Abnormal ascospore morphology in the *bud* mutant of *Neurospora tetrasperma*

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Abstract

A recessive ascospore mutant of *Neurospora tetrasperma*, named *bud*, was isolated from a wild-collected heterokaryotic strain with four different nuclear components. *bud* segregates as a single mendelian gene. When *bud* is homozygous, meiosis is apparently normal but postmeiotic events are not. Abnormal orientation of spindles at the postmeiotic mitosis often results in failed pair-wise association of nuclei and their irregular distribution along the length of the ascus prior to spore delimitation. Consequently, many asci cut out more than four ascospores; some contain no nuclei while others contain more than two. The most dramatic effect of *bud* is on ascospore delimitation itself. Many ascospores are irregularly shaped and are often interconnected, because of incomplete spore delimitation. Ascospores also show one or two lobes or bud-like extensions of varying sizes. Over 75% of ascospores from *bud* × *bud* remain white or tan and are inviable. The interaction of *bud* with a dominant Eight-spore mutant (*E*) was examined in both heterozygous and homozygous crosses. When both *bud* and *E* are heterozygous, *bud* has no effect on ascospore delimitation or on the phenotype of *E* because *bud* is recessive; many asci produce 5–8 ascospores just as in *E* × *E*. And when *bud* is homozygous and *E* is heterozygous, ascospore delimitation is less affected than when *E* is absent. Moreover, when both *bud* and *E* are homozygous, the effect on ascospore development is less extreme than when *E* is homozygous singly.

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Index Descriptors: Ascus development; Abnormal ascospore delimitation; Ascospore mutant; Ascospore lobes; Budded ascospores; *Neurospora tetrasperma*.

1. Introduction

Asci of *Neurospora tetrasperma* are normally four-spored, with each ascospore receiving two nuclei that are of different mating types, *mat A* and *mat a*, also called mating-type idiomorphs, and hereafter written as A and a. Single-ascospore cultures are self-fertile as in homothallic species but distinct mating types are present as in heterothallics. Such species are said to be pseudohomothallic or secondarily homothallic (Dodge, 1927; Raju and Perkins, 1994). The four-spored ascus development that results in pseudohomothallism is precisely programmed (Figs. 1 and 2). Mating types almost always segregate at the first division of meiosis owing to tight linkage of *mat* to the centromere (Gallegos et al., 2000; Merino et al., 1996; Raju and Perkins, 1994). The second and third-division spindles overlap and align pairwise. Subsequently, nonsister nuclei of opposite mating type become enclosed in each ascospore (Raju, 1992a; Raju and Perkins, 1994).

Several genotypes are known in which ascus development is altered in *N. tetrasperma*. The dominant eight-spore mutant *E* causes many asci to produce 5–8 ascospores rather than the normal four. In these asci one or more large, binucleate, ascospores are replaced by pairs of small, uninucleate ascospores (Dodge, 1939; Dodge et al., 1950; Raju, 1992a). Eight-spored asci are also produced in outcrosses between certain *N. tetrasperma* wild-collected strains (Jacobson, 1995). In both Dodge's *E* strains and in certain outcrosses, eight-spored asci are formed because of altered spindle alignment at the second and third divisions and the failure of...
nuclei to associate in pairs prior to ascospore delimitation (Dodge et al., 1950; Jacobson, 1995; Raju, 1992a).

In the present study, we describe a new recessive mutant of N. tetrasperma that shows normal meiosis and a nearly normal postmeiotic mitosis, but in which ascospore delimitation is highly irregular. Ascospores are often incompletely cut out, leaving cytoplasmic interconnections between spores; many spores are lobed, and some spores contain one or more nuclei while others contain no nuclei at all. The interaction of Dodge’s E gene with bud partially alleviates the effects of bud and E on ascospore delimitation.

2. Materials and methods

2.1. Strains

The bud ascospore mutant was isolated from a heterokaryotic strain with four different nuclear components (P452; Table 1) that was collected by David Perkins in Dunnellon, Florida in March 1970. P452 produced two populations of perithecia: the majority (70–80%) contained normal four-spored asci and the remaining perithecia produced abnormal asci and ascospores with buds. Among 12 abnormal ascospores that were isolated from abnormal perithecia, only two germinated (P452-1 and P452-2, both bud A + bud a). These produced self-fertile cultures in which all perithecia again produced abnormal ascospores. Single-mating type, homokaryotic bud A or bud a strains were isolated from these two heterokaryotic self-fertile strains for further cytogenetic analysis of bud (see Jacobson, 1995; Raju, 1992a). The bud mutation was also

Fig. 1. Schematic diagram of ascus development in N. tetrasperma. (A) Anaphase I. Alleles of a centromere-linked gene (e.g., mat A and mat a) segregate at the first division of meiosis. (B) Telophase II. The two second-division spindles overlap, and are aligned nearly parallel to one another along the long axis of the ascus. (C) Interphase II. The nuclei in each proximal and distal half ascus are non-sisters arising from different spindles. (D) Telophase III. The spindles are again aligned in pairs in each half ascus. (E) Interphase III. The eight nuclei are aligned in four pairs, all lined up in single file. (F) The four pairs of nuclei (A + a) become enclosed in each of the four ascospores. (G) A mitosis in young ascospores results in four nuclei per ascospore. Additional mitoses occur in mature black ascospores.

Fig. 2. Ascus development in wild type N. tetrasperma. (A) Anaphase III. Condensed chromosomes are segregating on the two spindles that are aligned across each half ascus. (B) Interphase III, at about ascospore delimitation. (C) An ascus with four, young, and binucleate ascospores. (D) Two four-spored asci following a mitosis in the ascospores. (E) A partial rosette of maturing asci. (Ascus development in wild type × bud is similar to that in wild type × wild type.) Scale bars in (A–D) 10 μm; and (E) 100 μm.
recombined with Dodge’s E gene to examine possible interaction of the two mutations during ascus and ascospore development (see Table 1).

2.2. Crosses for perithecia

All crosses were made on synthetic crossing medium (Davis and de Serres, 1970) in 9 cm petri plates, supplemented with 1% sucrose and 2% agar, and incubated at 25°C (12 h light/12 h dark). Early cytological observations were made on dual-mating-type strains (e.g., P452, P452-1, and P452-2) that were allowed to self. Heterokaryotic strains that contain both bud A and bud a nuclei were sometimes inoculated at the center of the plate and allowed to grow and fruit. P452-1 and P452-2 strains were also used as fertilizing (male) parents with appropriate single-mating type protoperithecial (female) wild type and bud a parents. For these crosses, the female parent was first grown 2 or 3 days and then fertilized with the conidia from the dual-mating-type male parent. Subsequent work was done on crosses of homokaryotic bud A strains with homokaryotic bud a strains (Table 2). Homokaryotic strains were usually grown in separate plates for 2 or 3 days and fertilized reciprocally by spreading conidia and mycelial fragments on the surface of the already grown protoperithecial parent.

2.3. Cytology

Agar strips bearing developing perithecia between 3 and 6 days after fertilization (5-8 days after simultaneous inoculation) were fixed in 9:6:2 (v/v) ethanol, propionic acid, and 10% aqueous chromic acid. The fixed perithecia were hydrolyzed and their contents were dissected out and stained with an iron-hematoxylin procedure (Raju, 1978; Raju and Newmeyer, 1977). Maturing rosettes of ascites from 6 to 8-day-old perithecia were prepared without fixation or hydrolysis, and were lightly stained with (10-fold) dilute solutions of ferric acetate mordant and hematoxylin. An Olympus BH2 microscope was used for observations at 100–1000x.

3. Results

Preliminary observations on abnormal ascus development in the bud mutant from P452 were made by A.G.B. at Cornell University in 1972. Subsequent cytogenetic analysis of bud was done by N.B.R. at Stanford University. No effort was made to map the bud mutation to a specific linkage group. However, in various crosses of bud to wild type, bud segregated 1:1 as a single mendelian gene and progeny were obtained in both mating types with equal frequency indicating that the two are not linked.

Conidia of N. tetrasperma contain several nuclei, the modal class being 3 or 4 (Raju, 1992a). Conidial isolations from the original P452 strain from Dunnellon showed that it is a four-component heterokaryon (bud A + bud a + bud A + bud a). Of the 32 single-conidial cultures that were progeny tested, 9 contained a single component, 17 contained two types, four contained three types, and only two contained all four nuclear types (Table 3). All four genotypes were not equally represented in P452, however; bud A and bud A nuclei were more abundant than bud a and bud a.

Conidia from P452-1 Ala and P452-2 Ala (both bud A + bud a) were spread on agar medium containing sorbose, and 156 single-conidial colonies were transferred to crossing medium in 75 x 24 mm tubes. Within a week,
heterokaryotic, self-fertile (A+a) cultures produced perithecium and ascospores, whereas no perithecium were formed in single-mating type, self-sterile cultures. Among the 156 cultures tested, 94 were self-fertile (A+a), and 62 were self-sterile (58 A and 4 a). Again, the nuclear ratios were skewed in the two heterokaryons tested: mat A nuclei were more abundant than mat a (Table 4).

In another experiment, 43 apparently small ascospores from selfed perithecia of P452-1 (bud A + bud a) were isolated onto complete medium slants and heat shocked to induce germination. Of the 43 ascospores, 23 germinated and formed cultures: 14 were self-fertile (A+a), and 9 were self-sterile (4A and 5 a). Unlike the conidial isolates, which showed skewed mating type ratios, the two mating types were equally represented in this small sample of ascospore cultures. All nine self-sterile cultures were intercrossed among siblings by simultaneous inoculation of compatible pairs on synthetic crossing medium. All 20 crosses produced abnormal budded ascospores in most of the asci. The 14 self-fertile progeny also produced mostly budded ascospores.

Dodge’s E strains were used in crosses with bud strains to obtain bud E double mutants. Sixteen small, homokaryotic, ascospores were isolated from a cross of bud A × E a. One of the progeny, bud E a, was then crossed to wild type 85 A, and 18 small-ascospore progeny were analyzed to obtain bud E A (see Tables 1 and 2 for strains and crosses).

### 3.1. Cytological observations

Initial cytological observations were based on homozygous bud × bud perithecia that were a minority (25–30%) in the original P452 self-fertile strain. All other perithecia were either homozygous (bud+/bud+) or heterozygous (bud+/bud) for bud and contained normal four-spored asci. Subsequent, more detailed cytological observations were made on self-fertile, single-ascospore progeny (P452-1 and P452-2) that were homokaryotic for bud and heterokaryotic for mating type. Additional observations were made on bud × bud+, bud × bud, bud E × bud, and bud E × bud E using homokaryotic strains (Table 2). One thousand or more asci were examined for each cross.

#### 3.1.1. bud × bud+

When bud is heterozygous, ascus development is completely normal (like wild type); thus bud is recessive. Alleles of any gene near a centromere (e.g., mating types) segregate at the first division of meiosis, and consequently both A and a nuclei become enclosed in each of the four ascosomes. Thus, single-ascospore cultures are self-fertile (Fig. 1; Raju and Perkins, 1994). Meiosis and a postmeiotic mitosis are normal and normal numbers of mature, four-spored asc is produced (Fig. 2). Second and third-division spindles are aligned in pairs and overlap with each other (Fig. 2A). Pairs of nonsister nuclei then realign themselves in single file along one side of the ascus. During ascospore formation, the preformed ascospore-delimiting membranes invaginate around pairs of nuclei and the surrounding cytoplasm, and cut out four binucleate ascospores (Figs. 2B and C). A subsequent mitosis in young ascospores makes them 4-nucleate (Fig. 2D).

#### 3.1.2. bud × bud

When bud is homozygous, early ascus development and meiosis are nearly normal. The two second-division spindles also overlap and are nearly parallel to one another. Following meiosis, the four nuclei show a normal biseriate alignment. The two nuclei in each proximal or distal half-ascus are nonsisters, arising from two different spindles (Fig. 3A). Postmeiotic mitosis and the alignment of nuclei are abnormal in up to 50% of ascii. The four spindles at the third division (mitosis) are not always aligned in pairs, nor are they parallel to one another (Fig. 3B). This irregular alignment and orientation of spindles often results in abnormal distribution of nuclei in the ascus prior to spore delimitation (Fig. 3C). The individual nuclei of each “pair” are often irregularly aligned with respect to each other.

A more dramatic effect of bud is expressed during ascospore delimitation. In a majority of asci, the eight nuclei are sequestered into more than four ascospores, usually one or two normal-sized binucleate ascospores, and the remaining ascospores are irregularly shaped and incompletely cut out (Figs. 3D–F). Some of the apparently normal-sized spores contain just one nucleus or no nucleus at all, whereas some smaller spores

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<td>Single-conidial isolates from the original P452 strain from Dunnellon, and their inferred genotypes based on progeny tests</td>
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Genotypes of all the nuclear types in a heterokaryon are separated by “+” and enclosed in parentheses.

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<td>Single-conidial isolates (N = 150) from P452-1 and P452-2 (both bud A + bud a) and their genotypes</td>
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<td>(bud A + bud a) self-fertile</td>
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receive two nuclei. In up to 10% of asci, three or four nuclei are enclosed in one or two large ascospores, and the remaining nuclei are sequestered into smaller spores or left out in the cytoplasm. In a majority of asci, small, bud-like vesicles (lobes) are present on one or more ascospores (Figs. 3D–H). These do not grow from the surface of the spores after they are delimited, but are formed when the spores are cut out. The abnormal ascospores in ~25% of asci show cytoplasmic connections between adjacent ascospores. The interconnections serve as conduits for cytoplasmic continuity or nuclear passing. This was inferred from the distribution of nuclei, following a mitosis, into various ascospores and their lobes. A nucleus may sometimes migrate into a lobe or get caught at the juncture between two spores or between the main spore and a bud (Figs. 3E and F).

Ascospore maturation is clearly abnormal when bud is homozygous. Although nearly normal numbers of ascospores are ejected from homozygous bud perithecia, over 75% of the spores are white or tan, and inviable. In most of the intact asci, one or more ascospores are abnormal (lobed) and fail to mature (Figs. 3G and H). However, normal-size, non-lobed, black ascospores are fully viable.

3.1.3. bud E × bud E⁺

Because bud is recessive, the crosses heterozygous for both bud and E (bud E⁺ × bud E⁺) show only the eight-spore phenotype of the dominant E; many asci produced five to eight ascospores. In these asci, one or more large, binucleate, dual-mating-type ascospores are replaced by pairs of small, uninucleate, single-mating-type ascospores (see Raju, 1992a). None of the ascospores (large or small) is lobed or interconnected, apparently because bud is recessive (Fig. 4). We have therefore examined the interaction of bud with E in crosses where E is heterozygous and bud is homozygous (Fig. 5). Early ascus
development is normal through meiosis I, and the alignment of second and third-division spindles is similar to that of \( E \times E^+ \) (Dodge et al., 1950; Raju, 1992a). In about 50% of asci, spindles typically overlap at both second and third divisions; such asci presumably produce four large (normal) heterokaryotic ascospores. In the remaining asci, second-division spindles are in tandem, and third-division spindles are aligned equidistant or irregularly (Fig. 5A); such asci are believed to produce one or two large ascospores and several small ascospores. Whereas the large ascospores are without lobes or buds, some of the small spores are lobed and are often interconnected with adjacent spores (Fig. 5B).

In general, the effect of \( bud \) is less drastic when \( E \) is heterozygous than when \( E \) is absent (Figs. 3 and 5C). In the presence of \( E \), the second and third-division spindles in many asci are aligned farther apart (as in \( E^+ /C2 E^+ \)) and the well-separated nuclei are presumably sequestered into ascospores more effectively.

Fig. 4. A partial rosette of maturing asci from \( E \times bud \). Since \( E \) is dominant over \( E^+ \), and \( bud \) is recessive, only the eight-spore phenotype of \( E \) is expressed. Many asci produce 5–8 ascospores and none of them show \( bud \) phenotype. These asci are not distinguishable from those of \( E \times E^+ \). Scale bar: 100 \( \mu m \).

Fig. 5. \( E bud \times bud \). (A) Top ascus at metaphase III. The four spindles are aligned nearly equidistant in many asci, just as in \( E \times bud \). Bottom ascus shows several budded ascospores. (B) An eight-spored ascus showing several abnormal small ascospores. (C) A rosette of maturing asci. Approximately 25% of asci show one or more budded ascospores. However, the \( bud \) phenotype is much less severe than when \( E \) is absent in the cross. Scale bars in (A and B) 10 \( \mu m \); (C) 100 \( \mu m \).

Fig. 6. (A) A rosette of asci from \( E \times E \). Almost all asci abort when \( E \) is homozygous. (B) A rosette of asci from \( E bud \times E bud \). Several asci produce one or more mature ascospores; apparently, the interaction of the two mutants results in less severe effects on ascus development than when \( E \) alone is homozygous. Scale bar: 100 \( \mu m \).
3.2. *bud E × bud E*

Dodge's *E* gene produces many eight-spored asci when it is heterozygous (see Fig. 4), but ascospore production is greatly reduced when *E* is homozygous (Dodge, 1939; Raju, 1992a). Most of the asci undergo meiosis and a postmeiotic mitosis but they abort and degenerate just prior to or shortly after spore delimitation. Only a small fraction of asci (1–5%) contain up to eight maturing ascospores (Fig. 6A). However, when *E* is combined with *bud* and the double mutant is made homozygous, ascus abortion is less severe than when *E* alone is homozygous. Many asci cut out 2–6 ascospores, some of which are shaped abnormally. Ascospore cutting is more complete and bud or lobe formation is less pronounced than when *bud* alone is homozygous. Many more asci produce one or more ascospores that pigment and develop to a later stage than when *E* alone is homozygous (Fig. 6B).

4. Discussion

The *bud* mutant of *N. tetrasperma* is defective primarily in ascospore delimitation. Meiosis is normal, but spindle orientation at the post-meiotic mitosis and subsequent nuclear distribution along the ascus are irregular. At least two other *N. tetrasperma* mutants, *E* and *peak*, exhibit abnormal spindle orientation and nuclear distribution, but without grossly affecting ascospore delimitation per se. For example, the *E* gene results in many five- to eight-spored asci in which one to four binucleate, large spores are replaced by pairs of uninucleate, small spores. In these asci abnormal spindle behavior at the second and third divisions results in failed pairwise association of nuclei and abnormal alignment of nuclei along the length of the ascus. Yet, ascospore delimitation is normal and complete (Dodge et al., 1950; Raju, 1992a). A second example is the peak mutant of *Neurospora crassa*, which results in swollen, balloon-shaped asci in which the ascospores are not linearly ordered (Raju, 1988). When the peak mutant was introgressed from *N. crassa* into *N. tetrasperma*, spindle orientation and pairwise association of nuclei were clearly abnormal in the swollen asci. Consequently, many asci produce more than four spores, some of which are uninucleate. Asc with eight spores are frequent (Pincheira and Srb, 1969; Srb et al., 1973). Similar, but more pronounced spindle abnormalities are seen in certain intercrosses between unrelated *N. tetrasperma* strains that produce mostly eight-spored asc (Jacobson, 1995; Raju, 1992a). Ascospores in all the above examples are delimited completely and they are neither interconnected nor lobed (see Fig. 4). Thus, neither the abnormal orientation of spindles nor the abnormal distribution of nuclei can account for abnormal lobed or budded ascospores in *budbud* asci.

Thompson-Coffe and Zickler (1992, 1993) used immunofluorescence to show that microtubules and actin microfilaments play an important role during meiosis, postmeiotic nuclear distribution, and ascospore delimitation in *Sordaria macrospora*. We have shown here that meiosis is nearly normal, but that postmeiotic mitosis, subsequent nuclear positioning along the length of the ascus, and spore delimitation are abnormal in the *bud* mutant. Normal appearing spindles are formed even when their distribution and orientation at the postmeiotic mitosis are abnormal. This suggests that the spindle microtubules, responsible for chromosome segregation, per se are not abnormal in this mutant. We have not investigated the role of other components of the cytoskeleton in abnormal ascospore formation in the *bud* mutant.

Numerous abnormal ascospore mutants have also been described in the eight-spored species *N. crassa* (Raju, 1992b; Srb et al., 1973). For example, when *Round spore* is heterozygous, all asci produce eight round ascospores in the linear ascus rather than the normal spindle-shaped ascospores (Mitchell, 1966). In up to 10% of asci, one or two ascospores show a bud-like lobe, usually at the distal end of the ascus (N.B. Raju, unpublished). This result suggests that normal amounts of ascospore delimiting membrane is pre-formed but less of it is used for cutting out round spores than for spindle-shaped spores of the same volume. The unused spore-delimiting membrane is believed to form a lobe on one or more of the ascospores. Ascospore delimitation is complete for all other spores, and none of the eight spores are interconnected. Round ascospores are also produced in the colonial temperature-sensitive mutant *cot-2*. Ascospores are all normal when *cot-2* is heterozygous but they are all round when *cot-2* is homozygous (Barry et al., 1972). In addition, spore delimitation in the round-spored asci of *cot-2* is clearly abnormal; some ascospores are incompletely cut out and show buds or interconnections with the adjacent ascospores (N.B. Raju, unpublished).

*Banana* is an extreme mutation that fails to cut out individual ascospores. Raju and Newmeyer (1977) have shown that the eight nuclei, prior to spore delimitation, fail to line up in single file. Consequently, all eight nuclei are enclosed in a single giant ascospore that occupies about one-half of the ascus length. Since the giant ascospores require much less spore delimiting membrane than all eight normal ascospores combined, any remaining excess membrane might be expected to form lobes or buds on the giant ascospores of Ban. This was not observed. Instead, the excess spore-delimiting membranes form invaginations or vesicles inside the ascospore cytoplasm (Raju and Newmeyer, 1977).
4.1. Maintenance of bud in nature

Recessive sexual phase mutations have been shown to be sheltered in natural populations of the eight-spored heterothallic species \textit{N. crassa} (Leslie and Raju, 1985; Raju and Leslie, 1992). The presence of such a recessive mutation was revealed only by obtaining a mutant of opposite mating type, and then testing it in a backcross to the wild strain. In the present study, we have shown that both \textit{bud} and \textit{bud}– nuclei were equally represented in the conidial isolates from P452. Thus, \textit{bud} was apparently not at a marked selective disadvantage in the heterokaryotic vegetative phase. Because the mutant is recessive, only homozygous \textit{bud} combinations would be selected against during the sexual phase, just as with the omnipresent recessive sexual-phase mutations studied by Leslie and Raju (1985). However, \textit{N. tetrasperma}, being a four-spored species and an inbreeder, presents a very different situation. Heterozygous combinations of centromere-linked recessive mutations, once formed, would perpetuate generation after generation without showing any detrimental effects. It would therefore be of interest to isolate and compare recessive sexual-phase mutations in this pseudohomothallic species with those of \textit{N. crassa}.

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References


