DOI: 10.1534/genetics.107.079384

Note

Characterization of Interactions Between and Among Components of the Meiotic Silencing by Unpaired DNA Machinery in *Neurospora crassa* Using Bimolecular Fluorescence Complementation

Nirmala Bardiya,* William G. Alexander,* Tony D. Perdue,† Edward G. Barry,† Robert L. Metzenberg,‡ Patricia J. Pukkila† and Patrick K. T. Shiu*,[1]

**Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211, †Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599 and ‡Department of Biology, California State University, Northridge, California 91330*

Manuscript received August 16, 2007
Accepted for publication November 17, 2007

ABSTRACT

Bimolecular fluorescence complementation (BiFC) is based on the complementation between two nonfluorescent fragments of the yellow fluorescent protein (YFP) when they are united by interactions between proteins covalently linked to them. We have successfully applied BiFC in *Neurospora crassa* using two genes involved in meiotic silencing by unpaired DNA (MSUD) and observed macromolecular complex formation involving only SAD-1 proteins, only SAD-2 proteins, and mixtures of SAD-1 and SAD-2 proteins.

CHARACTERIZATION of protein–protein interactions is important for deciphering gene functions and cellular processes. Several methods are used to detect interactions between proteins, with co-immunoprecipitation and the yeast two- and three-hybrid systems being among the most popular (Phizicky and Fields 1995). Recently, other techniques have been developed to visualize protein complexes in living cells. These include fluorescence resonance energy transfer (FRET; Miyawaki *et al.* 1997) and bimolecular fluorescence complementation (BiFC; Hu *et al.* 2002). BiFC is based on the principle that two split fragments of the yellow fluorescent protein (YFP) do not emit fluorescent light by themselves. However, if two interacting proteins are individually tagged with different split YFP fragments, the protein interaction can reconstitute the fluorophore. This method allows the assessment of protein–protein interactions *in vivo* in the homologous host, as has been recently demonstrated in *Acremonium chrysogenum*, a filamentous fungus (Hoff and Kück 2005).

In this study, we asked whether BiFC could be applied to the model fungal organism *Neurospora crassa*. Several phenomena were first characterized in *N. crassa*, including meiotic silencing by unpaired DNA (MSUD) (Shiu *et al.* 2001). In MSUD, genes that are not paired during meiotic prophase I, as well as all homologous copies, are silenced during the sexual phase. MSUD is likely to involve the production of double-stranded (ds)RNA, which is mediated by the *sad-1*-encoded RNA-directed RNA polymerase (RdRP) (Shiu and Metzenberg 2002). Mutations in *sad-1* allow the expression of unpaired genes, which would otherwise be silenced during meiosis and the subsequent mitosis. SAD-1 is localized in the perinuclear region, where small interfering (si)RNAs are shown to reside in mammalian cells (Shiu *et al.* 2006). The proper localization of SAD-1, which requires the SAD-2 protein, is important for its function. With the use of green and red fluorescent proteins (GFP and RFP) as visualization tools, we discovered previously that SAD-1-GFP and SAD-2-RFP colocalize in the perinuclear region during sexual development (Shiu *et al.* 2006). Since the perinuclear region can represent the center of RNAi activity, we used BiFC to investigate interactions among SAD-1 molecules, SAD-2 molecules, and between SAD-1 and SAD-2 molecules.

SAD-2-YFP localizes in the perinuclear region: As a prerequisite for using the BiFC approach, we tested whether the YFP is suitable for localization studies in *N. crassa*. The YFP used in this study is a yellow-green variant of the *Aequorea victoria* GFP (Ormö *et al.* 1996). Using the pYFP plasmid (Figure 1), we fused the *sad-2* open reading frame (ORF) to the 5′ end of the YFP gene. The resulting plasmid, as well as other chimeric constructs used in the study, can complement the barren phenotype of a corresponding *Sad-x* × *Sad-x*

We dedicate this article to the memory of Robert Metzenberg.

[1]*Corresponding author:* Division of Biological Sciences, University of Missouri, 103 Tucker Hall, Columbia, MO 65211.
E-mail: shiup@missouri.edu

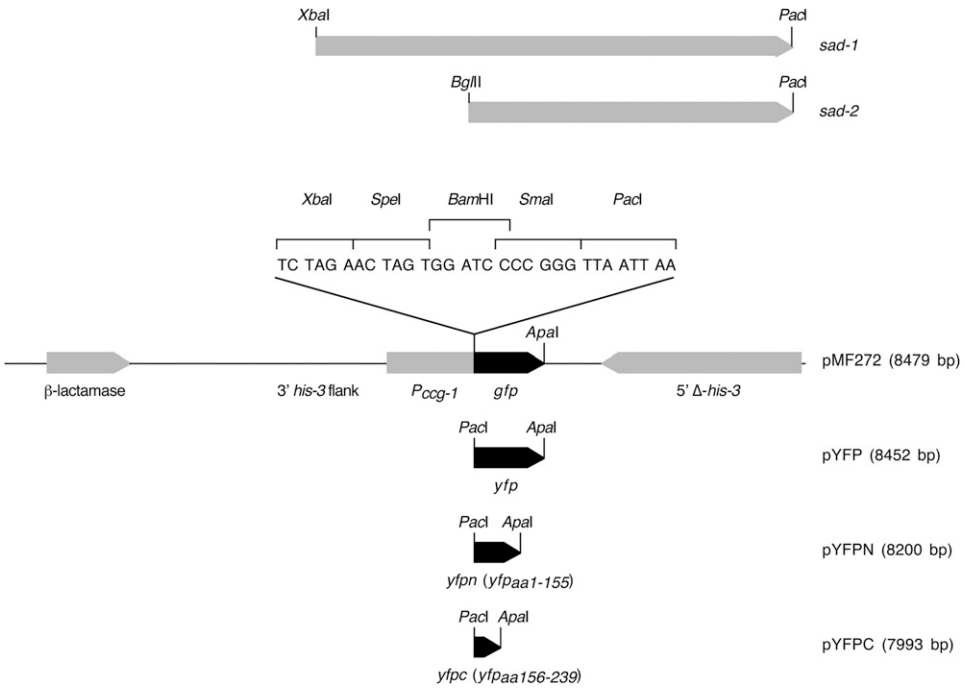

FIGURE 1.—Construction of YFP and split-YFP plasmids for the BiFC assay in *N. crassa*. To construct YFP and split-YFP plasmids suitable for expression in *N. crassa*, we began with pMF272, a Neurospora GFP vector (FREITAG *et al.* 2004). Using the *Pac*I and *Apa*I sites, we replaced the GFP gene with the *yfp*, *yfpn* (aa 1–155), or *yfpc* (aa 156–239) PCR fragments (Table 1); fragments were amplified from pYHN2 (HOFF and KÜCK 2005), a derivative of pEYFP-1 (Clontech, Palo Alto, CA). The resulting plasmids are pYFP (GenBank accession no. EF661030), pYFPN (EF661031), and pYFPC (EF661032), respectively. Restriction sites suitable for the linearization of the above YFP plasmids include *Apa*LI, *Ase*I, *Dra*I, *Nde*I, and *Ssp*I. Linearized plasmids were targeted to the *his-3* locus by gene replacement (Table 2; MARGOLIN *et al.* 1997). *sad-1* and *sad-2* were inserted respectively into the *Xba*I/*Pac*I and *Bam*HI/*Pac*I sites of various YFP plasmids.

cross (both SAD-1 and SAD-2 are required for meiosis), suggesting that the chimeric proteins are functional *in vivo*. Our result demonstrates that, similar to SAD-2-GFP and SAD-2-RFP (SHIU *et al.* 2006), SAD-2-YFP localizes in the perinuclear region throughout meiotic prophase (Figure 2A), suggesting that the pYFP plasmid we constructed can be used as a visualization tool for *N. crassa*.

Interaction among SAD-1 molecules: In preparation for the BiFC assay, we made Neurospora expression vectors containing defined fragments of the *yfp* gene. The pYFPN plasmid contains the first 465 bp of the *yfp* ORF (hereafter *yfpn*), which encodes amino acids 1–155 (Figure 1). The pYFPC plasmid contains the last 255 bp of the *yfp* ORF (hereafter *yfpc*), which encodes amino acids 156–239. When coexpressed in *N. crassa*, the *yfpn* and *yfpc* constructs do not emit detectable fluorescence (Figure 2B), suggesting that these split YFP fragments have a limited intrinsic ability to associate and form a functional fluorophore. To determine whether SAD-1

TABLE 1

Primers used in the amplification of *yfp* fragments

Primer	Sequence (5′–3′)	Uses
YFP97F	*Eco*RI *Pac*I RSIAT linker M1	5′ priming for *yfpn* and *yfp*
	CG-GAATTC-TTAATTAA-C-CGCTCCATCGCCACG-ATGGTGAGCAAGGGCGAGGAGCTGTTCACCG	
YFP561R	*Apa*I Stop C155	3′ priming for *yfpn*
	TCTT-GGGCCC-TTAGGCCATGATATAGACGTTGTGGCTG	
YFP562F	*Eco*RI *Pac*I KQKVMNH linker D156	5′ priming for *yfpc*
	CCG-GAATTC-TTAATTAA-C-AAACAGAAGGTCATGAACCAC-GACAAGCAGAAGAACGGCATCAAGGTG	
YFP816R	*Apa*I Stop K239	3′ priming for *yfpc* and *yfp*
	CGCG-GGGCCC-TTACTTGTACAGCTCGTCCATGCCGAG	

DNA amplification by polymerase chain reaction (PCR) was performed in a model PTC-100 Peltier thermal cycler (MJ Research, Waltham, MA), using the Expand long template PCR kit (Roche Applied Science, Indianapolis). When necessary, PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). Bacterial plasmid DNA was purified with the HiSpeed Plasmid Midi kit (QIAGEN, Valencia, CA). All DNA sequencing was performed by the University of Missouri DNA core (Columbia, MO).

TABLE 2

***Neurospora* strains used in this study**

Strain	Genotype
59-21	*Sad-1*$^{\Delta}$ *rid his-3*$^{+}$::*sad-1-yfpn A*
59-22	*Sad-1*$^{\Delta}$ *rid his-3*$^{+}$::*sad-1-yfpc a*
59-24	*rid his-3*$^{+}$::*sad-2-yfpn*; *inv Sad-2*^{RIP} *A*
59-25	*rid his-3*$^{+}$::*sad-2-yfpc*; *inv Sad-2*^{RIP} *a*
59-26	*rid his-3*$^{+}$::*sad-2-yfp*; *inv Sad-2*^{RIP} *a*
59-27	*Sad-1*$^{\Delta}$ *rid his-3*$^{+}$::*yfpn A*
59-28	*rid his-3*$^{+}$::*yfpc*; *inv Sad-2*^{RIP} *a*
81-01	*fl A*

Auxotrophic markers used in this study were obtained via the Fungal Genetics Stock Center (FGSC; McCluskey 2003). Descriptions of the genetic loci used in this study can be found in Perkins *et al.* (2001). Culturing and crossing media were prepared as previously described (Westergaard and Mitchell 1947; Vogel 1964). Growth, genetic techniques, and other routine manipulations were performed according to Davis and De Serres (1970). Homokaryons were isolated with the method of Ebbole and Sachs (1990).

interacts with other SAD-1 molecules, we covalently linked the *sad-1* gene to *yfpn* and to *yfpc*. The rationale was that the formation of SAD-1 macromolecular complexes involving both YFPN and YFPC fragments would result in fluorescence. Our results indicate that SAD-1-YFPN and SAD-1-YFPC in fact interact and that asci containing both proteins exhibit yellow fluorescence (Figure 2C). The SAD-1–SAD-1 interaction appears to localize in ~10 cytoplasmic spots per ascus. We have previously shown that while the majority of SAD-1-GFP proteins localize in the perinuclear region, they can also be found in several cytoplasmic spots (Shiu *et al.* 2006).

Interaction between SAD-2 molecules: We asked whether SAD-2 interacts with other SAD-2 molecules. In asci containing both SAD-2-YFPN and SAD-2-YFPC, yellow fluorescence can be seen in the perinuclear region and in ~10 cytoplasmic spots per ascus (Figure 2D). This result suggests that SAD-2 proteins also form macromolecular complexes in Neurospora.

Interaction between SAD-1 and SAD-2 in the perinuclear region: We examined asci expressing both SAD-2-YFPN and SAD-1-YFPC and asci expressing both SAD-1-YFPN and SAD-2-YFPC; we observed yellow fluorescence in the perinuclear region and in several cytoplasmic spots, as illustrated in Figure 2E. These data suggest that SAD-1 and SAD-2 likely interact physically *in vivo*. Although SAD-2-YFPN can reconstitute a functional fluorophore with SAD-2-YFPC (Figure 2D) and with SAD-1-YFPC (Figure 2E), it cannot do so with YFPC alone (Figure 2F), suggesting that intrinsic interactions between YFPN and YFPC are not contributing to the perinuclear fluorescence.

Concluding remarks: SAD-2 is properly localized in the perinuclear region, even in the absence of SAD-1 (Shiu *et al.* 2006). However, in the absence of SAD-2, SAD-1 is no longer localized in the perinuclear region,

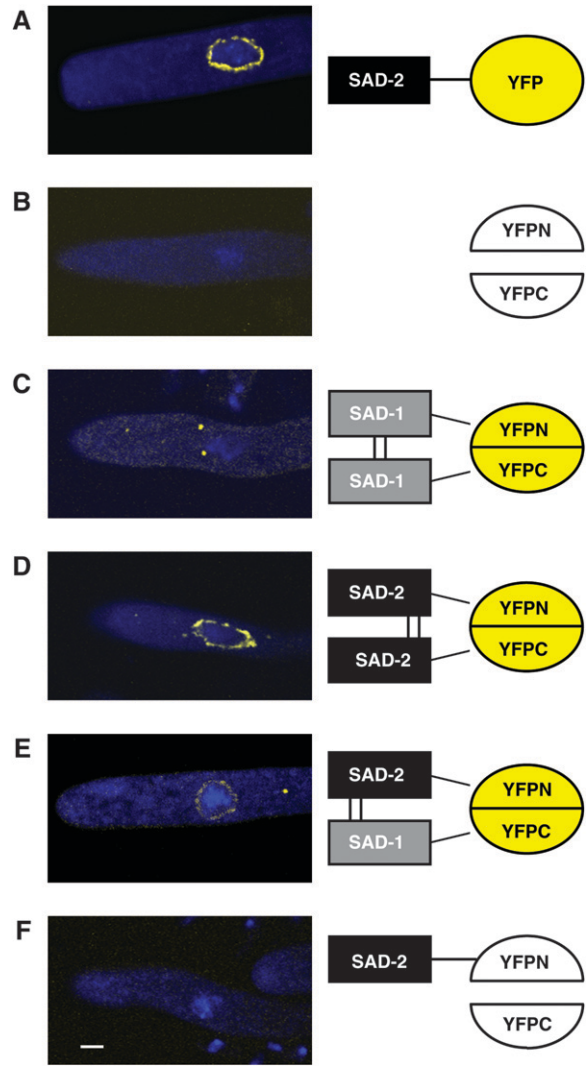

Figure 2.—Visualization of interactions between SAD proteins using BiFC. Micrographs illustrate asci in meiotic prophase expressing (A) *sad-2-yfp* (59-26 × 81-01), (B) *yfpn* and *yfpc* (59-27 × 59-28), (C) *sad-1-yfpn* and *sad-1-yfpc* (59-21 × 59-22), (D) *sad-2-yfpn* and *sad-2-yfpc* (59-24 × 59-25), (E) *sad-1-yfpc* and *sad-2-yfpn* (59-22 × 59-24), and (F) *sad-2-yfpn* and *yfpc* (59-24 × 59-28). Bar, 5 μm. Perithecia were fixed in freshly prepared 4% paraformaldehyde, 90 mM PIPES pH 6.9, 10 mM EGTA, 5 mM $MgSO_4$ for 20 min at room temperature. They were rinsed briefly in PBS, and the perithecial contents were teased out into a drop of 90% glycerol, 10% 100 mM potassium phosphate (pH 8.7), 10 μg/ml DAPI, and 100 mg/ml 1,4-diazabicyclo[2,2,2]octane. Contents of 3-5 perithecia were dispersed under a cover slip by gentle tapping, slides were sealed using clear nail polish, and viewed either immediately or after storage at −20°. Samples were imaged using a Zeiss LSM510 confocal laser scanning microscope. All images were collected using a PlanNeofluar 40× (NA1.3) oil immersion objective. Visualization of the YFP was achieved by use of a 514 nm Argon laser line for excitation and a BP530-600 emission filter. DAPI visualization was achieved by use of a 364 nm Argon laser excitation and a BP385-470 emission filter. All images were collected using standard Zeiss software.

suggesting that SAD-2 may function to recruit SAD-1 to this region, and that the proper localization of SAD-1 may be important for its activity. Here we have seen that SAD-1 complexes including only SAD-1-YFPN and SAD-1-YFPC are mainly localized in the cytosol, while complexes including SAD-2-YFPN and SAD-1-YFPC are found in the perinuclear region. We conclude that either SAD-1 does not form complexes involving other SAD-1 molecules at perinuclear sites or such complexes fell below our limits of detection. RNAi-related proteins have been shown to colocalize in the perinuclear region of Drosophila and mouse germ cells (Kotaja and Sassone-Corsi 2007; Lim and Kai 2007; Pane *et al.* 2007). Our results support the notion that the perinuclear region is the center of RNAi activity for meiotic silencing and that interactions among different SAD proteins could be important for their functions.

The YFP and split YFP plasmids we report here are based on the popular GFP plasmid (Freitag *et al.* 2004), thus facilitating swaps between different fluorescent vectors. Although other approaches, such as co-immunoprecipitation, could be used to demonstrate protein–protein interactions in the homologous Neurospora host, the BiFC assay we describe here provides a straightforward and sensitive alternative to such methods.

We are indebted to Birgit Hoff and Ulrich Kück for the pYHN2 plasmid and to Michael Freitag and Eric Selker for the pMF272 plasmid. We thank Sven Saupe for his helpful suggestions and Helene Metzenberg and the members of the Shiu Laboratory for their indispensable help. The postdoctoral fellowship of N.B. was supported by a University of Missouri Research Board grant (RB-05-124; P.K.T.S.). R.L.M., P.J.P, and P.K.T.S. were supported, respectively, by National Science Foundation (NSF) grants MCB0234421, EF0412016, and MCB0544237. R.L.M. was grateful to the NSF for allowing unspent funds to continue to be used past their official termination date.

LITERATURE CITED

Davis, R. H., and F. J. De Serres, 1970 Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17A:** 79–143.

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

Freitag, M., P. C. Hickey, N. B. Raju, E. U. Selker and N. D. Read, 2004 GFP as a tool to analyze the organization, dynamics and function of nuclei and microtubules in *Neurospora crassa*. Fungal Genet. Biol. **41:** 897–910.

Hoff, B., and U. Kück, 2005 Use of bimolecular fluorescence complementation to demonstrate transcription factor interaction in nuclei of living cells from the filamentous fungus *Acremonium chrysogenum*. Curr. Genet. **47:** 132–138.

Hu, C. D., Y. Chinenov and T. K. Kerppola, 2002 Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol. Cell **9:** 789–798.

Kotaja, N., and P. Sassone-Corsi, 2007 The chromatoid body: a germ-cell-specific RNA-processing centre. Nat. Rev. Mol. Cell Biol. **8:** 85–90.

Lim, A. K., and T. Kai, 2007 Unique germ-line organelle, nuage, functions to repress selfish genetic elements in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **104:** 6714–6719.

Margolin, B. S., M. Freitag and E. U. Selker, 1997 Improved plasmids for gene targeting at the *his-3* locus of *Neurospora crassa* by electroporation. Fungal Genet. Newsl. **44:** 34–36.

McCluskey, K., 2003 The Fungal Genetics Stock Center: from molds to molecules. Adv. Appl. Microbiol. **52:** 245–262.

Miyawaki, A., J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams *et al.*, 1997 Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature **388:** 882–887.

Ormö, M., A. B. Cubitt, K. Kallio, L. A. Gross, R. Y. Tsien *et al.*, 1996 Crystal structure of the *Aequorea victoria* green fluorescent protein. Science **273:** 1392–1395.

Pane, A., K. Wehr and T. Schüpbach, 2007 *zucchini* and *squash* encode two putative nucleases required for rasiRNA production in the *Drosophila* germline. Dev. Cell **12:** 851–862.

Perkins, D. D., A. Radford and M. S. Sachs, 2001 *The Neurospora Compendium: Chromosomal Loci*. Academic Press, San Diego.

Phizicky, E. M., and S. Fields, 1995 Protein-protein interactions: methods for detection and analysis. Microbiol. Rev. **59:** 94–123.

Shiu, P. K. T., and R. L. Metzenberg, 2002 Meiotic silencing by unpaired DNA: properties, regulation, and suppression. Genetics **161:** 1483–1495.

Shiu, P. K. T., N. B. Raju, D. Zickler and R. L. Metzenberg, 2001 Meiotic silencing by unpaired DNA. Cell **107:** 905–916.

Shiu, P. K. T., D. Zickler, N. B. Raju, G. Ruprich-Robert and R. L. Metzenberg, 2006 SAD-2 is required for meiotic silencing by unpaired DNA and perinuclear localization of SAD-1 RNA-directed RNA polymerase. Proc. Natl. Acad. Sci. USA **103:** 2243–2248.

Vogel, H. J., 1964 Distribution of lysine pathways among fungi: evolutionary implications. Am. Nat. **98:** 435–446.

Westergaard, M., and H. K. Mitchell, 1947 Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Bot. **34:** 573–577.

Communicating editor: M. S. Sachs