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SSP2, a sporulation-specific gene necessary for outer spore wall assembly in the yeast *Saccharomyces cerevisiae*

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Abstract Sporulation in yeast consists of two highly coordinated processes. First, a diploid cell that is heterozygous at the mating-type locus undergoes meiosis, in which one round of DNA replication is followed by two rounds of nuclear division. Second, the meiotic products are packaged into spore cells that remain within the mother cell. A large number of genes are induced specifically during sporulation, and their products carry out different sporulation-specific events. Expression of these sporulation-specific genes is controlled by several regulators which function at different stages of the sporulation program, resulting in a cascade of gene expression following induction of meiosis. Here we describe one sporulation-specific gene, *SSP2*, which is induced midway through meiosis. Ssp2 shows significant homology to the predicted product of a hypothetical ORF in *Candida albicans*. Homozygous mutant *ssp2* diploid cells fail to sporulate. In the mutant background, meiotic recombination and nuclear divisions remain normal; however, viability declines rapidly. Following meiosis, *ssp2* cells form the prospore membrane, but fail to form the outer layer of the spore wall. The Ssp2 protein localizes to the spore wall after meiosis II. In addition, the *ssp2* defect is also associated with delayed and reduced expression of late sporulation-specific genes. Our results suggest that *SSP2* function is required after meiosis II and during spore wall formation.

Keywords Yeast · Meiosis · Sporulation · Spore-wall formation

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Introduction

Sporulation in the yeast *Saccharomyces cerevisiae* occurs when **a**/α diploid cells are starved for nitrogen in the presence of a non-fermentable carbon source (Malone 1990; Kupiec et al. 1997). During this process, the diploid cell undergoes a single round of DNA replication, followed by two successive rounds of nuclear division, to produce four haploid meiotic nuclei. Meiotic products are packaged into four spores that remain in the mother cell, forming what is known as an ascus. During the first meiotic division, a number of events occur that ensure proper chromosome segregation (Baker et al. 1976; Hawley 1988; Kleckner 1996; Kupiec et al. 1997; Roeder 1997). Among these events are a high level of exchange between homologs, and synapsis of homologous chromosomes.

Unlike higher eukaryotes, yeast cells undergo a closed meiosis – the nuclear membrane remains intact throughout meiosis. Completion of meiosis II results in four nuclear lobes, by each spindle pole body (Moens 1971; Moens and Rapport 1971; Byers 1981). The haploid nuclei are then enveloped by the prospore membrane that is formed as an extension from the cytoplasmic outer plaque of the spindle pole body. The prospore membrane acts as a scaffold for spore wall formation, and is synthesized by the fusion of secretory vesicles that derive from the Golgi complex (Byers 1981; Neiman 1998). The prospore membrane bilayer that is closest to the daughter nucleus acts as the plasma membrane of the spore cell. The mature spore wall consists of four layers. The two innermost layers are indistinguishable from the vegetative cell wall and often appear as an electron-transparent layer (Katohda et al. 1984; Briza et al. 1988). The outermost layer consists of an insoluble crosslinked dityrosine coat, which renders the spores resistant to degradative enzymes and organic solvents. This layer is closely associated with the underlying chitosan layer (Briza et al. 1988, 1990a, 1990b).

A large number of genes are expressed specifically during sporulation, and their products carry out

different sporulation-specific processes. Expression of these sporulation-specific genes is controlled by a number of regulators that function at different stages of the sporulation program (Mitchell 1994; Kupiec et al. 1997; Chu and Herskowitz 1998). Several approaches have been used to identify genes that are essential for sporulation-specific events (Clancy et al. 1983; Percival-Smith and Segall 1984; Gottlin-Ninfa and Kaback 1986; Rockmill and Roeder 1988; Bishop et al. 1992; Burns et al. 1994; Kupiec et al. 1997; Nag et al. 1997; Chu et al. 1998; Ross-Macdonald et al. 1999; Primig et al. 2000). These studies have resulted in the isolation of mutants with defects at different stages of the sporulation process, and of genes that are expressed at specific times after induction of the sporulation process (reviewed in Kupiec et al. 1997; Nag et al. 1997; Chu et al. 1998; Ross-Macdonald et al. 1999; Primig et al. 2000). These studies have also revealed that between 1000 and 1600 genes undergo changes in their expression pattern during sporulation. However, many of the genes identified by monitoring sporulation-specific expression are not essential for the sporulation program itself (Gottlin-Ninfa and Kaback 1986; Bishop et al. 1992; Burns et al. 1994; Nag et al. 1997). Most studies involving meiotic mutants and sporulation-specific genes have concentrated on events that occur during the early stages of meiotic development, such as recombination and chromosome synapsis. Consequently, little is known about the events that occur midway through meiotic development, including the control of meiotic divisions and the mechanism of coordination between meiosis and spore formation.

A growing number of mutants have been identified that complete meiosis II, but fail to form spores (Moens et al. 1974; Briza et al. 1990b; Honigberg et al. 1992; Friesen et al. 1994; Krisak et al. 1994; Nag et al. 1997). Some of these mutants also exhibit defects in the completion of meiotic divisions. For example, *spo14* and *ssp1* mutants show delayed kinetics of meiotic divisions, and a significant fraction of cells do not complete both meiotic divisions (Honigberg et al. 1992; Nag et al. 1997). Spo14 possesses a phospholipase D (PLD) activity that is essential for meiotic development, and the prospore membrane is not formed in the mutant background (Rose et al. 1995; Rudge et al. 1998). However, studies with several *spo14* mutants with alterations in different regions of the protein suggest that PLD activity alone is not sufficient for sporulation. In *spo14* and *ssp1* cells, viability declines rapidly in the meiotic medium and nuclei fragment after meiosis II (Honigberg et al. 1992; Nag et al. 1997). It is believed that the lack of prospore membrane formation leads to nuclear fragmentation, followed by cell death. However, the cause of nuclear fragmentation and cell death is not clearly understood. Two sporulation-specific kinases, encoded by the genes *SMK1* and *SPS1*, with homology to MAP kinases and Ste20 kinase, respectively, are required for the normal progression of late events of the sporulation program. In these two mutants, although meiotic divisions occur normally, mature spore walls are not formed (Friesen et al. 1994; Krisak et al. 1994). Most of these mutations affect spore wall synthesis or maturation, while a few are known to disturb the coordination between meiotic nuclear divisions and spore packaging.

In an attempt to identify new sporulation-specific genes, Nag et al. (1997) followed differential expression of genes during sporulation and vegetative growth. In their studies, several novel sporulation-specific genes were identified. Recently, Chu et al. (1998) and Primig et al. (2000) used DNA microarrays bearing nearly every yeast gene to monitor gene expression patterns during meiosis and mitosis. Most of the genes identified by monitoring differential expression of genes during vegetative growth and sporulation were also identified by the microarray analysis. Here we describe the functional analysis of one previously uncharacterized sporulation-specific gene, *SSP2*.

The *ssp2* cells fail to form mature spores. In the *ssp2* background, meiotic divisions follow wild-type kinetics; however, the nuclei fragment after continued incubation in the sporulation medium and the viability of *ssp2* cells declines rapidly in the sporulation medium. The Ssp2 protein localizes to the spore wall, and electron microscopic analysis also indicates that both the prospore membrane and the electron-transparent layers are formed in the *ssp2* cells; however, the outer spore walls are not formed.

Materials and methods

Yeast strains

Unless mentioned otherwise, all strains were derived from the SK1 background. The construction of DNY184 (*MAT*α *leu2::hisG his4-B lys2 ho::LYS2 ura3 cyh*^{r}) and DNY185 (*MAT***a** *leu2::hisG his4-B lys2 ho::LYS2 ura3 can1*) were described previously (Nag et al. 1997). DNY191 was constructed by mating DNY184 and DNY185. PSY5 is identical to DNY191, except it has a homozygous deletion of *SSP2*. The *ssp2* phenotype was also studied in the AS4 (*MAT*α *trp1 arg4 tyr7 ade6 ura3*) × AS13 (*MAT***a** *leu2-Bst ura3 ade6*) background (Nag et al. 1989). The diploid strain PSY17 was derived from AS13 and AS4 haploid strains containing the *ssp2*Δ mutation. For comparative analysis, *SSP2* was cloned from DNY184, and from FY23, which is a close derivative of the S288C background (Winston et al. 1995).

Plasmids

The vector plasmids used in this study were pRS306, pRS316 (Sikorski and Hieter 1989) and the T-A cloning vector from Invitrogen (Carlsbad, Calif.). All restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.). The *URA3* disruption derivative of YOR242c was constructed as follows. An 858-bp PCR fragment (extending from –8 to +850 of the *SSP2* coding sequence; +1 indicates position A in the *SSP2* start codon) was amplified with *Taq* polymerase (Perkin-Elmer, Boston, Mass.), using DNY184 DNA as a template, and cloned into the T-A cloning vector. The *URA3* gene was inserted into the *Ssp*I site present within the *SSP2* coding sequence. The resulting plasmid was digested with *Bam*HI and *Xba*I before yeast transformation. The plasmid pPS6 was constructed by cloning the PCR fragment containing the *SSP2* gene into the *Kpn*I + *Bam*HI-digested pRS306.

The cloned fragment is 2216 bp long (–543 to +1672). Cloning of *SSP2* was done in two steps, by PCR using *Pfu* polymerase (Stratagene, La Jolla, Calif.). First, a 962-bp fragment (+712 to +1672) including portions of the *SSP2* coding region and its downstream region was inserted as a *Kpn*I-*Xho*I fragment into *Kpn*I+*Xho*I-digested pRS306, to generate pPS5. The sequences of the primers used were +712 5′-CCAGAATGGATATTCCTCGAGAGCACA-3′ +738 and +1672 5′-ACGGGGTACCTGCTCACACCAAAGATAGTATGGTAGC-3′ +1646. In the next step, another PCR fragment/(–543 to +738) containing the rest of the *SSP2* coding region and 543 bp of sequence upstream of the *SSP2* coding region was inserted into the *Xho*I+*Bam*HI-digested pPS5, to generate pPS6. The PCR fragment was digested partially with *Xho*I and completely with *Bam*HI before ligation with *Xho*I+*Bam*HI-digested pPS5. The partial digestion was necessary due to the unexpected presence of the *Xho*I site near the 5′ end of the *SSP2* gene. The primers used were +738 5′-TGTGCTCTCGAGGAATATCCATTCTGG-3′ +712 and –543 5′-ACGCGGATCCAGAAAGCGGCCAGAAGTATATGAAAGCCAAGT-3′ –512. The plasmid pPS18 was derived from pPS6 and had an internal deletion of a 588-bp (*Eco*RI-*Xho*I) fragment of *SSP2*. The plasmid pPS4 is the same as pPS6, except that FY23 genomic DNA was used as a template, and the fragment was 198 bp shorter than that present in pPS6. The plasmid pPS23 had three copies of the coding sequence for the influenza virus hemagglutinin (HA) epitope (Wilson et al. 1984) inserted just before the *SSP2* stop codon, and was constructed as follows. The *SSP2* stop codon was changed to a *Cla*I site. Three copies of sequence encoding the HA epitope were introduced at the *Cla*I site by inserting the *Cla*I-digested PCR product (amplified with the primers 5′ HA: 5′-CCCATCGATTCTTACCCATACGATGTTCCTG-3′, and 3′ HA: 5′-CCCATCGATAGCTCCACCGCGGTGGCGGGCCG-3′) using plasmid SK P/X HA (Neiman et al. 1997) as a template. The plasmid construction was confirmed by DNA sequencing.

Genetic techniques

Standard genetic methods and media were used (Rose et al. 1990). All chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise stated. Sporulation was carried out at 30°C as described by Nag et al. (1997). Briefly, a single colony was inoculated into 3 ml of YPD. After overnight growth at 30°C, the culture was diluted 1000-fold in 250 ml of presporulation medium and incubated at 30°C until the cell concentration reached $2–4\times10^7$/ml. Cells were then washed with 50 ml of potassium acetate (10 g/l) before suspending them in 250 ml of sporulation medium (10 g/l potassium acetate containing one-fifth of the nutritional requirements) at a final concentration of $1.5–2\times10^7$ cells/ml. The presporulation medium contained 5 g/l yeast extract, 10 g/l peptone, 6.7 g/l yeast nitrogen base without amino acids, 10 g/l potassium acetate, and 0.05 M potassium biphthalate, and the pH was adjusted to 5.5. Assays for cell viability and genetic recombination were carried out as described by Nag et al. (1997).

Northern analysis

Total RNA was isolated from 25-ml aliquots collected at different time intervals after induction of sporulation using the glass bead-phenol extraction procedure (Nag et al. 1997). At the same time an aliquot was fixed with ethanol to monitor nuclear divisions. Northern analysis was carried out as described before (Nag et al. 1997), and 35 μg of RNA was used for each sample. A *Bsr*G1-*Xho*I fragment containing an 880-bp segment of the *SSP2* coding sequence was used as a probe for Northern hybridization analysis.

Fluorescence microscopy

Meiotic nuclear divisions were followed by staining with the DNA-specific dye DAPI (4′, 6-diamidino-2-phenylindole). Samples were sonicated for 10–15 s, mixed with an equal volume of absolute ethanol containing DAPI (1 μg/ml), and stored at 4°C. Cells were then washed with 1 ml of water and visualized by fluorescence microscopy. About 200 cells were counted for each time point. Differential interference and fluorescence microscopy were performed with a Zeiss Axiophot microscope.

Electron microscopy

Yeast cells were sporulated as described above. Fixation of these samples for electron microscopy was performed essentially as described by Byers and Goetsch (1991) with some modifications. Cells from approximately 10 ml (2×10^7 cells/ml) of each sample were collected by centrifugation at each time point. The cells were washed with 5 ml of water and then incubated in 5 ml of pretreatment buffer [0.2 M TRIS-HCl buffer (pH 9.0), 20 mM EDTA, 1 M NaCl, with 3.9 μl of β-mercaptoethanol added per ml] for 5 min at room temperature. Cells were then pelleted and washed twice with 0.7 M sorbitol and then transferred to 5 ml of cacodylate-buffered glutaraldehyde [a mixture of equal volumes of 30 μg/ml glutaraldehyde and 0.2 M cacodylate-calcium buffer (0.2 M sodium cacodylate and 10 mM $CaCl_2$, pH 6.50)] for 30 min at room temperature. Samples were then rinsed twice with 0.1 M phosphate-citrate buffer (0.085 M KH_2PO_4 and 15 mM sodium citrate, pH 5.8) and stored overnight at 4°C. Cells were then treated with filtered Glusulase (NEN Dupont, Boston, Mass.) for approximately 2 h, pelleted and washed with 5 ml of water. Cells were resuspended in 5 ml of aqueous potassium permanganate (40 mg/ml) and incubated for 3 h at 4°C. The cells were then washed five times with 5 ml water and then post-fixed in aqueous uranyl-acetate (20 mg/ml) at 4°C overnight. Cells were pelleted, resuspended in 0.3 ml of water and embedded in an equal volume of low-melting-point agarose (40 mg/ml) (SeaPlaque low melting temp. agarose; FMC Bioproducts, Rockland, Me.), mixing thoroughly before allowing the agar to solidify. The agar blocks were cut into small pieces and the samples were subjected to a graded series ethanol dehydrations up to absolute ethanol. Samples were infiltrated with Spurr's resin (Electron Microscopy Sciences, Fort Washington, Pa.) over 2 days and polymerized at 65°C for 2 days under vacuum.

Ultra-thin sections were cut on a Leica Ultracut UCT ultramicrotone using a Diatome histoknife (Diatome, Ft. Washington, Pa.). Thin sections were stained with an aqueous solution of saturated uranyl acetate for 30 min at room temperature, and with Reynolds' lead citrate (Reynolds 1963) for 5 min. Samples were examined in a Zeiss 910 electron microscope operating at 80 kV.

Dityrosine assay

A fluorescence assay was used to monitor dityrosine formation in sporulating yeast cells. Patches of cells were replica-plated onto nitrocellulose filters and then transferred onto sporulation plates. After allowing cells to sporulate for 2 days, the filters were treated with 400 μl of lysis buffer (350 μl of 0.1 M sodium citrate/0.01 M EDTA, 70 μl glusulase, 15 μl β-mercaptoethanol) in a glass petri dish. After incubation at 37°C for 3–5 h, lysis of asci was monitored under the microscope. After incubation, filters were soaked in 300 μl of concentrated ammonia and photographed under UV light (254 nm).

Calcofluor staining

Aliquots (5 ml) of cells were collected at different time points after induction of meiosis, and stained as described by Pringle (1991). After brief sonication, cells were fixed with 0.55 ml of 37% formaldehyde. After fixation, cells were washed with water and resuspended in 5 ml of Calcofluor white (Fluorescent Brightner 28; Sigma) solution (1 mg/ml) and incubated at room temperature for 10 min. Cells were washed five times with water and then suspended in 5 ml of water and viewed under the microscope.

Immunofluorescence staining

Immunofluorescence staining was performed as described by Pringle et al. (1991). The samples were collected at different time points and fixed with formaldehyde as described by Nag et al. (1997). After formation of spheroplasts by treatment with Zymolyase, fixed cells were applied to polylysine-coated coverslips, and incubated with mouse anti-HA antibody (Babco, Berkeley, Calif.) overnight at 4°C. Cells were washed and then treated with goat anti-mouse antibody conjugated to fluorescin isothiocyanate (Babco). The coverslips bearing stained cells were then placed on a slide containing a drop of mounting medium [0.9 ml/ml glycerol, 1 mg of *p*-phenylene diamine (pH 9.0)/ml, and 0.5 μg of DAPI/ml) and photographed with epifluorescence illumination using a 100× objective.

Results

SSP2 is essential for sporulation

YOR242c was found to be induced midway through meiosis in both differential-display and DNA microarray analyses. To investigate whether this ORF is necessary for the sporulation process, we made a disruption allele by inserting the *URA3* gene within the coding sequence of YOR242c. The homozygous diploid mutant cells failed to sporulate, but did not exhibit any mitotic growth defects. This result suggested that YOR242c is essential for sporulation. The gene was named *SSP2* (for *s*porulation *sp*ecific). The ORF is 1,116 bp long and is predicted to encode a 43,106-Da protein containing 371 amino acids. The expression pattern of *SSP2* was also studied by Northern analysis and compared with the kinetics of meiotic nuclear division. *SSP2* is induced between 4 and 6 h after induction of meiosis (Fig. 1). The transcription pattern suggests that *SSP2* is induced at the time of initiation of nuclear divisions (see below). This expression pattern is also consistent with the results of the differential-display experiment and the DNA microarray analysis.

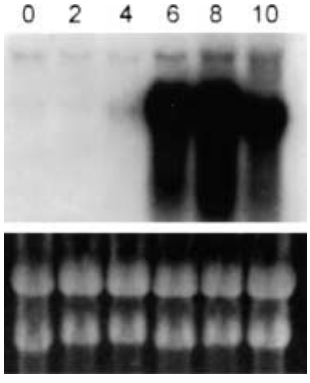

Fig. 1 Northern analysis of *SSP2* expression during sporulation. The *numbers at the top* indicate the time (in hours after induction of sporulation) at which each sample was collected. The *lower panel* shows the ethidium bromide-stained gel prior to blotting. A *Bsr*GI-*Xho*I fragment containing the *SSP2* coding sequence was used as the probe

The *SSP2* sequence in the SK1 background differs from that in the S288C strain

During the cloning of a PCR fragment (generated by *Pfu* polymerase) containing *SSP2* from SK1 cells, the presence of a few unexpected restriction sites was detected. This result led us to clone and sequence the gene from both SK1 and S288C (FY23) strains, in order to identify potential sequence differences. The source of the DNA used for yeast genome-sequence analysis was S288C (Goffeau et al. 1997). Three independent cloning experiments were performed to minimize the possibility of errors introduced during the PCR amplification. The *SSP2* sequence derived from the FY23 strain was identical to the published sequence. However, sequencing data for *SSP2* from the SK1 strain indicated the presence of some sequence differences between the *SSP2* genes from SK1 and S288C. The GenBank Accession No. for the *SSP2* sequence in the SK1 background is AF418016.

Most of the sequence dissimilarities lay outside the coding region (Table 1). In addition, the differences within the coding region were primarily silent changes. Arginine at position 62 in FY23 was changed to histidine in SK1, and aspartic acid at position 88 was changed to glycine. Microarray analysis using the whole genome has also revealed the existence of a large amount of genetic variation (deletions and polymorphisms) among W303, SK1 and S288C strains (Primig et al. 2000).

For further phenotypic analysis, a deletion mutant was constructed by deleting a 588-bp fragment from the coding region (codons 49–242) of the *SSP2* gene. Diploid cells homozygous for the deletion mutation behaved like the disruption mutant. Since the *SSP2* sequence varies in different strains, the deletion allele was introduced into the AS4/AS13 background (Nag et al. 1989). Diploid cells containing a homozygous *ssp2* deletion mutant allele of all strain backgrounds behaved similarly. We also introduced the plasmid

Table 1 DNA sequence and amino acid variation in *SSP2* in strains S288C and SK1

Nucleotide position[a]	Strain[b]	
	S288C	SK1
–441	C	T
–402	T	C
–211	G	T
–122	A	T
–11	A	T
+185–186	GT (R_{62})	AC (H_{62})
+263	A (D_{88})	G (G_{88})
+1553	C	T
+1583	G	A
+1623	A	G

[a] Position +1 corresponds to the A in the *SSP2* start codon. The *SSP2* coding sequence runs from +1 to +1116

[b] Alterations in the amino acid sequence are indicated in *parentheses* in the single-letter code. The amino acid position is indicated as a *subscript*

containing the wild-type gene from FY23 (S288C background) into PSY5 (an SK1-derived *ssp2*/*ssp2* diploid strain) and the wild-type gene from the SK1 strain into the AS4/AS13-derived *ssp2* diploid strain. In both cases the mutant phenotype was rescued, suggesting that the sequence heterogeneity does not affect the function of the gene. The remaining experiments were carried out in the SK1 background using the deletion mutant strain.

Commitment to meiotic recombination remains normal in the homozygous *ssp2* diploid, but viability declines rapidly in sporulation medium

To identify the nature of the defect in the *ssp2* strain, we monitored commitment to meiotic recombination in the mutant background and compared it with that of the wild-type strain. Induction of meiotic recombination was monitored using *his4-X* and *his4-B* alleles; the mutations in these alleles map near the 5′ and 3′ ends of the *HIS4* gene, respectively. The results are shown in Fig. 2A. Meiotic recombination occurred normally in the *ssp2* background; both wild-type and mutant cells showed about a 100-fold increase in intragenic recombination over the mitotic level. A defect in meiotic recombination was not expected, since *SSP2* is induced midway through meiosis and recombination occurs early in meiosis.

To monitor the viability of the sporulating cells, samples were withdrawn from the sporulation medium at various times and plated on complete synthetic medium. The viability of *ssp2* cells declined rapidly in the sporulation medium (Fig. 2B). The drop in viability appears to occur at the time when the cells become committed to meiotic development. About 5% of the original population remained viable after 24 h in the sporulation medium.

Nuclear divisions are normal in the *ssp2* cells

Since *SSP2* is induced at the time of nuclear division, we monitored the kinetics of nuclear divisions in the mutant background and compared it with that of wild-type cells. To follow nuclear divisions, cells were withdrawn from the sporulation medium and stained with the DNA-specific dye DAPI. Both bi-nucleate and tetra-nucleate cells began to appear at the same time in wild-type and *ssp2* cells (Fig. 2C). About 80% of the cells had completed meiosis I after 9 h in the sporulation medium, and there was no apparent difference in the kinetics of nuclear division between wild-type and mutant cells. These results suggest that the *ssp2* mutation has no effect on meiotic nuclear divisions. However, the nuclei in the mutant cells became fragmented after continued incubation in the sporulation medium (Fig. 3). Nuclear fragmentation became conspicuous after 10–12 h in the sporulation medium.

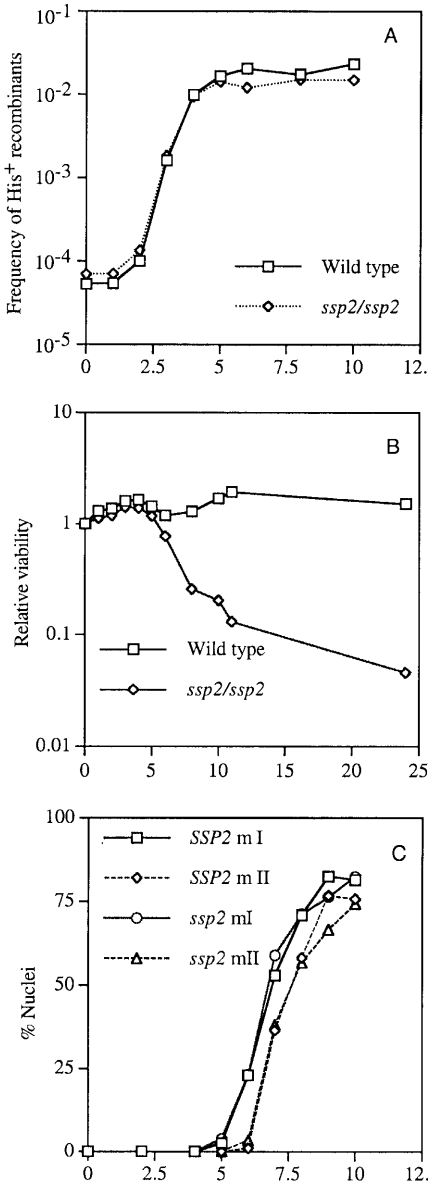

Fig. 2A–C Commitment to meiotic recombination (**A**), cell viability (**B**) and meiotic nuclear divisions (**C**) in wild-type and homozygous *ssp2*/*ssp2* diploids. The value at each time point is the mean of three independent experiments. **A** Meiotic recombination was monitored using *his4-X* and *his4-B* alleles, which carry mutations near the 5′ and 3′ ends of the *HIS4* gene, respectively. The recombination frequency was calculated as the ratio of the number of His^+ cells to the total number of colony-forming units at the time indicated. **B** Cell viability. The relative viability was obtained by dividing the total number of colony-forming units at the time indicated by the total number of colony-forming units at time 0. **C** Meiotic nuclear divisions in wild-type and *ssp2*- diploid cells. Cells were collected at the indicated time points (in hours) after induction of meiosis. The percentage of cells completing meiosis I and meiosis II was determined by fluorescence microscopy of cells stained with DAPI. The percentage of cells completing meiosis I was determined by dividing the sum of bi-, tri-, and tetranucleate cells by the total number of cells. The fraction of cells completing meiosis II was calculated by dividing the sum of tri-, and tetranucleate cells by the total number of cells, and at least 200 cells were counted for each time point

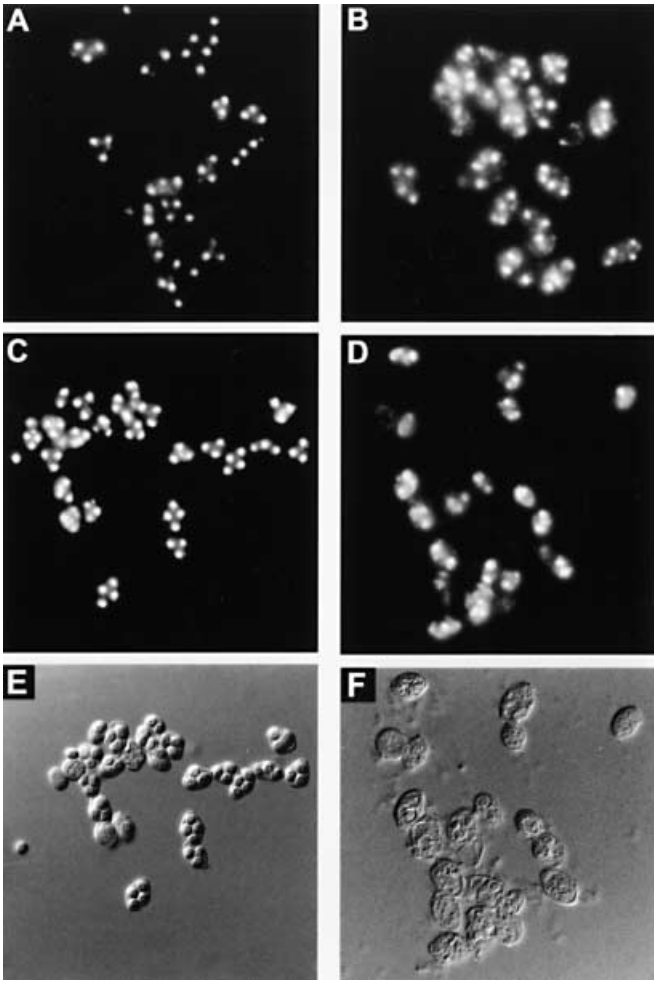

Fig. 3A–F Light microscopy of wild-type (**A**, **C** and **E**) and homozygous *ssp2* (**B**, **D** and **F**) diploid cells undergoing sporulation. Cells were collected at different time points after induction of sporulation, and fixed with ethanol, stained with DAPI as described in Materials and methods, and visualized by fluorescence (**A–D**) or differential interference (**E** and **F**) microscopy. **A, B** Sample taken at 11 h. **C–F** Sample taken at 24 h. Note that in wild-type cells (**C** and **E**), spores contain well-defined nuclei, whereas in the mutant backgrounds, nuclear integrity is disrupted

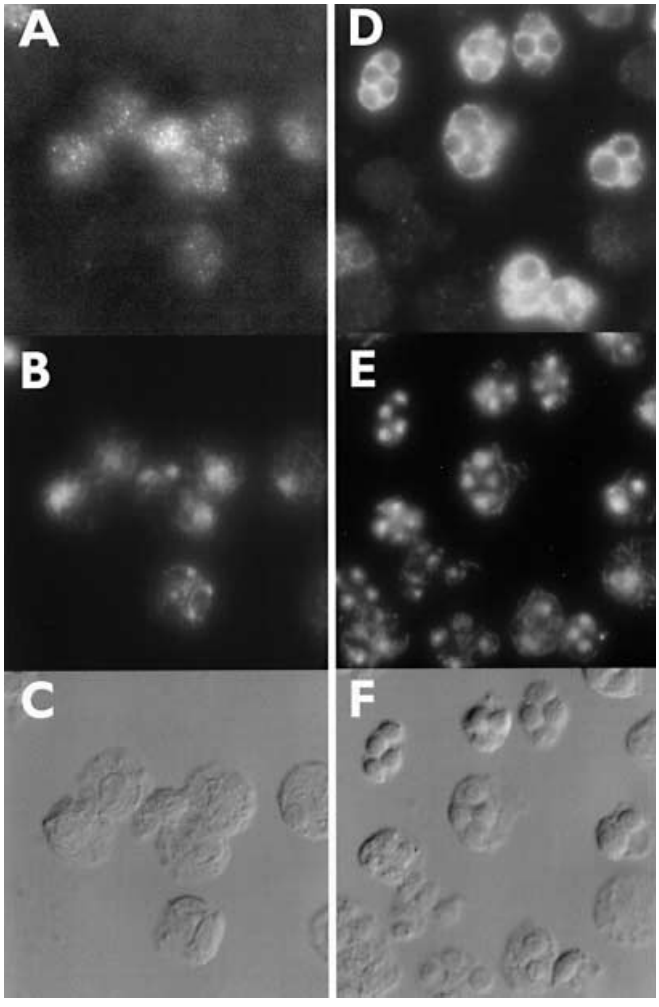

Fig. 4A–F Localization of Ssp2 in meiotic cells. The *ssp2*/*ssp2* cells containing the *SSP2::HA* fusion construct on an *ARS-CEN* plasmid (pPS23) were sporulated, and cells were collected and processed for immunofluorescence microscopy. Formaldehyde-fixed cells were treated with a mouse anti-HA primary antibody followed by an fluorescein isothiocyanate-conjugated anti-mouse secondary antibody, stained with DAPI, and examined for FITC fluorescence (**A** and **D**) and DAPI fluorescence (**B** and **E**), and observed by differential interference microscopy (**C** and **F**). **A–C** Cells collected after 7 h in the sporulation medium. **D–F** Sample obtained after 9 h in sporulation medium

Ssp2 localizes to the spore wall

Since the kinetics of nuclear division remain normal in the *ssp2* background, it is possible that Ssp2 is necessary for spore wall formation. It is also possible that Ssp2 is a structural protein of the spore wall. To elucidate the possible role of *SSP2* during sporulation, we performed localization studies using a fusion protein in which three copies of the influenza virus hemagglutinin (HA) epitope were inserted at the C-terminal end of Ssp2. Double-labelling experiments with the DNA-specific dye DAPI and anti-HA antibody indicated that Ssp2 localizes to the spore wall after meiosis II (Fig. 4). The Ssp2-HA fusion protein forms a ring structure surrounding the haploid nuclei. This staining pattern was not observed in the control strain transformed with the empty vector. In samples collected after 7 h in the sporulation medium, when cells are undergoing meiotic divisions, weak staining was observed distributed throughout the cell (Fig. 4A–C). The antibody staining was conspicuous as a ring structure only after meiosis II, at the time of spore wall formation (Fig. 4D–F).

The antibody signal was not observed in cells collected at 12 or 24 h, when mature spores are formed. The lack of antibody signal in cells containing mature spores is most likely to be due to difficulty in permeabilizing the spores. This localization result suggests that Ssp2 may be required for spore wall synthesis during sporulation. Antibody localization at the spore wall may also be due to the presence of Ssp2 in the ascal cytoplasm – because mature spores are difficult to permeabilize, and condensation of the ascal cytoplasm around

the spores can lead to an apparently peripheral localization of a protein that is, in fact, present in the ascal cytoplasm. However, we believe that Ssp2 localization to the ascal cytoplasm is unlikely since in most cells the antibody staining was found in a ring structure at the time of spore wall formation (before mature spores are formed) after meiosis II, instead of being dispersed throughout the ascal cytoplasm.

The outer layer of the spore wall is not formed in the *ssp2* mutant

The above results also indicate that the *ssp2* defect occurs after nuclear division, during spore packaging. The nuclear fragmentation phenomenon could be due to absence of the prospore membrane. In the absence of the prospore membrane, nuclear dimensions might not remain constrained, thus resulting in the apparent loss of nuclear integrity. To understand the nature of the spore-morphogenesis defect in the *ssp2* mutant, we analyzed mutant cells collected at different time points after induction of sporulation by electron microscopy . The results are shown in Fig. 5. A typical wild-type ascus is shown in Fig. 5B. Within the ascus, spores are separated from the ascoplasm by multilayered spore walls. In wild-type cells, the prospore membrane grows as it surrounds a nuclear lobe. Development of the prospore membrane was observed in both wild-type and *ssp2* cells (Fig. 5C and D).

The *ssp2* cells formed the prospore membrane and the electron-transparent layer, but failed to form the outer spore wall (Fig. 5E). The deposition of dityrosine-containing macromolecules in the outer spore wall confers a bluish fluorescence in the presence of ammonia when spores are exposed to ultraviolet radiation (Briza et al. 1990b). To confirm the results of ultrastructural analysis, we carried out the fluorescence assay for the presence of this dityrosine-containing outer layer. The characteristic blue fluorescence was present in wild-type cells but was absent in *ssp2* cells (data not shown), further suggesting that the outer spore wall is not formed in *ssp2* cells.

The outermost dityrosine-containing layer is deposited over a chitosan-containing layer (Briza et al. 1990b). In our electron-microscopic analysis, these two layers appeared to be absent in most of the *ssp2* cells. The presence of the chitosan layer is essential for deposition of the dityrosine-containing layer. To detect the chitosan layer, we stained sporulating cells with Calcofluor white, which specifically stains chitin (Fig. 6). In wild-type cells, Calcofluor staining was observed before spore wall maturation (Fig. 6B). Such staining, however, was not observed in mature spores, as the stain was unable to permeate through the mature outer spore wall (Fig. 6C; Christodoulidou et al. 1999). In *ssp2* cells, circular structures were visible in mutant cells after Calcofluor staining (Fig. 6D and E), suggesting that the chitosan layer may also be synthesized in the *ssp2* background.

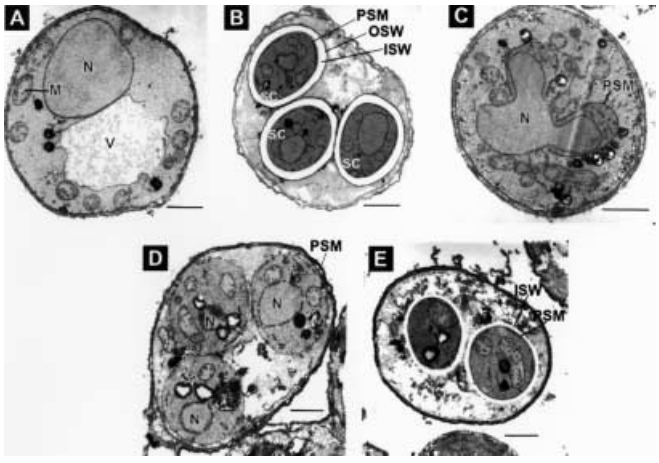

Fig. 5A–E Ultrastructure of sporulating wild-type and *ssp2* cells. Cells were collected at different times after induction of meiosis and processed for electron microscopic analysis as described in Materials and methods. **A** A typical *ssp2*/*ssp2* cell collected immediately (0 h) after transfer to sporulation medium. **B** A typical wild-type ascus collected after 10 h in the sporulation medium. **C**–**E** *ssp2*/*ssp2* mutant cells collected at different stages of spore morphogenesis: after 7 h (**C**) and 10 h (**D** and **E**). Note that both prospore membrane (**C** and **D**) and electron-transparent layers (**E**) are formed in the mutant cells, but the outer layer is not formed. An ascus containing two immature spores is shown in **E**. However, mother asci containing more than two spores were also observed. PSM, prospore membrane; ISW, inner spore wall (the inner electron-transparent layer); OSW, outer spore wall; N, nucleus; M, mitochondria; SC, spore compartment; V, vacuole. *Bars* 1 μm

The chitosan layer appeared disorganized in 24-h samples. This apparent disorganization is likely to be a result of cell death (discussed below). Since the chitosan layer was apparently present in mutant cells (Fig. 6), Ssp2 may serve as a spore-wall structural protein necessary for deposition of the outermost dityrosine layer. However, we can not rule out the possibility of a role for Ssp2 in formation of the chitosan layer, since the circular structures observed in mutant cells were not as brightly stained as they were in the wild-type background. The observed ring structures could be due to background Calcofluor staining of immature spores in *ssp2* cells. These results suggest that Ssp2 is required for spore wall formation, and, more specifically, that formation of the outer spore wall is affected by the *ssp2* mutation.

SSP2 is required for normal expression of late sporulation-specific genes

Another feature of spore wall-defective mutants is that the expression of late sporulation-specific genes is either delayed and/or reduced (Friesen et al. 1994; Krisak et al. 1994; Ufano et al. 1999; Tevzadze et al. 2000). To determine whether *ssp2* mutant cells exhibit a similar phenotype, we analyzed the expression patterns of a middle (*SPO12*, Malavasic and Elder 1990), a mid-late (*DIT1*, Briza et al. 1990b; 1994) and a late (*SPS100*, Law

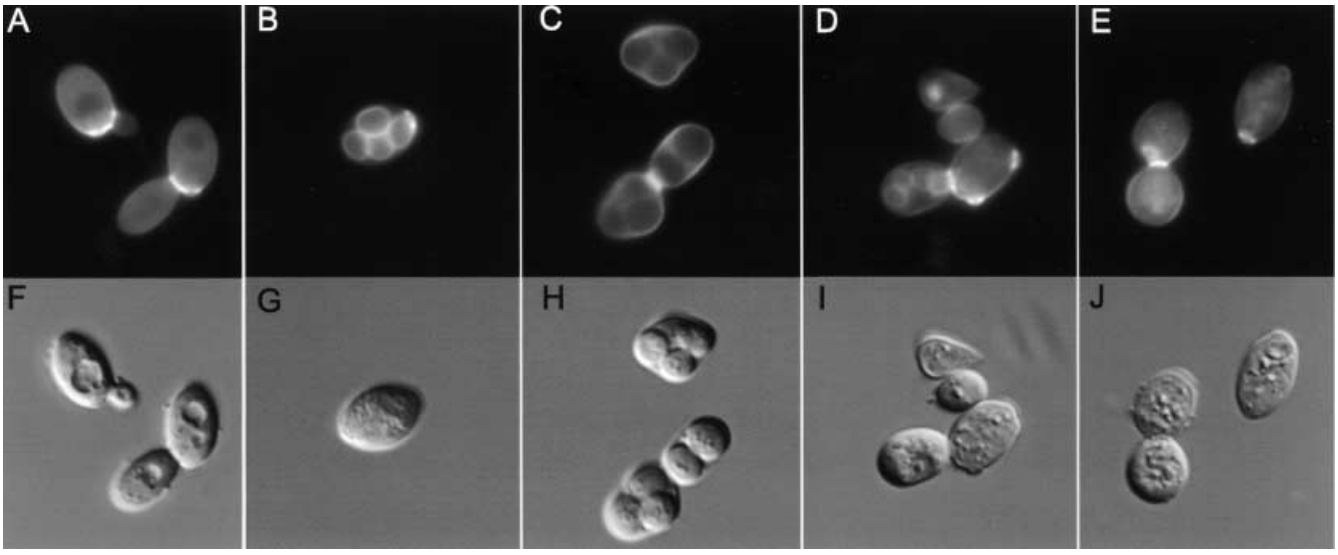

Fig. 6A–J Calcofluor staining of wild-type and *ssp2*/*ssp2* diploid cells undergoing meiosis. Cells were collected at different time points after induction of sporulation, stained with Calcofluor white and visualized by fluorescence (**A–E**) or differential interference microscopy (**F–J**). Samples were prepared from *ssp2* cells at 0 h (**A** and **F**); wild-type cells at 11 h (**B** and **G**); wild-type cells at 24 h (**C** and **H**); *ssp2* cells at 11 h (**D** and **I**); and *ssp2* cells at 24 h (**E** and **J**)

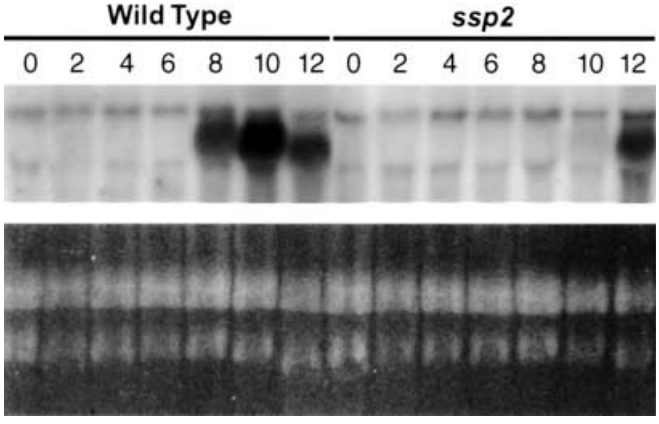

Fig. 7 Expression pattern of *DIT1*, a mid-late sporulation-specific gene, in wild-type and in *ssp2* cells during sporulation. The *numbers above the lanes* indicate the time (in hours) of sample collection. The *lower panel* shows the ethidium bromide-stained gel before blotting. An 1.8-kb *Hin*dIII fragment containing the *DIT1* ORF was used as the probe

and Segall 1988) gene by Northern analysis. The results are shown in Fig. 7. Although the expression pattern of *SPO12* remained unaltered in the *ssp2* mutant cells (data not shown), the timing and level of expression of *DIT1* were altered in the mutant background. Meiotic expression of *DIT1* was delayed by about 2 h. The level of *SPS100* mRNA was below the limits of detectability by Northern analysis (data not shown). These results suggest that *SSP2* is required for proper expression of late meiosis-specific genes. The normal expression pattern of the middle genes suggests that early steps of spore wall formation can proceed without the *SSP2* function, and that a later step in spore wall formation is affected in the *ssp2* background. The ultrastructural analysis indicating that only the outermost layer is affected in the *ssp2* cells supports this hypothesis.

Discussion

This report describes the functional analysis of a novel meiosis-specific gene, *SSP2*, that is essential for the sporulation program in yeast. Comparative protein sequence analysis indicated that a region of 207 amino acids near the C-terminal end of Ssp2 has significant homology (40% identity) to a protein encoded by a hypothetical ORF, Ca38F10.06, of the pathogenic diploid yeast *Candida albicans,* which is not known to have a sexual lifecycle. *C. albicans* does not have a haploid life cycle, and diploid cells proliferate asexually by budding or by formation of hyphae (Pla et al. 1996). Diener and Fink (1997) have identified a functional homolog of *DMC1*, *DLH1*, in *C. albicans*. *DMC1* is a highly conserved meiosis-specific gene in *S. cerevisiae*, with an essential role in meiotic recombination. High-level expression of *DLH1* rescues the meiotic defect in the *dmc1* background. However, *DLH1* expression can not be detected under conditions that induce *DMC1* in *S. cerevisiae*. Comparative genome sequence analysis between *C. albicans* and *S. cerevisiae* also revealed the presence of homologous genes implicated in different meiosis-specific processes (Tzung et al. 2001). These results suggest that *C. albicans* retains several components that are required for the sporulation program.

SSP2 is expressed at the time of initiation of nuclear divisions, when the cells become committed to meiotic development. Commitment to meiosis occurs after recombination and chromosome synapsis, and immediately before nuclear divisions (Kupiec et al. 1997). The observation that meiotic recombination remains normal in the mutant background is consistent with this expression pattern, since recombination is an early

meiotic event. Since meiotic recombination was monitored at one locus and intragenic recombination occurs mostly by gene conversion events, it is possible that the *ssp2* mutation may have some effect on reciprocal exchange or on gene conversion events at other loci.

Although the kinetics of nuclear divisions remained normal in *ssp2* cells, viability declined rapidly in the sporulation medium. Only 5% of the original population remained viable after 24 h in the sporulation medium. It is likely that cells that entered meiosis died and the remaining viable cells failed to enter the sporulation program. An alternative possibility is that during spore packaging, the prospore membrane might not package some nuclei. The haploid nuclei that failed to be packaged by the prospore membrane could have migrated to the daughter bud upon transfer to the growth medium. Such migrations of daughter nuclei have been demonstrated in *spo14*-arrested cells (Honigberg et al. 1992; Honigberg and Esposito 1994). The remaining viable cells might have resulted from these daughter cells. However, the latter possibility seems unlikely since none of the 50 colonies (formed by the 24-h sample of *ssp2* cells) tested were maters, and all of them were His^-.

After meiosis II, the haploid nuclei are packaged by the prospore membrane. The prospore membrane acts as the plasma membrane of the spore cell, and is synthesized de novo in the mother cell. Ultrastructural analysis of *ssp2* mutant cells showed normal growth of the prospore membrane during spore packaging. Our results indicate that Ssp2 is necessary for the formation of the mature outer spore wall, since the first two layers of the spore wall are formed in mutant cells (Fig. 5) and Ssp2 localizes to the spore wall (Fig. 4). The results of Calcofluor staining, to detect the presence of the chitosan layer, are also consistent with the inference from the electron microscopic studies, i.e., the formation of the outer spore wall is affected in *ssp2* cells. The Calcofluor-stained rings appeared disorganized in cells collected at late time points. These aberrant structures were not observed in mutant cells collected after 11 h in the sporulation medium when mature spores are formed in the wild-type background. The mutant cells exhibited disorganized chitin staining in 24-h samples, at a time when nearly 95% of the cells were dead. It is likely that these disorganized structures are formed following cell death during prolonged incubation in the sporulation medium. However, we can not eliminate the possibility that the loss of viability actually results from these disorganized structures.

A rapid decline in cell viability in the sporulation medium has also been observed with several other spore wall-defective mutants. For example, in *spo1*, *spo14* and *ssp1* mutant cells, nuclei fragment soon after meiosis II and viability declines rapidly in the sporulation medium (Honigberg et al. 1992; Nag et al. 1997; Tevzadze et al. 2000). The results presented here, and recent results obtained with *ssp1* mutant cells (Moreno-Borchart et al. 2001), suggest that the loss of viability is not the consequence of a defect in prospore membrane formation. In *ssp2* cells, even though the prospore membrane is formed, cells die rapidly in the sporulation medium.

The reduction in viability appears to begin long before the onset of spore wall formation. A defect in formation of the outer layer of the spore wall does not appear to be responsible for cell death, since several other spore wall-defective mutants (e.g., *dit1*, *dit2, smk1* and others) that fail to form the outer spore wall appear to die slowly (Briza et al. 1990b; Friesen et al. 1994; Krisak et al. 1994). One possible mechanism of cell death is that the *ssp2* mutation may affect a fundamental step during meiotic divisions that can not be detected by DAPI staining. For example, *spo1* mutants exhibit a similar phenotype, and *SPO1* is required for duplication of the spindle pole body (Tevzadze et al. 2000). Similarly, Spo14 is also believed to be involved in spindle pole body function (Rudge et al. 1998).

It is also likely that there are two classes of post-meiotic mutants that affect spore wall formation. One class of mutants die rapidly, while the others die slowly upon prolonged incubation in the sporulation medium. *ssp1*, *spo14*, *spo1*, and *ssp2* may belong to the former class, while *smk1* and *swm1* fall into the second class. However, several of these mutants were analyzed in different strain backgrounds and under different sporulation conditions. Therefore, differences in the rates of loss of viability among the post-meiotic mutants may also be due to differences in the strain background and the sporulation conditions.

In summary, meiotic divisions and spore morphogenesis appear to be highly coordinated. Improper completion of an earlier step may lead to a defect in later steps in the sporulation process. This idea is also supported by the severe reduction in expression of late sporulation-specific genes in the *ssp2* mutant. Future work should reveal the precise role of *SSP2* in the sporulation process.

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