss et al., 1976). In *Neurospora*, sensitivity to cyclosporin A depends on a 20-kDa cyclosporin A-binding protein called cyclophilin CyP20 (Tropschug et al., 1988, 1989; Rassow et al., 1995). The *csr-1* gene encodes both the cytosolic and mitochondrial forms of this protein. Because mutation in *csr-1* confers resistance to cyclosporin A (Tropschug et al., 1989), we explore the possibility of using this locus for targeted gene placement. Our rationale is that the introduction of any gene of interest into the *csr-1* locus would introduce a drug resistant phenotype, therefore creating a positive selection for targeted transformants.

2. Materials and methods

2.1. Vector construction

The *csr-1* gene (GenBank Accession No. J03963), physically mapped between *met-6* and *al-2* on linkage group (LG) I, is designated as open read frame (ORF) NCU00726.2 according to the Broad Institute *Neurospora* genome website (http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html). To obtain the "left flank" for the *csr-1* knock-in vector, we used primers CSR-822351F and CSR-824250R (Table 1) to amplify a 1900-bp fragment 5′ of the *csr-1* ORF (Fig. 1a). Both of these primers contain an artificial *Apa*I site, allowing the PCR product to be inserted into the *Apa*I site of the pBluescript II KS (−) (Stratagene, La Jolla, CA), the starting plasmid for our *csr-1* knock-in vector. To obtain the "right flank", we used primers CSR-825975F and CSR-827023R to amplify a 1049-bp fragment 3′ of the *csr-1* ORF. The CSR-825975F primer contains an artificial *Sac*II site while the CSR-827023R primer is located next to an endogenous *Sac*I site. Accordingly, this PCR product was introduced into the *Sac*I/*Sac*II sites of the plasmid. Our final *csr-1* knock-in vector, based on pBluescript II KS (−) and containing both the left and right flanks described above, is called pCSR1 (GenBank Accession No. DQ907525; Supplemental Fig. S1).

To introduce the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*) into pCSR1, we ligated a 1412-bp *Hpa*I–*Hpa*I *hph* fragment (containing the *Aspergillus nidulans trpC* promoter; Carroll et al., 1994) from pCB1004 to an *Eco*RV-cut pBluescript II KS (−) vector. A *Cla*I–*Sac*II fragment from the resulting plasmid was then ligated to a *Cla*I–*Sac*II-cut pCSR1 in order to yield the pCSR1::hph plasmid (GenBank Accession No. DQ907526). For transformation of *Neurospora*, pCSR1::hph DNA was linearized with *Bss*HII (Table 2). pCSR1 and pCSR1::hph are available through the Fungal Genetics Stock Center (FGSC; McCluskey, 2003).

2.2. Strains, media, and culturing methods

The *Neurospora* strains used in the study are Oakridge wild-type *a* (FGSC 2490; Fungal Genetics Stock Center) and *mus-51*$^{\Delta}$*::Bar*$^{+}$; *his-3 a* (FGSC 9538). Descriptions of the genetic loci used in this study can be found in Perkins et al. (2001). Culturing media were prepared as previously described (Vogel, 1964). Growth, preparation of mycelia, genetic techniques, and other routine manipulations were performed as described by Davis and de Serres (1970). A sorbose–glucose–fructose medium was used for restrictive colonial growth (Brockman and de Serres, 1963).

2.3. Nucleic acid methods

Cloning and other molecular techniques were done according to Sambrook and Russell (2001). *Neurospora* genomic DNA was purified using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Amplification of *Neurospora* DNA (100 ng) was performed in a model PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA), using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN); PCR was performed according to the manufacturer's instructions, with the following parameters: denaturation at 94 °C, annealing at 55 °C, and elongation at 68 °C. For DNA sequencing, PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). Bacterial plasmid DNA was isolated with the HiSpeed Plasmid Midi Kit (Qiagen). Vector sequences and insertional events were verified by DNA sequencing, which was performed by the University of Missouri DNA core (Columbia, Missouri). Southern analysis was performed using the DIG High Prime DNA labeling and detection kit (Roche, Indianapolis).

2.4. Transformation of Neurospora

Cyclosporin A (Sigma, St. Louis, MO; catalog no. 30024), stored as a 500× stock solution (2.5 mg/ml in 100%

Table 1
Primers used in this study

Primer	Sequence (5′–3′)	Use
CSR-822351F	GGCC-GGGCCC-AACACCTCCGTCGCCATAAACTCC	Amplification of the left flank
CSR-824250R	CCTC-GGGCCC-TGCAGCCATTGACGACATTGC	Amplification of the left flank
CSR-825975F	CCCA-CCGCGG-TGGATTTCCTGCGCTGCACAC	Amplification of the right flank
CSR-827023R	GGCCCCTGGTTTACTGAGGGC	Amplification of the right flank
CSR-822316F	CCGCGGTAGTCGTTGTTGGAAG	Screening for transformants
CSR-827066R	GTACATCAAGGCGAACCTACGTCC	Screening for transformants

The *Apa*I (GGGCCC) and *Sac*II (CCGCGG) sites are underlined. Primers were named according to their positions in contig 7.2.

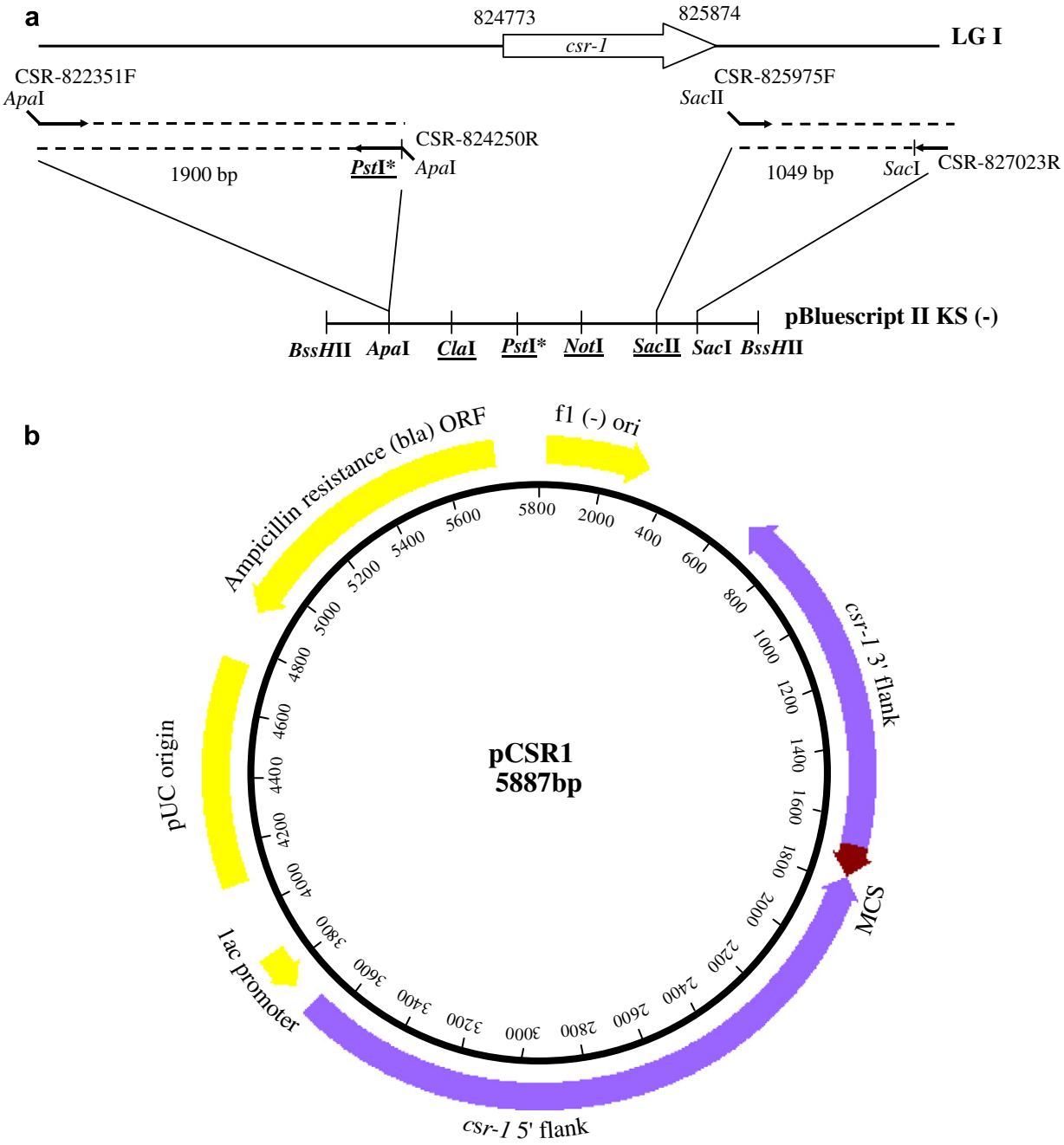

Fig. 1. (a) Construction of the *csr-1* knock-in vector, pCSR1. Nucleotide numbers are utilized according to contig 7.2 of the *Neurospora* genome. PCR primers used to amplify the *csr-1* flanking sequences are shown as arrows. Restriction sites suitable for cloning are underlined. **Pst*I cuts twice. (b) A circular map of pCSR1.

ethanol) at −20 °C, was added to the autoclaved media at 1× concentration. Transformation by electroporation was performed as previously described (Margolin et al., 1997), with the exception that 90 μl of washed conidia (2×10^8) and 10 μl of DNA (500 ng) were used in a 1-mm cuvette. Following electroporation, 750 μl of ice-cold 1 M sorbitol was added to the cuvette. The electroporated conidia were allowed to regenerate in 2 ml of Vogel's medium for 2 h in a 30 °C shaker. Aliquot (350 μl) of the mixture, together with 7.5 ml of pre-warmed (50 °C) soft top agar (1%) was then added to a plate containing 20 ml solidified bottom agar (1.5%). Both top and bottom agar contained 1× cyclosporin A. After incubation at 30 °C for 4–7 days, colonies were picked from the transformation plates (eight in total) to agar slants containing 1× cyclosporin A.

3. Results

3.1. Construction of a csr-1 knock-in vector, pCSR1

Because *csr-1* mutants are resistant to cyclosporin A, there is a great potential to use the *csr-1* locus as a targeted gene

Table 2
Restriction sites suitable for linearization of pCSR1

Restriction enzymes	# Cuts	Nucleotide position
*Ahd*I	1	4972
*Bss*HII	2	619, 3718
*Dra*III	1	237
*Nsp*I	1	4083
*Pci*I	1	4079
*Pst*I	1	362
*Sap*I	1	3963
*Sca*I	1	5452
*Xmn*I	1	5571

The start of pCSR1 is positioned according to the published sequence of pBluescript II KS (−).

placement site. We aimed to construct a knock-in vector targeting the *csr-1* locus. When introduced into the *Neurospora* genome via a double crossover, the knock-in vector should replace the *csr-1* gene with a user's gene of interest, thus making the transformant resistant to cyclosporin A. Based on the published *Neurospora* genome sequence, we created a *csr-1* knock-in vector named pCSR1 (Fig. 1b). This vector contains sequences flanking the c*sr-1* ORF (see Section 2.1), allowing homologous recombination in this region and subsequently the replacement of the endogenous *csr-1* copy.

3.2. Targeted gene placement in Neurospora using pCSR1

To show that a gene of interest can be easily targeted to the *csr-1* locus, we put our knock-in vector to a test. We inserted the hygromycin B phosphotransferase gene (*hph*) into the pCSR1 plasmid (Section 2.1). The *hph* gene confers resistance to hygromycin B in *Neurospora* (Staben et al., 1989). The resulting plasmid, named pCSR1::hph, was linearized with *Bss*HII and introduced to a wild-type strain (FGSC 2490) via electroporation. Cyclosporin A-resistant colonies were isolated and subjected to molecular analysis as well as the hygromycin-resistance test.

Because the positive transformants contain a smaller sequence at the *csr-1* locus (*hph* is shorter than the *csr-1* region it replaces), we were able to use PCR amplification to determine which of the candidates contain the inserted transgene (*hph*) (Fig. 2). Out of the 148 colonies we have examined, 34 colonies yielded a PCR product of 4439 bp that corresponds to a *csr-1* gene replacement event (Fig. 3a). This suggests that the *csr-1*-based transformation system yields a 23% success rate (Table 3). We have also tested the candidates for the presence of the hygromycin-resistant marker (*hph*). Our results indicate that there is a perfect correlation between the molecular (PCR amplification) and functional (hygromycin-resistance) test (Fig. 3b). This observation suggests that if the gene of interest has a scorable phenotype, one can easily identify a positive transformant with high confidence. From the molecular test, most of the positive gene placement transformants appear to be homokaryotic (Fig. 3a). This is in contrast to the *his-3* gene placement system (Margolin et al., 1997), in which the transformants are rarely homokaryons. The homokaryotic nature of *csr-1*$^{\Delta}$ transformants can be attributed to the fact that untransformed *csr-1*$^{+}$ nuclei are selected against based on their abilities to produce cyclosporin A-binding protein. Results from a Southern blot analysis indicate that only one copy of the transgene (*hph*) is present in the positive transformants (Supplemental Fig. S2), demonstrating the single-copy insertion nature of the *csr-1* system.

3.3. False-positive colonies may arise from spontaneous mutations of the csr-1 gene

Our transformation results indicate that 77% of the cyclosporin A-resistant colonies do not contain the gene

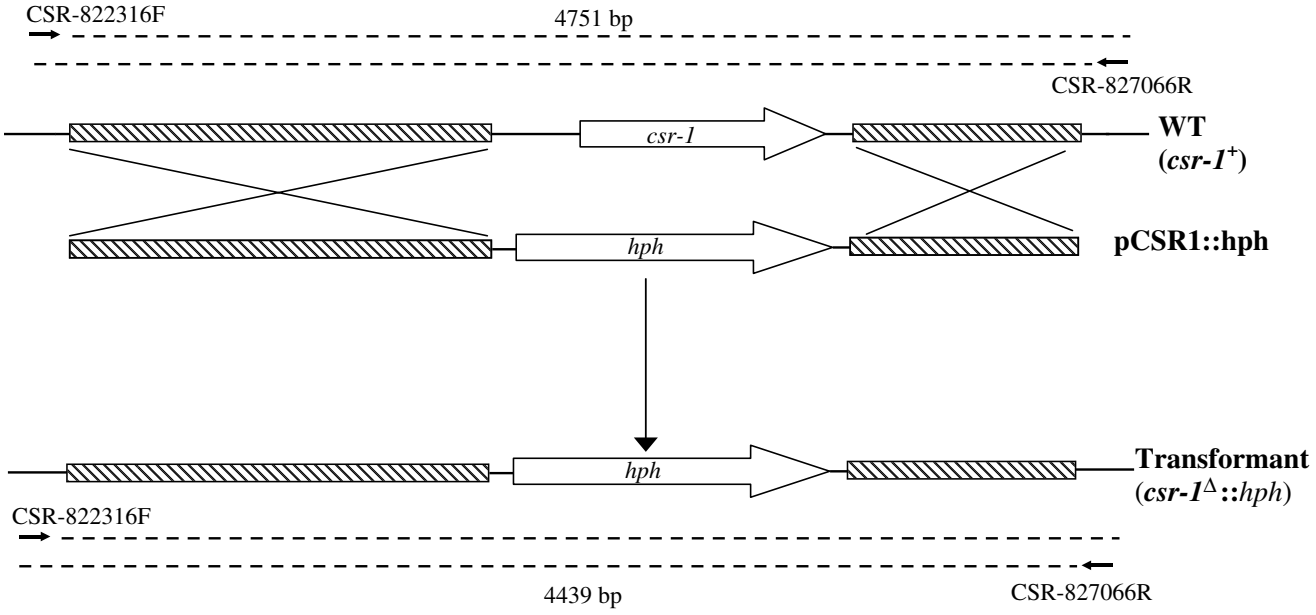

Fig. 2. *csr-1*-based gene placement system. A schematic diagram of recombination events at the *csr-1* locus. Left and right flanks are denoted by hatched boxes.

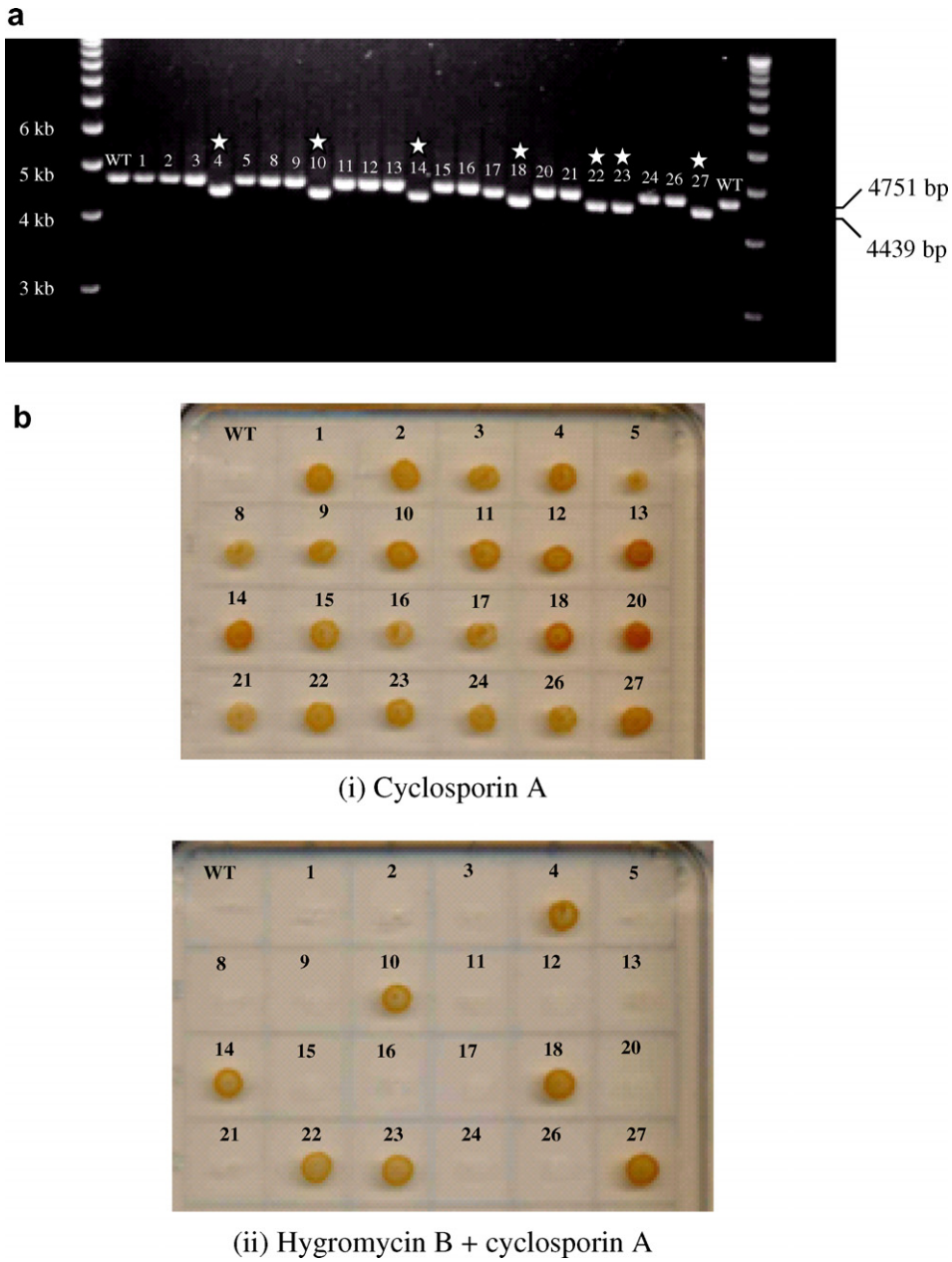

Fig. 3. Identification of positive primary transformants using molecular and functional assays. (a) A PCR-amplified fragment from a positive transformant is shorter than one from a wild type (WT) (4439 bp vs. 4751 bp). The presence of only the 4439-bp PCR products in the positive transformants (asterisked) suggests that they contain only transformed nuclei. (b) Identification of positive transformants by resistance to hygromycin B.

placement event. To determine if the transformation efficiency can be improved by using strains with enhanced homologous recombination, we repeated the experiment using a *mus-51* mutant as the recipient strain. *mus-51* and *mus-52* encode proteins important for the non-homologous end joining of double-stranded DNA breaks (Ninomiya et al., 2004). The elimination of any one of these genes leads to the suppression of random ectopic integration and subsequently the enhancement of homologous recombination. Our results indicate that the *csr-1* system has similar transformation efficiency in a *mus-51* background (7 out of 50 or 14%), suggesting that the leakiness of the system cannot be eliminated by the suppression of ectopic integration. The presence of false-positive colonies may be due to spontaneous mutations (at a rate of 1 ± 0.5 in 8×10^6 conidia) of genes important for the cyclosporin A sensitivity in *Neurospora*. Supporting this hypothesis, we have identified two false positives that contain complete or partial deletion of the *csr-1* gene (Fig. 4). Other false-positive colonies that yield a PCR product of similar length to that of wild type may represent point mutations at the *csr-1* locus. Alternatively, they may be the result of spontaneous mutations at other loci that are important to cyclosporin A sensitivity. These results, and those previous, suggest that spontaneous mutations, not efficiency of homologous recombination, are responsible for the leakiness of the *csr-1* placement system.

Table 3
The percentage of gene placement events at the *csr-1* locus using pCSR1::hph

Electroporation no.	No. of colonies on control[a] plates	No. of colonies on test plates	Positive transformants	Success rate
1	44	16	2	12.5 %
2	12	33	12	36.4 %
3	16	34	12	35.3 %
4	28	39	6	15.4 %
5	ND[b]	26	2	7.7 %
Total		148	34	23.0 %

[a] Control, electroporation with no DNA.
[b] ND, not determined.

4. Discussion

We describe here the development of a *csr-1* knock-in vector that is suitable for targeted gene placement in *Neurospora*. The *csr-1* gene encodes the cyclosporin A-binding protein CyP20, which is required for sensitivity to cyclosporin A, an antifungal compound. Cyclosporin A, together with CyP20, inhibits the activity of calcineurin, a calmodulin-regulated phosphatase required for hyphal growth (Prokisch et al., 1997). The insertion of a transgene to the *csr-1* locus creates a cyclosporin A-resistant phenotype in the transformants, allowing researchers to identify gene placement candidates easily.

Targeted gene placement is particularly crucial for studies in *Neurospora*, due to the fact that genes unpaired during meiosis are silenced (Shiu et al., 2001). Therefore, for proper gene expression during meiosis, a transgene is often required to be inserted into both mating partners, at an identical location. *Neurospora* researchers routinely use the *his-3* locus for targeted gene placement (Margolin et al., 1997). Although this system has served the community well, researchers often encounter situations where there is a need to introduce more than one gene into a fungus. The lack of a user-friendly alternative prompted us into the development of this new gene placement system. In *Neurospora*, many transformation

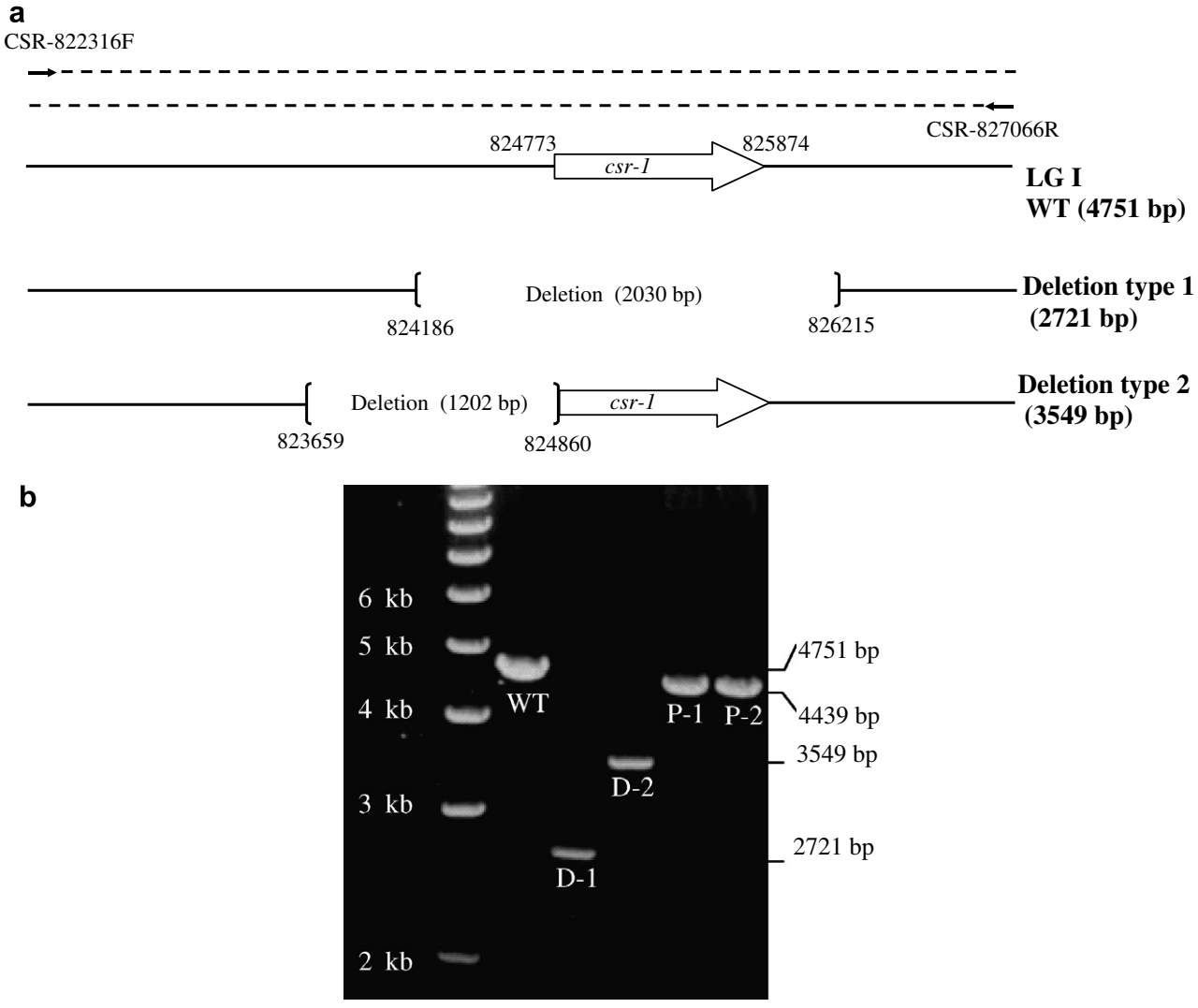

Fig. 4. Identification of spontaneous *csr-1* mutants. (a) The topography of two spontaneous mutants containing deletions at the *csr-1* locus. (b) Corresponding PCR-amplified products. WT: wild type; D-1, D-2: deletion mutants; P-1, P-2: positive transformants.

systems are available with a dominant selectable marker and do not require any specific mutations in the recipient strain. These dominant selectable markers, for example, include genes conferring resistance to benomyl (Orbach et al., 1986), bialaphos (Avalos et al., 1989), bleomycin (Austin et al., 1990), and hygromycin (Carroll et al., 1994). Although excellent for gene cloning experiments, these systems are generally problematic because the transgenes are introduced at random ectopic sites, often inserted in more than one site, and sometimes in multiple tandem copies. This property is highly undesirable, because insertion at different chromosomal sites most likely would result in different expression levels, making comparison of gene expression impossible. Also, the presence of multiple tandem repeats of a transgene is often correlated with vegetative silencing (Cogoni and Macino, 2000). The two most often-used single-copy gene placement loci in *Neurospora*, *his-3* and *am*, provide a good alternative to the aforementioned methods (Margolin et al., 1997; Miao et al., 1994). However, both the *his-3* and *am* locus require a pre-existing mutation in the target strain (*his-3* and *lys-1*, respectively). The *csr-1* gene placement method we describe here combines the best of both worlds, allowing users to target their gene of interest to a specific site, even in a wild-type background.

Other than being resistant to cyclosporin A, we did not observe any aberrant phenotypes in the gene placement transformants, suggesting the suitability of the *csr-1* system for general use. It is also worth noting that, in a *csr-1* replacement mutant, the inserted gene of interest is linked to a phenotype (resistance to cyclosporin A). Following a cross to another fungus, one can easily select a gene of interest by screening for cyclosporin A resistance, thus making its genetic manipulation easy. A gene targeted to the *his-3* locus, on the other hand, confers histidine independence and is not linked to any genetic marker (Margolin et al., 1997). Additionally, most of the *csr-1* placement transformants are homokaryotic. The elimination of the need to perform homokaryon isolation (Ebbole and Sachs, 1990) is an added bonus for the *csr-1* gene placement system. Selection of cyclosporin A resistance using pCSR1 yields 23% of positive candidates, a high enough success rate that entails a PCR screening of a relatively small number of transformants, even in the absence of a scorable phenotype for the transgene. To increase the likelihood of obtaining the desired transformant, one can consider utilizing pCSR1::hph for gene placement. Dual selection for both hygromycin and cyclosporin A resistance using pCSR1::hph should yield a 100% success rate.

Another potential gene placement system that would not require any pre-existing marker is the *mtr* locus (Koo and Stuart, 1991). *mtr* encodes the neutral amino acid permease. This permease controls the uptake of neutral amino acids and their derivatives, such as 4-methyltryptophan (MT) and *p*-fluorophenylalanine (FPA). While MT and FPA are toxic to wild-type *Neurospora*, the *mtr* mutant is resistant to these compounds because of its uptake defects. Gene replacement at the *mtr* locus has been widely used for the study of homologous recombination (Schroeder et al., 1995; Ninomiya et al., 2004). The construction of an *mtr*-knock-in vector containing adequate multi-cloning sites can potentially turn that locus into a gene placement system. However, several drawbacks of the *mtr* mutants could make this locus less desirable for general use in *Neurospora*: (1) *mtr* ascospores have low viability and germination rates (Perkins et al., 2001), and (2) *mtr* mutants do not work well with other auxotrophic markers that require supplementation of neutral amino acids. In comparison, the *csr-1* locus does not have such complications and thus represents a more logical choice for targeted gene placement in *Neurospora*.

Cyclophilins are found in all organisms so far studied, including bacteria, fungi, plants, and animals (Wang and Heitman, 2005). In fungi, some cyclophilins are dispensable for viability while others are important for virulence (Dolinski et al., 1997; Wang et al., 2001; Viaud et al., 2002). Because of its potential benefit, it could be worthwhile to determine whether a cyclosporin A-resistance based gene placement strategy can be applied to other fungal organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb. 2006.12.011.

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