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MOLECULAR AND CONFOCAL MICROSCOPY COMPARISONS OF WILD TYPE AND TEMPERATURE SENSITIVE ALLELES OF THE *ASPERGILLUS NIDULANS* MORPHOGENETIC LOCUS *HYPA*

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Saskatoon

by

Yu Sha

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ABSTRACT

Aspergillus nidulans is a filamentous fungus whose cells have highly polarized growth. This requires many genes including *hypA*, which affects hyphal morphogenesis by promoting tip cell growth and suppressing growth in basal regions. The *hypA* locus was cloned previously by complementing the *hypA1* phenotype. The *hypA* orthologues in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are essential, whereas *hypA* deletion strains in *Aspergillus nidulans* are viable but morphologically abnormal.

The *A. nidulans hypA* locus has two temperature sensitive alleles, *hypA1* and *hypA6*. These alleles were generated independently, but using the same mutagen, and were identified and characterized by different groups. Although the *hypA1* strain has been shown to sporulate at restrictive temperature, the *hypA6* strain was described as having a restrictive arrest phenotype, which suggested it was a lethal defect and was at odds with the report that *hypA* was dispensable for growth. To resolve this discrepancy, my study compared the *hypA1* and *hypA6* strains using morphometry, and compared their genetic lesions. I show that the *hypA1* and *hypA6* phenotypes are indistinguishable and mutation events in their coding regions are identical.

Both *hypA1* and *hypA6* strains were able to sporulate at restrictive temperature as shown by scanning electron microscopy. Their cell morphologies were indistinguishable after growth under restrictive conditions, as were their repolarization kinetics when restrictive-grown cells were shifted to permissive temperature.

Sequencing the *hypA* locus from a *hypA6* strain, and comparing it to a *hypA*1 strain showed that they had identical lesions (G329R), which is consistent with the morphometric analysis. Unexpectedly, the *hypA*1 and *hypA6* strains shared additional non-conservative amino acid substitutions, K885F and E932K, with their wildtype parent A28 compared to the strain used for complementing *hypA1*. The repair of these two amino acid changes can not rescue *hypA1* or *hypA6* phenotype at 42°C.

The *S. cerevisiae* homologue of *hypA* protein is TRS120p, which is involved in endomembrane trafficking. FM4-64 endomembrane staining of *hypA1* and *hypA6* strains showed they had aberrant endomembrane arrays at restrictive versus permissive temperature, which recovered the wildtype pattern after a return to permissive conditions. This is consistent with the similarities between the *hypA* and *TRS120* sequences.

The disparity between the published descriptions of the *hypA1* and *hypA6* restrictive phenotypes has been resolved, and the allele renamed *hypA1/A6*.

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LIST OF ABBREVIATIONS

TAE	Tris-acetate-EDTA
4-NQO	4-nitro quinoline oxide
bp	base pair
BSA	bovine serum albumin
CM	complete medium
DEPC	diethyl-pyrocarbonate
DIC	differential interference contrast
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FITC	fluoresecin isothiocyanate
FM4-64	amphiphilic stytyl dye (N- (3- triethylammoniumpropyl)- 4- (6- (4- (diethylamino) phenl) hexatrienyl) pyridinium dibromide)
GFP	green fluorescence protein
kbp	kilo base pair(s)
kDa	kilodalton
MM	minimal medium
MOPS	3-(N-morpholino) propanesulfonic acid
PABA	p-aminobenzoic acid

PCR	polymerase chain reation
PEG8000	polyethylene glycol 8000
rpm	revolutions per minute
SDS	sodium dodecyl sulfate (sodium lauryl sulfate)
SE	standard error
SEM	scanning electron microscope
TE	Tris-HCl-EDTA
TEM	transmission electron microscope
TRAPP	transport protein particle
Tris	tris(hydroxymethyl) aminomethane
UV	ultraviolet

1. INTRODUCTION

1.1 Aspergillus nidulans life cycle

Aspergillus nidulans is a filamentous fungus with highly polarized cell growth. *A. nidulans* goes through its life cycle predominantly by asexual reproduction and before its sexual stage was discovered, it was recognized as deuteromycete, an artificial grouping used for convenience to accommodate a large number of fungi that seldom or never produce sexual stages but are genetically ascomycota. In the 1960s a common soil ascomycete, *Emericella nidulans,* was found to produce the sexual fruiting bodies, cleistothecia, that germinated to produce *Aspergillus nidulans* colonies (reviewed in Deacon, 1997). *A. nidulans* has since been reclassified into Ascomycotina (class Plectomycetes). Like other deuteromycetes, *A. nidulans* starts its asexual life cycle with a spore (conidium), that germinates (§1.1.1), grows into vegetative mycelium (§1.1.2), and then produces a new generation of conidia, sporulating for dispersal and short term survival.

1.1.1 Germination

When conditions permit, especially in the presence of a reduced carbon source like glucose (Osherov and May, 2000), plus moisture and temperature in the range of 0-45°C, wild type *A. nidulans* spores will break dormancy and germinate. This

process includes water uptake, resumption of metabolic activities, and nuclear division (d'Enfert, 1997). Two growth modes are adopted in this process: first isotropic spore swelling and then polarized germ tube extension.

During isotropic growth, the spore increases two or more times in diameter by water uptake. New cell wall is inserted in all directions (Van Etten *et al*., 1983), since wall thickness remains approximately constant, even though when the spore diameter doubles, its surface area increases four-fold. Eventually, wall deposition becomes restricted to a localized region, resulting in a transition from isotropic growth to polarized growth and in the formation of a germ tube. Germination is complete when polar growth is established and the germ tube is at least half the length of the spore.

1.1.2 Hyphal vegetative growth

There are two major types of vegetative growth amongst fungal groups: yeasts and filamentous hyphae. Filamentous growth is better adapted to solid substrates whereas yeast growth is optimal for liquids, although both forms can grow on both types of media under experimental situations. Yeasts form colonies of uninucleate cells on solid media, whereas filamentous fungi form a multinucleate branched mycelium. Mycelial growth requires the establishment of additional sites of polarization. Filamentous and yeastlike fungi have conserved growth mechanisms that differ in how cell and nuclear division are coordinated.

Typical of the ascomycete and basidiomycete fungi, the hyphae of *Aspergillus nidulans* are divided at regular intervals by cross-walls called septa. However, septation is not essential for hyphal growth, since Zygomycete hyphae lack septa

except associated with sporulation (Deacon, 1997) and aseptate mutants of *A. nidulans* can form extensive mycelia (Harris *et al*., 1994). Following the completion of mitosis, cytokinesis occurs in cells. Filamentous fungi undergo cytokinesis by forming crosswalls termed septa. Although light and electron microscopy studies have found that septum formation in higher fungi is similar to cytokinesis in animal cells than the phragmoplastic division process of higher plants (Gabriel, 1984; Harris *et al*., 1994), it differs in that the cytokinesis does not happen until the third nuclear division (Harris *et al*., 1994), and septa have pores in the centre through which the cytoplasm and even the nuclei can migrate towards the growing hyphal tip. Strictly speaking, therefore, septate hyphae do not consist of cells but of interconnected compartments. In wildtype *A. nidulans,* septation creates multinucleate hyphal compartments. These compartments are often called cells even though ascomycete septa are perforate, because they are functionally distinct. Both cell length and number of nuclei per cell are variable, but they are positively correlated (Kaminskyj and Hamer 1998). Factors involved in septation site selection include the position of mitotic spindles (Clutterbuck, 1970; Momany and Hamer, 1997a; Wolkow *et al*., 1996), and septin arrays (Westfall and Momany, 2002). Sites capable of forming septa appear to be established during tip growth (Kaminskyj 2000), prior to activation by a nearby mitosis.

The first septation event occurs when the germling has reached a critical size (Wolkow *et al*., 1996), and the septum is positioned at the spore-germ tube junction (Harris *et al*., 1994, Wolkow *et al*., 1996). After septation, the tip cell maintains its ongoing synchronous mitotic cycle (Rosenberger and Kessel, 1967), which is coupled

by septation (Clutterbuck, 1970; Wolkow *et al*., 1996; Momany and Hamer, 1997a), but nuclei in the basal cell are quiescent until branching (Kaminskyj and Hamer, 1998). For the normal vegetative growth, nuclei are evenly spaced along the hyphal axis.

1.1.2.1 Tip growth

Apical growth was proposed as the mechanism for hyphal extension by Ward (1888) and Reinhardt (1892) from their observations of growing hyphae. Hyphal morphogenesis is established by the growing tip, making it the key to fungal biology (Robertson, 1965; Gow, 1989; Heath 1990).

Tip growth utilizes a set of interactive processes that restrict growth to a narrow zone in the hyphal apex. Overall, these processes include deposition of wall skeletal polysaccharides and matrix components including β-glucans (Gooday, 1971), and apex-directed cytoplasmic migration and growth direction control (Kaminskyj and Heath 1996). In wildtype strains of *A. nidulans*, these processes lead to the formation of cylindrical hyphae with a diameter of about 3 μm.

Cell wall building materials and/or enzymes necessary for tip growth are transported in vesicles to the hyphal apex by the cytoskeleton. At the apex, the vesicles fuse with the cell membrane, a process called exocytosis. During exocytosis, the vesicle membranes integrate into cell membrane, for cell membrane expansion and to release matrix components. Exocytotic vesicles are produced by Golgi equivalents (also called dictyosomes) in basal and near-apical regions of the hyphal

cytoplasm. True fungi do not have stacked Golgi membranes, like animals and plants, but rather they have single cisterna that have an analogous function (Morré, 1990).

Hyphal diameter is precisely regulated, and coordinated with growth rate and growth direction, since hyphae are approximately cylindrical despite varying extension rates and direction changes (Kaminskyj *et al*., 1992; López-Franco *et al*., 1994; Riquelme *et al*., 1998). The plasma membrane is evenly pressed to the wall and lies immediately inside the hyphal wall. This suggests that either membrane insertion is coordinated with hyphal extension, or that there is a retrieval mechanism for excess membrane (Grove and Bracker, 1970; Heath *et al*., 1971; Howard and Aist, 1979; Heath *et al*., 1985; Roberson and Fuller, 1988). Recent research using a membrane-selective dye, FM4-64, suggests that some fungi can undergo endocytosis so that excess membrane could be recycled if necessary. However, endocytotic intermediates have not been seen with electron microscopy in *Aspergillus nidulans* (Fischer- Parton *et al*., 2000), nor in *Neurospora crassa* (Torralba and Heath, 2002). Equally, exocytotic intermediates are rarely visualized in fungi using electron microscopy (Kaminskyj and Heath 1996), so more research is needed into fungal plasma membrane dynamics.

During hyphal tip growth, the near-apical cytoplasm migrates forward relative to the lateral cell walls (McKerracker and Heath, 1987) which can be envisioned as a tube-dwelling amoeba without enclosure by a posterior plasma membrane (Reinhardt, 1892). Hyphal cytoplasm must therefore be capable of contractility. Like other amoeboid cells, hyphal cytoplasm contains F-actin (McKerracher and Heath, 1987;

Heath, 1990; Jackson and Heath, 1992), and can undergo active contractions that are Ca^{2+} mediated (McKerracher and Heath, 1986b; Jackson and Heath, 1992; Kaminskyj *et al*., 1992), and predominantly unidirectional toward the tips (McKerracher and Heath, 1986b; López-Franco and Bracker, 1996; Jackson and Heath, 1992). Differential attachment of the cytoplasm to the cell membrane and wall, which is tip enriched in oomycete (Kaminskyj and Heath, 1995) and *A. nidulans* (Kaminskyj, unpublished) hyphae, appears to contribute to directed migration.

Since the proposal of apical growth as the mechanism for hyphal extension (Ward, 1888; Reinhardt, 1892), the mode of tip growth of hyphae has been an object of study. In 1959, Robertson did key experiments on apical growth, from which he suggested that the normal process of apical growth involves two separate phenomena: extension of a plastic, deformable tip and rigidification of the wall behind the extending tip. Based on Robertson's results, Wessels (1981) proposed a "steady state model".

In Wessels' model, the newly formed wall at the extreme tip is viscoelastic. It flows outwards and backwards as new components are added at the tip, and the wall rigidifies progressively by the formation of extra bonds behind the tip. This is consistent with Robertson's original idea of a plastic, deformable tip wall that is rigidified behind the apex, but takes into account the new concept of molecular architecture.

The steady state hyphal tip growth model does not account for how an extending hyphal tip with a deformable wall can resist turgor, which has the potential to rupture the delicate tip. Turgor can be one to many atmospheres of pressure (Kaminskyj *et*

al., 1992), and it is difficult to envisage growth rates being precisely balanced to avoid bursting at the tip. Jackson and Heath (1990) proposed that the actin arrays in the apex of tip-growing cells provide internal structural support. When they disrupt actin caps with cytochalasin E, hyphal growth rate increased, after which the tip swelled and burst. When hyphae were stressed mechanically or osmotically, the weakest region of the tip was not the extreme apex, where the wall is thinnest and the actin cap is densest, but on the shoulders of the apex. Presumably this is a point of minimum composite strength where the actin arrays are less dense but the wall has not yet matured completely.

Inherent in apical growth is the forward transport of a range of vesicles containing enzymes and wall building materials synthesized by the endomembrane system, involving endoplasmic reticulum and Golgi equivalents. These vesicles are transported to the hyphal apex, where they form or interact with the Spitzenkörper (Grove and Bracker, 1970; Howard, 1981; Roberson and Fuller, 1988), after which they are competent to fuse with the plasma membrane.

The tip growth process has been modelled mathematically (Bartnicki-Garcia *et al*., 1989; Bartnicki-Garcia, 2003), they postulate that hyphal shape is determined by the location of the Spitzenkörper, but this model does not provide mechanistic insights.

1.1.2.2 Cytoskeletal filaments

Organelles can exhibit significant movement within fungal hyphae. This is not merely due to their being carried along by the bulk flow of cytoplasm keeping station

with the extending tip, since the movement of organelles in opposite directions within the same hypha has been observed frequently (e.g. Buller, 1933; McKerracher and Heath, 1987). The mechanisms producing organelle movement are not fully understood, but it is apparent that organelles are not independently motile. Diverse evidence (Steinberg, 1998) shows that organelle motility requires components of the cytoskeleton: microtubules and/or actin microfilaments and their associated motors (Xiang, *et al*., 1994; 1995a, 1995b; McGoldrick, *et al*., 1995), and uses energy supplied by ATP hydrolysis (Morris, 1976; Okley and Morris, 1980; Gow, 1995).

The recent discovery of the intermediate filaments in fungi (Momany and Hamer, 1997b), the septin homologues, increases the diversity of fungal cytoskeletal filaments. However, intermediate filament associated motor proteins have not been identified (Heath, 1995).

1.1.2.2.1 Actin filaments

Microfilaments are non-covalent polymers of globular 43-kD actin monomers. In eukaryotic cells, the actin cytoskeleton underlies numerous processes, including cell motility, cytoplasmic streaming, cytokinesis and organization of the cell surface and extracellular matrix (Bray, 2000).

Actin is enriched at the apical region in all tip-growing cell types examined (Heath, 1994; Geitmann and Emons, 2000), including *Aspergillus nidulans* (Harris *et al*. 1994). The apical actin arrays have been shown to reinforce the extensible wall at the tip of *Saprolegnia* hyphae (Jackson and Heath, 1990). F-actin is also likely to be involved in targeting exocytic vesicles to the apical secretion site and to regulate

fusion between vesicles and the plasma membrane (Howard, 1981). Disruption of the apical actin arrays using microbeams or actin-specific poisons disrupts tip shape and/or integrity (Heath, 1990; Jackson and Heath, 1993; Torralba *et al*., 1998). This suggests that the actin cytoskeleton is required for normal apical growth and hyphal tip shape generation and maintenance. Actin is also involved in septation, which is another form of wall deposition (Harris *et al*., 1994; Momany and Hamer, 1997a).

Cytoskeleton-based motility requires a cytoskeletal filament and its associated motor protein. The actin-associated motor protein is myosin. *A. nidulans* has one actin gene (Fidel *et al*, 1988) and one myosin (McGoldrick *et al*., 1995), both of which are essential. *A. nidulans* myosin is abundant at hyphal tips (McGoldrick *et al.,* 1995), where it colocalizes with actin. In the freeze-substitution prepared *Fusarium acuminatum* hyphae, actin is closely associated with vesicles and organelles (Howard, 1981; Grove and Bracker, 1970). In *A. nidulans*, the use of cytochalasin A causes disruption of microfilaments and inhibition of hyphal growth (Harris *et al*. 1994; Torralba *et al*., 1998). In *Neurospora crassa*, treating growing hyphal tips with cytochalasin causes morphological abnormalities at hyphal tips, leading to apical branching and curling (Riquelme *et al*., 1998).

1.1.2.2.2 Microtubules

Microtubules are non-covalent polymers of alpha and beta tubulins, which each has a molecular weight of around 50-55 kD. A third member of the tubulin superfamily, gamma tubulin (Oakley and Oakley, 1989), functions as a microtubule organizing center, where microtubules are nucleated to initiate polymerization.

Microtubule arrays form the mitotic and meiotic spindles, and are important for establishing cell polarity and vesicle trafficking. Cytoplasmic microtubule assembly is nucleated from microtubule organizing centers such as those associated with nucleus-associated organelles. Fungal cytoplasmic microtubules are widely distributed in cells (Hoch and Staples, 1985; Howard, 1981), and are orientated parallel to the longitudinal axis of the hypha.

Microtubule associated motors are kinesins and dyneins (reviewed in Vale *et al*., 1985). Since microtubules are formed from end to end association of alpha / beta dimers, their structure is polarized. *In vivo*, the beta subunit associates with gamma tubulin at the nucleation site, and the alpha subunit is exposed at the "free" end. *In vitro*, isolated microtubules can be induced to polymerize under appropriate conditions (Derksen and Emons, 1990). Polymerization proceeds at different rates at the two ends with polymerization occuring substantially faster at the end with the free alpha subunit, which is called the plus end. Most kinesins are plus-end directed motors, whereas dyneins are minus-end directed (Xiang *et al*., 1995b).

In *A. nidulans*, the *nudA* gene (Morris, 1976) encodes cytoplasmic dynein heavy chain, and *nudG* encodes cytoplasmic dynein light chain. However, nuclear distribution in *A. nidulans* is complex, involving at least two other gene products (*nudC* and *nudF*, which mutually interact; Willins *et al*., 1997).

In fungal tip growth, the function of the microtubules is less clear than that of the actin filaments. In some instances, tip growth arrest was observed after application of anti-microtubule drugs (Howard and Aist, 1980; Mizukami and Wada, 1983). In *A. nidulans*, however, experiments using microtubule depolymerising agents show that

apical elongation is not at all, or only to a small degree affected (Torralba *et al*., 1998). Also in other fungi, for example *Cladosporium cucumerinum* and *Trichoderma* (Peterbauer *et al*., 1992; Pedregosa *et al*., 1995), apical growth of the hyphae continued in spite of the depolymerization of microtubules, although at a reduced rate.

In most tip-growing cells, nuclear migration and positioning are microtubule-dependent (Aist, 1995). In *A. nidulans*, nuclear movement was inhibited by mutations in the beta tubulin gene and by the microtubule inhibitor benomyl (Oakley and Morris, 1980; Oakley and Rinehart, 1985). However, Oakley and Rinehart (1985) also found that mitochondrial movement was unaffected by either benomyl or beta tubulin mutations, suggesting that mitochondrial transport in *A. nidulans* may be actin-based. Different taxa employ characteristic mechanisms for transportation of certain organelles. In the Zygomycete, *Basidiobolus ranarum,* the use of UV irradiation to depolymerise cytoplasmic microtubules did not affect nuclear migration even after most of the microtubules surrounding the nucleus had been depolymerised (McKerracher and Heath, 1986a).

In *Aspergillus nidulans*, strains with beta tubulin mutations could still undergo relatively normal hyphal extension, which suggests that microtubules are not essential for tip growth in this system (Oakley and Morris, 1981). So the ways in which microtubules are involved in tip growth remains a subject of controversy (Srinivasan *et al*., 1996).

1.1.2.2.3 Intermediate filaments

Animal cells contain a diverse array of filaments intermediate in size between microtubules and F-actin, with a width of about 10 nm. Intermediate filaments appear to be primarily structural components (Bray, 1992) involved in positioning of organelles such as nuclei, vesicles, or in specialized cellular junctions.

Until relatively recently (Momany and Hamer, 1997b; Momany *et al*., 2001; Westfall and Momany, 2002), intermediate filaments were not widely reported in fungal cytoskeletons, apart from neck ring filaments in *Saccharomyces cerevisiae* (Byers and Goetsch, 1976) and structures in *Neurospora crassa* (Rosa *et al*., 1990; Alvarez *et al*., 1991; McConnell and Yaffe, 1993). In animal cells intermediate filaments are characteristically highly resistant to fixation damage, thus their rarity in the fungal ultrastructural literature is probably accurate.

Since the discovery of intermediate-sized filaments, now called septins, in *S. cerevisiae* by Byers and Goetsch (1976), septin homologues have been found in many fungi (reviewed in Momany and Taylor, 2000). Septin homologues have been found in *Aspergillus nidulans* (Momany and Hamer, 1997b; Momany *et al*., 2001) and have been shown to be essential for fungal morphogenesis (Westfall and Momany, 2002). There is a good possibility that intermediate filaments contribute to fungal cytoskeletal polarization, likely through interaction with F-actin (Gow, 1989).

1.1.2.3 Endomembrane system

Fungal tip growth requires localized fusion of exocytic vesicles with the apical cell membrane. These exocytic vesicles are produced by the endomembrane system whose function is to maintain certain organelle-associated synthetic functions and proper distribution of newly synthesized molecules (Nickel and Brügger, 1999).

The "endomembrane system" describes the structural and developmental continuity of internal membranes that characterizes the cytoplasm of eukaryotic cells (Morré and Mollenhauer, 1974). The endomembrane system consists of the nuclear envelope (NE), rough and smooth endoplasmic reticulum (rER, sER), which are distinguished by whether they have associated ribosomes, the Golgi apparatus, and transfer vesicles. Animal and plant cells have Golgi bodies consisting of stacked cisterna. In contrast, fungal Golgi equivalents (also called dictyosomes) are individual cisterna that fulfill the same functions (reviewed in Gupta and Heath, 2002).

During tip growth, secretory vesicles are directed to the hyphal tip. Before they fuse with cell memebrane, they interact with a vesicle / cytoskeleton assemblage called the Spitzenkörper whose behaviour determines the growth and the shape of the hypha. The Spitzenkorper has been known since it was first observed by Girbardt (1955) in living hyphae in 1957 (reviewed in López-Franco and Bracker, 1996), but its functions are still enigmatic.

The walls of fungal hyphae are derived in large measure from secretory vesicles. The contents of these vesicles provide matrix and secreted enzymes, while the vesicle membranes provide the new plasma membrane and integral enzyme complexes that synthesize new cell wall fibrils (Girbardt, 1969; Grove and Bracker, 1970; Grove *et*

al., 1970; Bartnicki-Garcia, 1973). These vesicles are produced by the ER-NE complex, delivered to Golgi equivalents, and then transported to the tip where ultimately they fuse with the plasma membrane.

In budding yeast, *Saccharomyces cerevisiae*, membrane transport is polarized toward the bud where growth occurs. Golgi (equivalent) structures cluster at sites of pronounced polarized growth, including mating projections, near the site of bud emergence, and in growing buds (Preuss *et al*., 1992). Putative exocytic vesicles are also concentrated at growth sites. These observations suggest that spatial control of vesicle targeting might operate at two levels: formation of vesicles near the growth site and localization of a vesicle targeting apparatus at the site of vesicle fusion.

Similarly in filamentous fungi, endomembrane elements including Golgi-like components have been observed associated with apical vesicles and oriented parallel with the long axis of the cell toward the growing apex (Howard, 1981). To provide energy for these processes, mitochondria have been found clustered immediately behind the Spitzenkörper region and also lying parallel to the long axis of the cell.

The associations between vesicles and endomembrane components suggest that the vesicles are derived endogenously from the endomembrane system, whose polarized arrangement is important in the hyphal polar growth.

1.1.2.4 Spitzenkörper

The Spitzenkörper (German for "tip body") was first noticed with light microscopy in the growing hyphal tip of higher fungi as a phase-dark structure (Girbardt, 1969; reviewed in López-Franco and Bracker, 1996), which electron

microscopy (Grove and Bracker, 1970) showed was composed of an apical cluster of vesicles (AVC; Bartnicki-Garcia *et al*., 1968; Brenner and Carroll, 1968) lying within a meshwork of microfilaments (Grove and Bracker, 1970). This apical aggregation of vesicles appears to predict future growth direction of the hyphal tip (Girbardt, 1969; López-Franco and Bracker, 1996; Bartnicki-Garcia, 2003). Thus the Spitzenkörper appears to be a marshalling point where tip directed exocytic vesicles accumulate, and it is the source for the vesicles that will go on to fuse with the cell surface. So the Spitzenkörper is considered to function as vesicle supply centre (VSC) during hyphal tip growth (Bartnicki-Garcia *et al*., 1989).

The vesicles of Spitzenkörper are derived from the endomembrane system (Grove *et al*., 1968; 1970) in the subapical and near apical regions of growing hyphal tips. This interpretation is supported by evidence that components of the endomembrane system such as Golgi equivalents are involved in transforming membranes from endoplasmic reticulum-like to a plasma membrane-like appearance, concomitant with the production of secretory vesicles (Grove *et al*., 1968; 1970). Fusion profiles showing continuity between vesicle membrane and plasma membrane indicate that the vesicle membrane is contributed to the plasma membrane at the apex (Grove *et al*., 1970; McClure *et al*., 1968), and that the vesicle contents are released into the wall region in a process comparable to exocytosis or vesicle-mediated secretion (Grove *et al*., 1970; Mollenhauer and Morré, 1966). So in part, hyphal tip growth may be viewed as the result of a highly polarized system of exocytosis.

1.1.2.5 Other regulatory components in hyphal growth

Besides cytoskeletal filaments, other factors have important roles in hyphal morphogenesis. Calcium ions are believed to be involved in regulating the polymerization and regulate the cytoskeleton and vesicle fusion rates (Grinberg and Heath, 1997; Hyde and Heath, 1997). The pH gradient has also been implicated in hyphal growth of filamentous fungi (Robson, *et al*., 1996; Bachewich and Heath, 1997). Molecular studies have demonstrated that the maintenance of hyphal polarity in *N. crassa* requires the cAMP-dependent protein kinase (Bruno *et al*., 1996). A Rho protein effecter encoded by *sepA* was found to regulate septation and to be required for the maintenance of cell polarity in *A. nidulans* (Harris *et al*., 1997). Also in *A. nidulans*, the different level of Ras activity was required for the process to proceed through different stages of development (Som and Kilaparthi, 1994). So the normal hyphal growth of filamentous fungi needs a coorperative regulation of many factors.

1.1.2.6 Hyphal branching

Polarization refers to the establishment of a limited exocytic site for germ tube and ongoing hyphal tip growth, and also to the establishment of new branches typically from basal cells. Branch sites are formed in subapical regions of *A. nidulans* hyphae, away from the tip in undisturbed cells. These branches then extend in a polarized manner like tip growth in the original hypha. Although the mechanisms responsible for the cytoplasmic decision to form a branch are unknown, a branch, once established, employs the same growth mechanisms as the original apex. Thus,

the basal cell must reorient its growth axis to establish the branch (Trinci, 1978). It is most likely that the mechanisms required for forming a new branch site are related to those required for polarization during germination.

Computer simulation and observations by Reynaga-Peña *et al*. (1997) are consistent with that the dynamics of the Spitzenkörper may be responsible for hyphal branching. This supports the idea that once a branch formed, it uses the same growth mechanisms as the original apex, and that branch Spitzenkörper are formed *de novo* rather than by division of the original Spitzenkörper. A morphologically similar Spitzenkörper has not observed in Oomycetes, but an apical accumulation of vesicles equivalent to Spitzenkörper has been described (Jackson and Heath, 1993). However, the "polarizing principle" that organizes the original or branch Spitzenkörper is unexplained.

Two models for branch initiation have been proposed. In one model branch initiation depends on local accumulation of factors originating near the new branch, from somewhere within the colony. Trinci (1974) and Prosser & Trinci (1979) proposed that tip growth vesicles are the key elements whose accumulation triggers branching. Similarly, Reynaga-Peña *et al*. (1997) suggested that a new branch Spizenkörper was formed by aggregation of exocytic vesicles rather than division and subapical transport of part of the original Spitzenkörper. In the other model, the focus is on events that are controlled independently at the tip. Calcium has been thought as a factor important in tip growth and branching (Heath, 1990; Grinberg and Heath,

1997; Hyde and Heath, 1997; Torralba *et al*., 2001). Watters *et al*. (2000) suggest that branch determination may require a combination of these two basic possibilities, with one or the other predominating under different circumstances.

1.1.2.7 Septation

Filamentous fungi undergo cytokinesis by forming perforate crosswalls termed septa. Light and electron microscopy studies have found that septum formation in higher fungi is more similar to cytokenesis in animal cells than the phragmoplastic division process of higher plants (Harris *et al*., 1994). Although the cells of *A. nidulans* are multinucleate, cytokinesis in this organism shares many features with other organisms, except that hyphal cells do not separate following division, which Fiddy and Trinci defined as "duplication cycle" (Fiddy and Trinci, 1976; Trinci, 1978; reviewed in Harris, 1997).

When spores of *A. nidulans* germinate they give rise to a germ tube. Wolkow *et al*. (1996) found that a single nuclear division is sufficient to activate septum formation, provided a critical size has been attained. Under typical growth conditions, in wild type cells, attainment of this minimum cell size requires three or more rounds of nuclear dividion (Harris *et al*., 1994). The first septum is placed at the basal end of the germ tube, separating the cell asymmetrically into a basal cell with a relatively constant length, and tip cell whose length varies from 20 μm to >100 μm (Wolkow *et al*., 1996). After formation of the first septum, subsequent rounds of nuclear division are followed by rounds of septum formation (Clutterbuck, 1970), creating multinucleate basal cellular compartments.

Septum formation requires the assembly of a septal band which is composed of actin, septins and formins functioning in a mutually dependent manner redundant (Harris, 2001). Septa in vegetative hyphae, except those defining foot cells for sporulation, have a central pore, through which cytoplasm and maybe even nuclei can pass. One suggestion for the role of septa in mycelia is as a structural support to maintain the rigidity of the hypha (Gull, 1978) when turgor pressure is low, although they are not required by zygomycete fungi, which also live in terrestrial habitats. More important, septa can protect the hyphae from excessive loss of cytoplasm in case of trauma (Yuan *et al*., 2003).

1.2 *Aspergillus nidulans* hyphal morphogenetic genes

During germination, *A. nidulans* spores switch from isotropic growth during spore swelling to polarized growth producing narrow tubular hyphae. Two processes are involved in this switch: polarity establishment, i.e., choosing the spot where new material will be deposited, and polarity maintenance, i.e., the continued deposition of wall material at the growing tip.

In addition to cytoplasmic factors such as cytoskeletal filaments, endomembranes and ion gradients, there are several classes of morphogenetic mutants that might reveal new aspects in hyphal morphogenesis (Kaminskyj and Hamer, 1998; Harris *et al*., 1999; Momany *et al*., 1999). The analysis of the regulation of cell polarity has been facilitated by genetic insights first demonstrated by the analysis of polarity mutants in budding yeast (reviewed in Drubin, 1991) and fission yeast (reviewed in Verde *et al*., 1995), which allow the identification of genes whose products are

important for the establishment and maintenance of polarized growth. Mutants of genes required for either of these steps will cause defects in hyphal morphogenesis. Several groups have instituted genetic screens to identify hyphal morphogenesis genes in *A. nidulans* (Kaminskyj and Hamer, 1998; Harris *et al*., 1999; Momany *et al*., 1999). The investigation of these genes can help further understand the mechanism of fungal hyphal morphogenesis or tip growth.

hyp morphogenetic mutants were isolated by Kaminskyj and Hamer (1998). Five *A. nidulans* morphogenesis loci, *hypA*- *hypE* (hypercellular), were identified by screening a collection of temperature sensitive mutants for strains that were wild type at 28°C but had nonlethal morphogenetic defects at 42°C. These *hyp* mutants are distinct from cell cycle mutants (Morris, 1976), septation mutants (Harris *et al*., 1994), and conidiation mutants (Wieser *et al*., 1994) by having normal mitosis, formation of septa and sporulation. Except *hypC*, the *hyp* strains have delayed germination at restrictive temperature which perhaps is due to defects in cell polarization. Similarly the reduced hyphal extension rates at 42°C may indicate defects in the maintenance of cell polarity. All the experimental results suggest that *hyp* loci may be important in *A. nidulans* hyphal morphogenesis, but their functions are not fully characterized.

Harris *et al*. (1999) and his colleagues are working on another group of *A. nidulans* hyphal morphogenesis genes, *pod* genes. Except *podA1* which was from the strain 8-160 in the temperature sensitive collection, and later found allelic to *hypA1* and renamed as *hypA6* (Kaminskyj, unpublished data), the restrictive phenotype of all the *pod* mutants had enlarged spores in a depolarized manner and accumulation of

nuclei, indicating *pod* genes prevent the spore germination at restrictive temperature. Their experiments showed that *podC* and *podD* genes are required only for the establishment of hyphal polarity, while *podB* is not only involved in both establishment and maintenance of polarity, but also alters the normal pattern of cell-wall deposition by affecting cytoskeletal organization.

Momany and her colleagues are studying genes involved in the synthesis and localization of cell wall material in *A. nidulans*. Since hyphal tip growth requires polarized cell wall insertion and wild type wall strength, they screened a collection of temperature sensitive *A. nidulans* mutants for a swollen cell phenotype seeking genes for the cell wall synthesis and localization of cell wall materials. Eight *swo* genes are involved in polarity establishment, maintenance and hyphal morphogenesis. Among this collection, *swoE* was obtained from the same strain as *podA1*, the strain 8-106. So *swoE* is *podA1* (*hypA6*). Recent discoveries showed that *swoA* encodes protein *O*-mannosyltransferase *pmtA* (Shaw and Momany, 2002) which involves in ER secretory system, and *swoF* encodes an *N*-myristoyl transferase (Shaw *et al*., 2002) which myristoylates a substrate needed for proper polarity establishment and maintenance. However, *swoH* likely encodes a nucleoside diphosphate kinase which is important in nucleic acid metabolism, rather than hyphal morphogenesis.

1.2.1 Morphogenetic locus *hypA*

The *hypA* locus was mapped to chromosome 1 with a close linkage to *pabaA6* (recombination rate 4%) and another morphogenetic locus, *sepA1* (recombination rate

2%), which encodes a formin required for actin organization (Harris *et al.,* 1997). *hypA* was cloned and shown to encode a predicted protein of 161.9 kDa (Kaminskyj and Hamer, 1998).

At restrictive temperature 42°C, *hypA* temperature sensitive strains have short, wide tip cells and basal cells, indicating that *hypA* is involved in both establishment and maintenance of hyphal polarized growth, and has a dual role in two kinds of cells: maintaining the tip cell polar growth and limiting growth in basal cells (Kaminskyj and Hamer, 1998). Although the temperature sensitive cells displayed a distinct phenotype at 42°C, they remained alive and could complete their asexual life cycle and sporulate, suggesting *hypA* is not essential in *A. nidulans* even though it is very important in hyphal morphogenesis.

BLAST analysis showed the similarities of HYPA to its orthologues are 21% identical and 38% positive in *Candida albicans*, 21% identical and 36% positive in *Ashbya gossypii*, 36% identical and 53% positive in *Neurospora crassa* and 24% identical and 40% positive in *Schizosaccharomyces pombe* in the region of 1-1474 in *A. nidulans* HYPA. None of these orthologues have been characterized. More importantly, *hypA* has a related sequence in *Saccharomyces cerevisiae*, *TRS120* (20% identical; 37% positive), which was shown to be an essential regulatory component of the TRAPP II complex that mediates Golgi transit (Sacher, 1998). And *S. cerevisiae TRS120* temperature sensitive mutant arrested with abnormal Golgi bodies at restrictive temperature. The mutant phenotypes of *N. crassa*, *S. pombe* and *S.*

cerevisiae at restrictive temperature can be rescued by *A. nidulans* wild type *hypA*. Orthologues in both *S. cerevisiae* and *S. pombe* (Dai, 2002) are essential, but surprisingly, *A. nidulans hypA* is not (Shi *et al*., submitted).

1.2.2 *hypA* temperature sensitive alleles

hypA has two temperature sensitive mutant alleles, *hypA1* and *hypA6*, which were selected from independently created 4-NQO mutagenized collections in different studies (Kaminskyj and Hamer, 1998; Harris *et al*., 1994). Strains with these alleles display similar phenotypes at restrictive temperature 42°C: short, wide germlings with abnormal pattern of branches. *hypA1* is a single base pair replacement, causing a non-conservative amino acid change in the deduced protein (Shi *et al*., submitted). Published reports (Harris *et al*., 1994) suggested that, compared with *hypA1* mutant, *hypA6* allele seemed to have more severe defects in establishing polarity and surviving at 42°C. We suspected as a result that its lesion was in a more critical part of the gene. The sequencing of these two mutant alleles and comparison of their phenotypes can help find out the relationships between the specific lesions in *hypA* gene and the resultant phenotype, and further explain the functions of *hypA*.

1.3 Objectives

Although both *hypA1* and *hypA6* temperature sensitive phenotypes suggested that *hypA* is involved in *A. nidulans* hyphal morphogenesis, it is still not clear how it functions. The study of the relationship between gene sequence and the hyphal

growth pattern by comparison of the *hypA1* and *hypA6* temperature sensitive allele sequences and their phenotypes at restrictive temperature can help characterize *hypA* function.

A. nidulans hypA is highly related to *S. cerevisiae TRS120,* which can complement *TRS120* mutant phenotype at restrictive temperature. *TRS120* is required for Golgi transit in *S. cerevisiae*, suggesting *hypA* may function through endomembrane system. The recent studies of endocytosis and vesicle trafficking in living fungal hyphae using FM4-64, a membrane-selective dye (Fischer-Parton *et al*., 2000) makes it possible to observe the endomembrane arrangement in living *A. nidulans* cells and document the endomembrane changes in *hypA* temperature sensitive strains.

The function of microtubules in hyphal growth varies with different physiological conditions and cell types. Although we have evidence showing in *A. nidulans* microtubules function as a transport track for nuclear movement during cell growth (Xiang, 1994), Bartnicki-Garcia and colleagues (Bartnicki-Garcia *et al*., 1989) also proposed that a growing scaffolding of microtubules propels the Spitzenkörper forward, although experimental evidence of a role for cytoplasmic microtubules in *A. nidulans* is rare.

In this study, the phenotypic comparison of *hypA1* and *hypA6* alleles at restrictive temperature and shifting temperatures was performed in order to identify their abilities to establish and maintain polarity in hyphal growth. The membrane-selective dye FM4-64 was used to stain the endomembranes in both *hypA1* and *hypA6* mutant cells at restrictive temperature and study the endomembrane arrangement. Since the

disruption of cytoplasmic microtubule assembly by fungicide benomyl causes directional changes in hyphal growth, the microtubule arrangement in both mutant cells was investigated at 42°C using strains containing GFP-tagged alpha tubuline. Finally, at genetic level, both *hyphA1* and *hypA6* were sequenced for finding out their lesion sites.

2. MATERIALS AND METHODS

Unless specified, all chemicals were reagent grade and obtained from VWR (www.vwrsp.com) or Sigma (www.sigmaaldrich.com). Custom primers were synthesized from Gibco BRL (www.lifetech.com) or Cortec (www.cortec.com). Water was deionized to ≥ 10 MegOhm using a Barnstead filter (Sybron corporation) or double distilled, and sterilized as appropriate. *Aspergillus nidulans* strains were grown as described in Kaminskyj (2001). The permissive temperature in this study was 28°C, and the restrictive temperature was 42°C.

2.1 *Aspergillus nidulans* strains and growth conditions

Aspergillus nidulans strains used in this study are listed in Table 2.1. An *A. nidulans* strain harboring the *alcA* promoter followed by GFP (green fluorescent protein) fused in frame with the full-length *tubA* coding sequence (Han *et al*., 2001) was used to construct *hypA1* and *hypA6* strains with GFP-tagged microtubules. Strains were grown on complete medium (CM) as described in Kaminskyj (2001) and supplemented for nutritional auxotrophies as needed. The CM contains 1% D-glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, 0.01% vitamin solution, and is buffered to pH 6.5. For inducing cleistothecia after mating, heterokaryons were grown on minimal medium (MM) as described in Kaminskyj (2001). Media were solidified with 1.5% agar. MM "dropout" plates that have all the supplements but one of the possible

Table 2.1 *Aspergillus nidulans* strains used in this study

strains	Genotype	Source
GR5	*pyrG89*; *wA3*; *pyroA4*	Fungal Genetics Stock Center (www.fgsc.net)
ASK30	*hypA1*; *wA3*; *pyroA4*	Kaminskyj and Hamer (1998)
ASK39	*hypA1*, *biA1*, *pabaA6*	Kaminskyj and Hamer (1998)
ASH80	*hypA6*, *pabaA6*; *pyroA4*	Harris *et al*. (1999)
GFP-*tubA*	*GFP-tubA-pyr4; pyrG89; pyroA4; wA3*	Han *et al*. (2001)
ASY1	*pyrG89*; *hypA6*, *pabaA6; wA3*; *pyroA4*	generated in this study
ASY2	*hypA6*, *pabaA6*; *GFP-tubA-pyr4*; *pyroA4*	generated in this study
ASY3	*hypA1*; *GFP-tubA-pyr4*; *pyroA4*	generated in this study

auxotrophies were used to determine the nutritional phenotype of progeny strains. CM containing 1% glycerol as sole carbon source was used to induce *alcA* expression of GFP- *tubA* (Han *et al*., 2001).

2.2 *Aspergillus nidulans* cultures

Aspergillus nidulans strains were maintained on CM at 28°C. Following sporulation, conidia were harvested in distilled water and stored at 4°C. Long-term storage was in 15% glycerol at -80°C. Spores were used within 1 month of storage at 4°C.

Aspergillus nidulans coverslip cultures were grown in liquid CM containing 100 μg / mL ampicillin. For cultures grown at 28°C, 5 mL of a 5 x 10^3 spores / mL suspension was dispensed into a 60 x 15 mm plastic Petri dish containing a 22 mm square glass coverslip. For 42°C cultures, the inoculum was 5 x 10^4 spores / mL. In "downshift" experiments, germlings were grown at 42°C for 12 to 14 h and then shifted to 28°C. In "upshift" experiment germlings were grown at 28°C for 12 to 14 h, and then shifted to 42°C.

2.3 Mating *Aspergillus nidulans* strains

Aspergillus nidulans strains were constructed for use in transformation, and to examine GFP tagged microtubule arrays in a *hypA1* or *hypA6* background. *Aspergillus nidulans* has no mating type, and self matings are common. To facilitate crossing between strains, parents were chosen to have different nutritional auxotrophies. The first stage in mating involves formation of a heterokaryon, requiring hyphal fusion.

Heterokaryon formation between parent strains is induced by growing them in mixed culture in rich medium, and selected by transfer to minimal medium where only fusions between parental strains survive.

Heterokaryons were constructed by inoculating 10^4 conidia of each mating strain at 28°C into 2 mL of CM in a 24-well tissue culture plate (Sarstedt, Inc., www.sarstedt.com). After 2 d, a mycelial mat had grown on the surface, which was transferred to a MM plate until conidiation. The plate was sealed with Parafilm and incubated in the dark at room temperature for 3 weeks to allow formation of sexual fruiting bodies, called cleistothecia. Detecting cleistothecia produced by outcrossing, rather than self-crossing, was facilitated by choosing parental strains with different spore colors. Progeny from an outcross should have both parental spore colors, plus recombinants if possible.

Cleistothecia were isolated and cleaned as described by Kaminskyj and Hamer (1998). Ascospores were isolated by crushing cleistothecia in 200 µL distilled water and plating dilutions onto appropriately supplemented media. After confirming outcrosses by the progeny spore color segregation, ascospores were spread on CM agar which can support growth of all auxotrophies to isolate colonies produced by single ascospores for phenotype analysis. Colonies were inoculated on replicate plates and grown at 28°C and 42°C to identify those with the *hypA1* / *hypA6* temperature sensitive phenotype. Nutritional auxotrophies were determined using replicate MM plates containing all but one supplement.

Progeny with the *hypA1* / *hypA6* temperature sensitive phenotype were confirmed using coverslip cultures. Inheritance of GFP-*tubA* was determined using epifluorencence microscopy with a FITC filter combination.

2.4 Microscopy

2.4.1 Epifluorescence microscopy

The preparation of *Aspergillus nidulans* samples for epifluorescence microscopy followed Kaminskyj and Hamer (1998). Coverslips with adherent germlings were fixed at room temperature for 5 min with 3.7% formaldehyde in 50 mM potassium phosphate buffer, pH 7.0, containing 0.2% Triton X-100, and then rinsed twice in distilled water. Cells were stained for 1 min in 1 µg / mL each Hoechst 33258 for nuclei and Calcofluor (Polysciences, Warrington, PA, USA) for cell walls, followed by rinsing twice in distilled water. Coverslips were mounted in antifade solution (50 mM sodium phosphate buffer pH 8.8, 50% glycerol, 0.1% n-propyl gallate), air dried, and sealed with nail polish. Cells were observed using an Axioplan Universal BH-2 epifluorescence microscope (Olympus, Zeiss) and DPLANAPO 100x objectives (oil immersion, N. A. 1.3) with a UV filter combination. Images were captured with a Sensys digital camera (Roper Scientific, www.inovis.com) operated by MetaVue software (Universal Imaging, www.imagel.com).

For morphometic comparision, at least 200 cells were counted for each replicate. Number of tips per germling are shown as the mean value ± standard error of the mean. Statistical analyses used Statview 1.3.1 (www.statview.com).

2.4.2 Confocal microscopy

For confocal microscopy, cells were imaged using a Zeiss META 510 laser scanning confocal microscope (www.zeiss.com) equipped with 25x N. A. 0.95 and 60x N.A. 1.4 water immersion Plan APO objectives. For all studies, laser power was 1.5 – 3.0% of full intensity. Cells continued to grow after imaging, demonstrating that they remained viable.

2.4.2.1 FM4-64 fluorescence examination of endomembrane

Endomembranes were stained with the membrane-selective dye FM4-64, and imaged using confocal microscopy. Wild type and mutant strains were grown on agar media. A 1 cm x 1 cm piece agar bearing the leading hyphae was excised with a scalpel and placed cell-side up on a slide. Cells were stained with 20 µL of 25 µM FM4-64 in liquid CM (Fischer-Parton *et al*. 2000) and covered with a glass coverslip. Cells were prepared immediately before use.

FM 4-64 was imaged using 514 nm excitation. The absorption maximum is 506 nm (www.probes.com). FM4-64 fluorescence was detected using an LP650 barrier filter, that is, a filter that transmits wavelengths longer than 650 nm.

2.4.2.2 GFP-tagged microtubule examination

Aspergillus nidulans strains with GFP-tagged microtubules can be imaged using the same fluorescence and emission parameters as for FITC: an excitation wavelength of 488 nm and a band pass barrier filter of 505-530 nm. Spores of wild type *A. nidulans*

GFP-*tubA*, *hypA1* GFP-*tubA* and *hypA6* GFP-*tubA* strains were grown on CM glycerol agar (Han *et al*. 2001). A 1 cm x 1 cm piece of agar bearing the leading hyphae was excised and mounted in liquid growth medium.

2.4.3 Scanning electron microscopy (SEM)

Aspergillus nidulans strains were grown on agar media, and then fixed in osmium tetroxide vapor by inverting plates over a 20 µL drop of 4% aqueous osmium tetroxide for 20 min to overnight at room temperature.

The agar bearing the fixed cells was cut into 5 mm squares and frozen at -80°C for several hours. Samples were freeze-substituted (Howard and Aist, 1979) in anhydrous acetone at -80°C for at least 12 hr, at -20°C for at least 12 hr and several hours at 4°C. Samples were critical point dried in a E3000 Series II Critical Point Drier (Polaron Controls Ltd., sum.uab.es/), mounted on aluminum stubs, gold sputter coated in a Edwards Sputter Coater S150B (www.emitech.co.uk), and examined with a Philips SEM 505 (Philips Electron Optics, Holland, www.cis.tugraz.at/felmi/sem505.htm). Images were captured on Polaroid 665 Black and White instant Pack Film (www.polaroid.com).

2.5 Effects of benomyl

The effects of benomyl on wild type and *hypA1*and *hypA6* mutant strains were tested in three aspects: the effects on spores during germination at 42°C, on upshifted hyphae, and on hyphae at 28°C.

For the effects on germination of *hypA* temperature sensitive mutants at 42°C, spores were inoculated on solid medium with a 0.5 μL drop of 10 mg / mL benomyl put on the agar 2 mm away from the edge of the spore inoculation and incubated at 42°C for 14 hr. A 1 cm square agar bearing the peripheral hyphae growing toward benomyl was excised and placed cell-side up on a slide. Cells were examined using phase contrast confocal microscopy.

To observe the effects of the inhibitor on hyphae at 28°C, conidia were grown on solid medium for about 14 hr at 28°C to allow the formation of wild type hyphae. The inhibitor was then applied about 2 mm away from the colony periphery, and hyphae were incubated for another 3 hr at 28°C before being processed for microscopy.

For observing the effects of the inhibitor on *hypA* temperature sensitive mutants hyphae at 42°C, the mutant spores were first grown at 28°C 14 h for the formation of wild type hyphae, then upshifted to 42°C and incubated 3 h with inhibitor put 2 mm away from the leading hyphal edge as described above, followed by microscopy.

2.6 Genomic DNA preparation

Aspergillus nidulans genomic DNA was isolated as described by Yelton *et al*. (1984). About 10^9 ASH80 (*hypA6*) spores were grown in 50 mL liquid CM at 28°C in a Gel Slick-treated flask shaken at 250 rpm for overnight. The mycelium was harvested by filtering through sterile cheesecloth, frozen at -80°C for half an hour and lyophilized in a Labconco Freezone 6. The dried mycelium was ground into powder in an Eppendorf tube with a sterile toothpick, then extracted in 500 μL of a cocktail containing 50 mM EDTA

(pH 7.5), 0.2% SDS, 2 µL / mL DEPC, and incubated at 68°C for 10 min. Extracted mycelia were centrifuged at 15000 x *g* for 5 min, and the supernatant was transferred to a fresh Eppendorf tube.

Proteins were removed by adding 30 µL ice cold potassium acetate solution (3M potassium, 5M glacial acetic acid, pH 4.8), incubating on ice for 5 min, and centrifuging at 15000 x *g* for 5 min.

The supernatant was extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) by vortexing and centrifuged at 15,000 x *g*. Genomic DNA was precipitated with 600 µL isopropanol. The crude DNA was air dried at room temperature, resuspended in 200 µL distilled water and incubated at 68°C for 10 min. RNAs were removed by adding 10 µL ice cold 10 M LiCl, and the DNA was precipitated in 95% ethanol at -20°C, centrifuged at 15000 x *g* for 5 min, and air dried. This DNA pellet was resuspended in 100 µL distilled water and kept at -20°C.

2.7 PCR

2.7.1 Primer design

The *Aspergillus nidulans hypA* sequence is deposited in GenBank as accession number AF001273. The primers used for amplifying *hypA* alleles (Table 2.2) were designed to increase specificity as described by Robertson and Walsh-Weller (1998). Twenty-two primers were used in amplifying and sequencing the *hypA6* allele, some of which were designed for earlier studies (Shi, unpublished). The potential efficiency of the PCR amplification was assessed using DNA Amplify program (Gene Codes Corporation, www.genecodes.com).

Table 2.2 Primers used in PCR amplifications

Primer name	Sequence (5' – 3')
-949 - -931	ccgagctcggagcgacaccatagcgta
536-516	gtaccagtgcccttggattc
326-347	ccccctatatcgcacctagaac
1027-1012	caagccgctcactgac
404-423	acggagctcaccggcgatac
1358-1340	ccggatccttgctgccgatgccttaga
819-838	cgcttgcaagcacaaacggc
1311-1293	ggaggaacctatggacagc
1293-1311	ccggatcctgtccataggttcctcc
2204-2186	ccggtaccaatctgggagcgcttcaca
2207-2226*	ggtggtgtcctgcggtttac
2669-2648*	ggggtacaccctcgctctcaag
2527-2541*	cccgttcacgaaatc
3053-3038*	cctcgagaatcataac
2904-2922*	cctggagctcgacattctc
3455-3438*	gggcaatctccagacttg
3285-3301*	gcgcaacgttcactatc
3455-3438*	gtctcacggagagtgtatc
3418-3434	ccggatccttaccgcaacggtaa
4372-4356	ccggtacctcggcagaggatcg

3967-3989*	gggcaatcgtgaagggacgattg
4972-4950*	ccagcaaaagccatctcagaatg

The nucleotides underlined are extra added to *hypA* sequence and used to facilitate previous molecular cloning. The numbering begins with the translation start codon ATG. And reverse primers have larger to smaller numbering. Primers with stars were generated in this study.

2.7.2 PCR amplification

In polymerase chain reactions, the 50 µL reaction mixture contained approximately 100 ng DNA template, 1x TNK50 buffer (10 mM Tris-HCl pH8.0, 5 mM NH_4Cl, 1.5 mM $MgCl_2$, 50 mM KCl; Blanchard *et al*. 1993), 0.2 mM dNTPs, 0.2 µM each primer and 2.5 U *Taq* DNA polymerase (Promega, www.promega.com).

The PCR program used in the Techne Genius thermal cycler (http://www.alab.com.pl/techne_sample.html) was 5 min at 94°C, 30 cycles of 1 min at 94°C (denaturation), 1 min at 55°C- 60°C (annealing with the actual temperature depending on the primer), 1 min at 72°C (primer extension) and 10 min at 72°C for one cycle. The completed reaction was held at 4°C.

Since the *Taq* DNA polymerase does not have 3'-5' proofreading exonuclease activity, each fragment was amplified at least twice in independent experiments. These PCR products were used as sequencing templates.

Gene regions where the putative lesions were located were amplified using *Pfx* polymerase (www.invitrogen.com) which has the 3'-5' proofreading exonuclease. The 50 µL *Pfx* polymerase PCR reaction mixture contained 1x *Pfx* amplification buffer®, 0.2 mM each of dNTPs, 1 mM $MgSO_4$, 0.2 µM each primer, 100 ng template DNA and 1 unit Platinum® *Pfx* DNA polymerase. The thermal cycle program followed the three-step cycling suggested by the manufacturer: a 2 min denaturation at 94°C, 35 cycles of 15 sec at 94°C for denaturation, 30 sec at 50-55°C for annealing and 1 min at 68°C for extension. After amplification, the reaction was maintained at 4°C.

Gene regions that contained the putative lesions of the *hypA* temperature sensitive alleles were amplified from wild type *hypA* by *Taq* or *Pfx* polymerase for sequencing and transformation.

2.7.3 PCR product purification

After the PCR amplification, amplicons were checked for the correct size using electrophoresis in 1% (w/v) agarose gel in TAE buffer. Fragments were stained with 1 μg / mL ethidium bromide and imaged using a Gel Doc 2000 (Bio-Rad Laboratories, www.bio-rad.com).

For sequencing or transformation, the PCR reactions where a sample showed it contained only one fragment were purified for sequencing using Concert Nucleic Acid Purification System (Gibco BRL® Life Technologies, www.lifetech.com), following the manufacturer's protocol. Where a sample showed more than one fragment had been amplified, the desired DNA fragment was excised, and the DNA was extracted using Concert Rapid Gel Extraction System (Gibco BRL® Life Technologies, www.lifetech.com). The purified DNA was resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) or distilled water and final concentration was measured using Beckman DU 7400 series UV spectrophotometer.

2.8 DNA Sequencing

2.8.1 Dye terminator cycle sequencing reaction

The PCR products were used directly for sequencing without cloning. The DNA fragments purified from PCR reactions were used as templates in dye terminator cycle sequencing reaction (Perkin Elmer Corp., www.appliedbiosystems.com), using fluorescently labeled dNTPs. The length of DNA templates for sequencing ranges from 400 bp to 1400 bp. Longer fragments require more labeled dNTPs, but since too much dye would interfere with the detecting efficiency of the sequencing machine, the volume of Big Dye® and buffer were adjusted according to the size of DNA templates. In a total volume of 10 µL PCR reaction, 25-50 ng DNA template were mixed with 1.5 - 2 µL Big Dye® containing the AmpliTaq DNA polymerase (Applied Bio System, www.appliedbiosystems.com) and labeled dNTPs, 2.25 - 3 µL 2.5x sequencing buffer® (Applied Bio System), and 0.16 µM primer in each PCR reaction tube.

The PCR reaction with the dye terminator was performed in a Perkin - Elmer DNA thermal cycler (the Perkin Elmer Corp., www.appliedbiosystems.com) using the dye terminator cycle standard program: denaturation at 80°C for 4 min followed by 24 cycles of 10 sec at 96°C, 5 sec at 50°C, 4 min at 60°C and held at 5°C. The ramping rate for all the temperature changes was 1°C per second.

2.8.2. Sequencing and data analysis

Products after PCR sequencing reactions were purified to remove unincorporated terminators, following the manufacturer's protocol. The PCR reaction contents was transferred to a 1.5 mL microcentrifuge tube containing 1 µL 3 M sodium acetate (pH

4.6) and 25 µL 95% ethanol and centrifuged at 13000 rpm for 30 min. The pellet was rinsed in 125 µL 70% ethanol and centrifuged at 13000 rpm for 1 min. The alcohol solution was removed with a micropipette and blotted with Kimwipe without disturbing the DNA pellet, and dried in a vacuum centrifuge at 13000 rpm for 15 min. Sequencing reactions were kept at -20°C until loading.

Sequencing used a ABI PRISM® 310 Genetic Analyzer (www.appliedbiosystems.com) with a 55 cm capillary. Data were analyzed using Sequencher™ 3.1.1 sequencing analysis software (Gene Codes Corporation, Inc. www.genecodes.com). Sequences were aligned and compared with the wild type *hypA* (GenBank AF001273) using ClustalW (http://www.ebi.ac.uk/clustalw/index.html).

2.9 Transformation

A. nidulans transformation followed the method of Osmani *et al*. (1987). About 10^9 freshly harvested *hypA6* strain ASY1 spores were germinated at 28°C in 50 mL of liquid CM with 100 µg / mL ampicillin in a sterile 250 mL Erlenmeyer flask coated with Gel Slick (FMC BioProducts, www.bioproducts.com) at 300 rpm for 5 -6 hr.

The germlings were harvested by centrifugation in 50 mL sterile conical tubes for 2 min at 4000 rpm, and were partially protoplasted in a solution containing 20 mL Solution I (0.8 ammonium sulfate, 100 mM citric acid, pH 6.0), 20 mL Solution II (1% yeast extract, 2% sucrose, 20 mM $MgSO_4$), 80 mg BSA, 100 mg Driselase (Interspex, www.wenet.net/~sharma/interspex), 300 mg Glucanex (Interspex), and 100 µL beta-glucuronidase for 2.5 hr at 28°C and 150 rpm.

The protoplasts were harvested, washed twice in ice cold Solution III (0.4 M ammonium sulphate, 1% sucrose, 50 mM citric acid) and resuspended in 1 mL 4°C Solution V (0.6 M KCl, 50 mM $CaCl_2$ and 10 mM MOPS buffer) and stored on wet ice over night.

For transformation, 100 µL of protoplast suspension were mixed with about 10 µg DNA fragment, 1 µg ARp1 DNA (Gems *et al*., 1991) and 50 µL Solution IV (25% PEG8000, 100 mM $CaCl_2$, 0.6 M KCl), and incubated on wet ice for 20 min. An additional 1 mL of solution IV was mixed into the protoplast suspension, and incubated at room temperature for another 20 min.

Four hundred microlitre aliquots of protoplast plus solution IV suspension were mixed with CM containing 0.6% agar and 1 M sucrose and plated over the same medium containing 1.5% agar. Plates were incubated at 28°C for 24 h to allow the protoplasts to regenerate their cell wall, and then transformants were selected at 42°C. Complementation was shown by the wild type colony growth at 42°C. Transformants were further confirmed by microscopic examination of coverslip cultures grown at 42°C.

3. RESULTS

Two temperature sensitive alleles of the *Aspergillus nidulans hypA* locus were generated by chemical mutagenesis (Harris *et al.,* 1994) of *A. nidulans* strain A28 (http://www.fgsc.net) in independent experiments (Harris *et al*., 1994; Kaminskyj and Hamer, 1998). Mutants with these alleles (*hypA1*, Kaminskyj and Hamer, 1998; *podA1*, Harris *et al*., 1999) were selected for having wildtype hyphal morphology when grown at 28°C, but reduced growth and abnormal hyphae at 42°C. Using genetic methods, Kaminskyj and Hamer (1998) showed that *podA1* was allelic to *hypA1*, so *podA1* was renamed *hypA6*.

Published descriptions of the two mutant phenotypes varied on key points, particularly that *hypA1* was described as being able to complete its asexual life cycle at 42°C (Kaminskyj and Hamer, 1998), whereas *podA1* (=*hypA6*) was said to have a temperature sensitive arrest within a day after germination (Harris *et al*., 1999). This suggested that the *hypA6* allele might cause a lethal defect at 42°C. However, work in progress by Shi and Kaminskyj (Shi *et al.,* submitted) had shown that a *hypA* deletion strain was viable but morphologically abnormal.

To reconcile these conflicting data, I undertook a detailed morphological comparison of *A. nidulans* strains carrying the *hypA1* and *hypA6* alleles. The *hypA* locus has been cloned and shown to encode a homologue of a member of the *Saccharomyces cerevisiae* TRAPP II complex, involved in Golgi transit. However, a complete biochemical analysis

of either gene has not been completed, and the function of the *hypA* gene product is unknown. Since the molecular lesion in *hypA1* has been identified, I sequenced the *hypA6* allele, to identify its defect and improve our understanding of the *hypA*'s function.

3.1 Phenotypic comparison of *hypA1* and *hypA6* strains

At 42°C, wildtype *Aspergillus nidulans* strains germinate in about 6 h, during which the single nucleus in the conidium has undergone at least one mitosis and the spore has swelled to double its initial size of ~ 3 μm in diameter. Germination is defined as initiation of polarized growth so that the germling is at least 50% longer than wide. In contrast, germination is delayed at 42°C in *hypA1* and *hypA6* strains, beginning at about 9 h, by which time the spores had swollen to three or more times their unhydrated diameter, and typically had undergone two or more rounds of mitoisis. Although this serves to distinguish one aspect of the wildtype and mutant phenotypes, it did not clarify the relationship between strains with the *hypA* temperature sensitive alleles.

3.1.1 Phenotypic characterization of *hypA* mutants

Strains with the *hypA1* and *hypA6* alleles were germinated at 42°C for 24 h, then fixed and stained with Calcofluor and Hoechst 33258 for cell walls and nuclei, respectively. Compared with wild type strains, both *hypA1* and *hypA6* germlings were significantly shorter and wider (**Figure 3.1**). In wild type cell, the width of hyphae was constant and about 3 μm. There were 3 ~ 4 nuclei in each basal cell with each nucleus about 2 ~3 μm in diameter (Deacon, 1997), separating by evenly spaced septa. The first septum formed at the conidium and germling junction as early as the completion of the

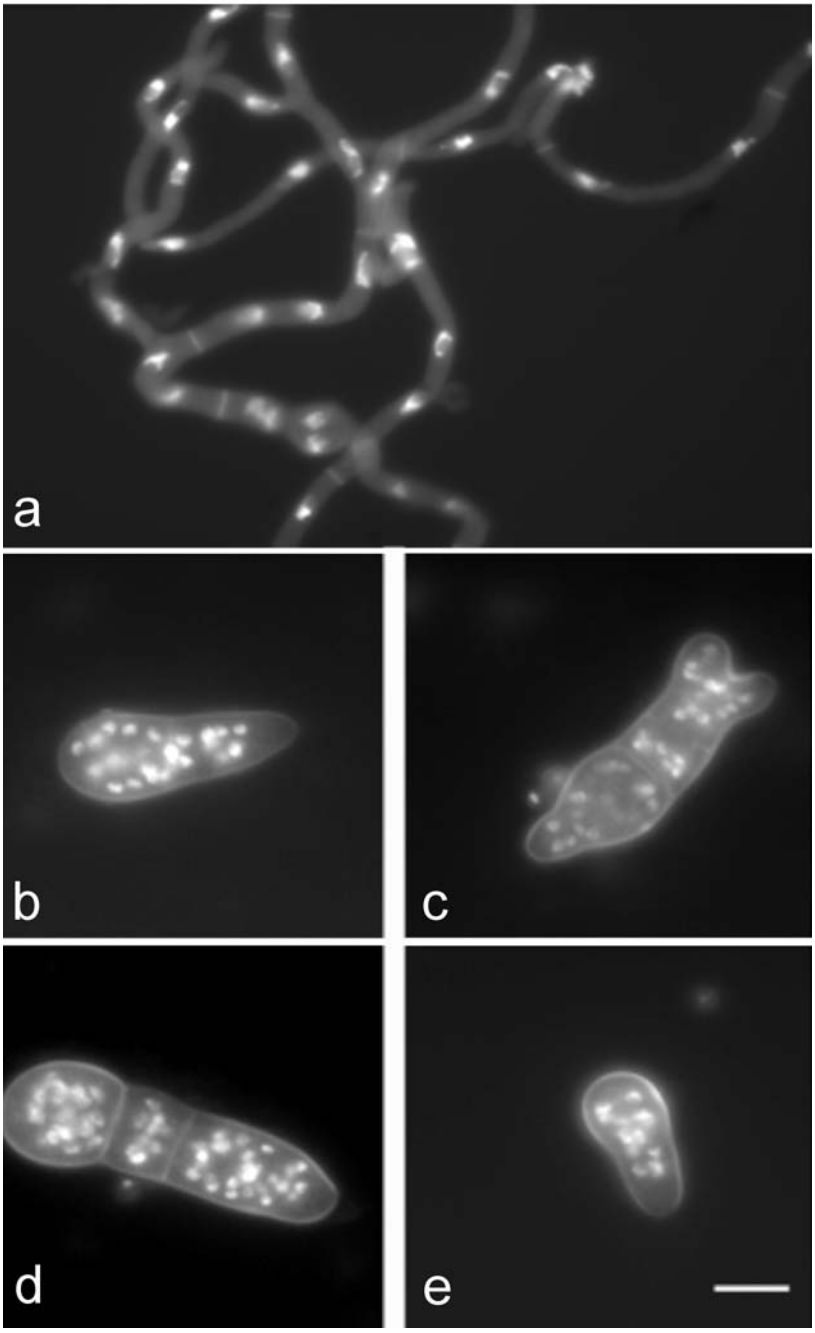
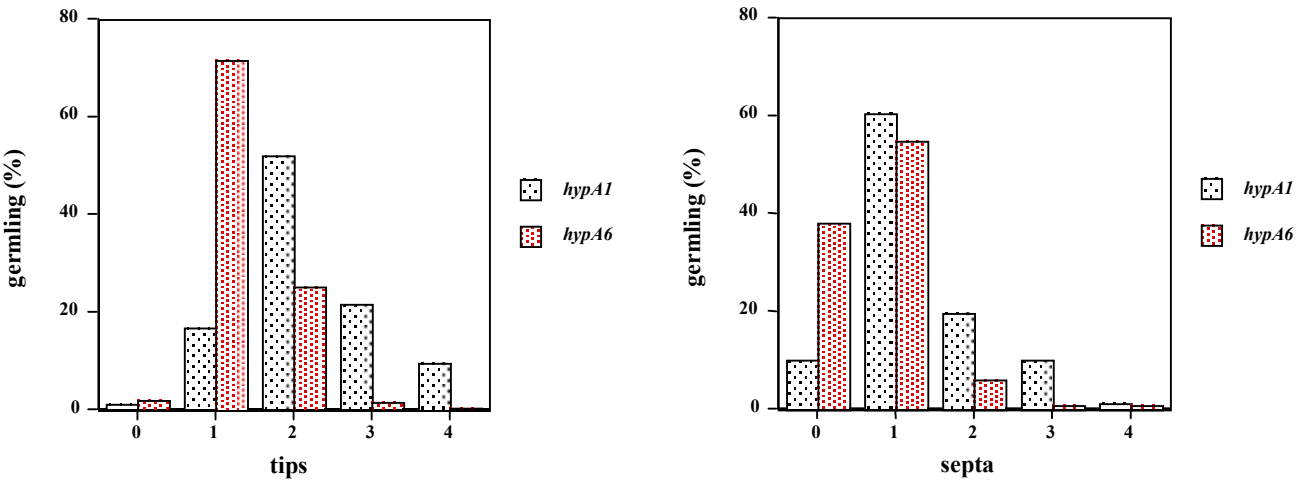


Figure 3.1 Phenotypes of *A. nidulans* wild type, *hypA1* and *hypA6* mutants at 42°C under epifluorescence microscopy. Wild type *A. nidulans* (a) and *hypA1* (b, c), *hypA6* (d, e) spores were incubated for 24 hr at 42°C. Cells were fixed and stained with Hoechst 33258 to visualize nuclei. The wild type hyphae were slender with regular septa and evenly distributed nuclei. The *hypA1* and *hypA6* mutant germlings were short, wide with irregular septa and more nuclei in basal cells. Bar: 10 μm.

third mitosis (Harris *et al*., 1994). Both *hypA1* and *hypA6* mutant grew similar to wild type at 28°C. But at restrictive temperature 42°C, they displayed similar phenotypes that were different from wild type. The hyphae were shorter, and wider ranging from about 5 μm ~ 15 μm. The nuclei were compact and smaller than those in wild type, and usually there were more than 10 nuclei in one basal cell. And septum was not spacing evenly. In some germlings, there was no septum produced even though the germling had more than 16 nuclei, while in others, the septa were produced so close that they separated some basal cells without any nucleus inside. The short and wide hyphae of *hypA1* and *hypA6* at 42°C indicated a delay in establishing polarity and a failure in maintaining it. The abnormal pattern of septa indicated the septum formation was also affected.

Microscopic comparison of the phenotype of *hypA1* and *hypA6* strains grown at 42°C for 24 h showed that they were quantitatively as well as qualitatively similar. Strains with the *hypA1* and *hypA6* allele were compared with each other for their growth after 24 h at 42°C. The total number of tips or septa was summed for at least 200 germlings, divided by the number of germlings observed, and expressed as mean number ± standard error of the mean. Table 3.1.1 shows the average numbers of tips and septa in samples of *hypA1* and *hypA6* germlings grown at 42°C for 24 h. Since after 24 h incubation at 42°C, some cells show a sign of dying with the cell membrane separated from cell wall, which was detected by FM4-64 staining, the cells counted include not only living cells, but also those are dying and dead already. Although the average numbers of both tips and septa in each germling are different, the statistic analysis of

Table 3.1.1 The percentage and average number of tips and septa per germling of *hypA1* and *hypA6* mutants incubated at 42°C for 24 h



	Tips / germling		Septa / germling	
Strains	ASK30 (*hypA1; pyroA4*)	ASK80 (*hypA6, pabaA6; pyroA4*)	ASK30 (*hypA1; pyroA4*)	ASK80 (*hypA6, pabaA6; pyroA4*)
Average±SE	2.17±0.06	1.27±0.03	1.32±0.06	0.70±0.04
(n)	(n=213)	(n=258)	(n=213)	(n=258)
P value*	0.815		0.649	

* Comparison by *t-test* statistical analysis

t-test did not show significant difference between *hypA1* and *hypA6* strains regarding the numbers of tips and septa per germling (p=0.815 for tip / germling; p=0.649 for septa / germling).

3.1.2 *hypA1* and *hypA6* strains can complete their asexual life cycle at 42°C.

Harris *et al*. (1999) described *hypA6* strains as having a temperature sensitive arrest within a day of germination at 42°C, implying that *hypA6* had a lethal restrictive phenotype. In contrast, Kaminskyj and Hamer (1998) reported that *hypA1* strains were able to conidiate at 42°C and produce viable spores.

To compare the ability to sporulate at restrictive temperature, *hypA1* and *hypA6* strains were germinated and grown at 42°C for several days and then prepared for scanning electron microscopy. Although both *hypA1* and *hypA6* mutants displayed defective hyphal phenotypes at 42°C, they could complete their asexual life cycle and sporulate. Using scanning electron microscopy (SEM), conidia of the *hypA6* strain and those of the *hypA1* strain were similar in both shape and diameter (**Figure 3.2**). The conidiophores in both strains were similar to those in wild type at 42°C, but the other structures for conidium production such as metulae and phialides were not observed (obscured by the spore mass). Compared to 28°C, strains of wild type, *hypA1* and *hypA*6 had reduced asexual sporulation at 42°C. However, spores of both *hypA1* and *hypA6* strains were viable and had the restrictive phenotype at 42°C. In addition, *hypA6* colonies were found to have grown after 2 days incubation at 42°C. The above results indicated

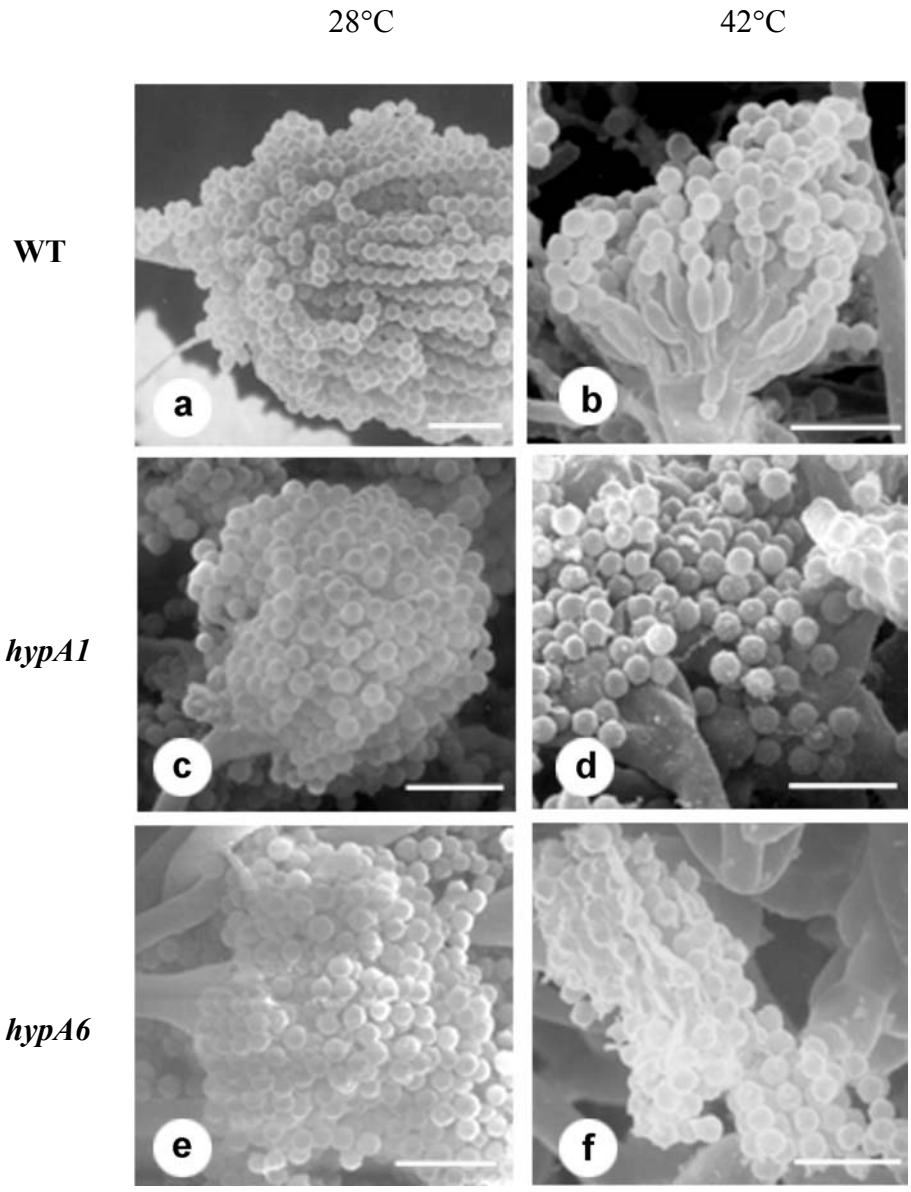


Figure 3.2 The sporulation of *A. nidulans* wild type, *hypA1* and *hypA6* mutants at 42°C. Spores were inoculated on solid medium, incubated for 2 weeks at 42°C, and examined under SEM. Although they had reduced asexual sporulation compared to 28° (left row), the conidia of *hypA1* (d) and *hypA6* (f) strains that were produced at 42°C were viable, and similar to wild type in shape. Bar: 10 μm

that *hypA6* could also complete the asexual life cycle at restrictive temperature, like *hypA1*, supporting that *hypA* is not essential in *A. nidulans*, and that the *hypA1* and *hypA*6 phenotypes are indistinguishable using these criteria.

3.1.3 Temperature shifting analysis

The *hypA1* temperature sensitive strains have distinctive polarity-related phenotypes when grown at permissive temperature then shifted to restrictive temperature, and *vice versa* (Kaminskyj and Hamer, 1998). These were compared for *hypA1* and *hypA6* strains in this study. *hypA1* and *hypA6* strains grown at permissive temperature 28°C were upshifted to restrictive temperature 42°C for 3 h before microscopic observation. In the downshift experiment, conidia were incubated at 42°C for 14h then downshifted to 28°C.

3.1.3.1 Hyphal tip growth ceases in upshifted temperature

When 28°C grown *hypA1* cells were shifted to 42°C for 3 h, their tip cells ceased growth (Kaminskyj, 2000) and their basal cells adopt the restrictive phenotype, including swelling and eventually branching. As shown in **Figure 3.3**, *hypA6* strains produce a qualitatively similar phenotype to *hypA1*under these conditions.

Within 3 h after upshifted to the restrictive temperature, the apical cell growth of both mutant strains ceased and the basal cells began to swell. In contrast to basal cells, the hyphal tip cells of both mutants were depleted of cytoplasm under differential interference contrast optics. There was an obvious separation caused by a septum between the cells with and without cytoplasm. Corresponding to the hyphae stained with Hoechst 22358, the nuclei in some of these tip cells were undergoing degradation, while

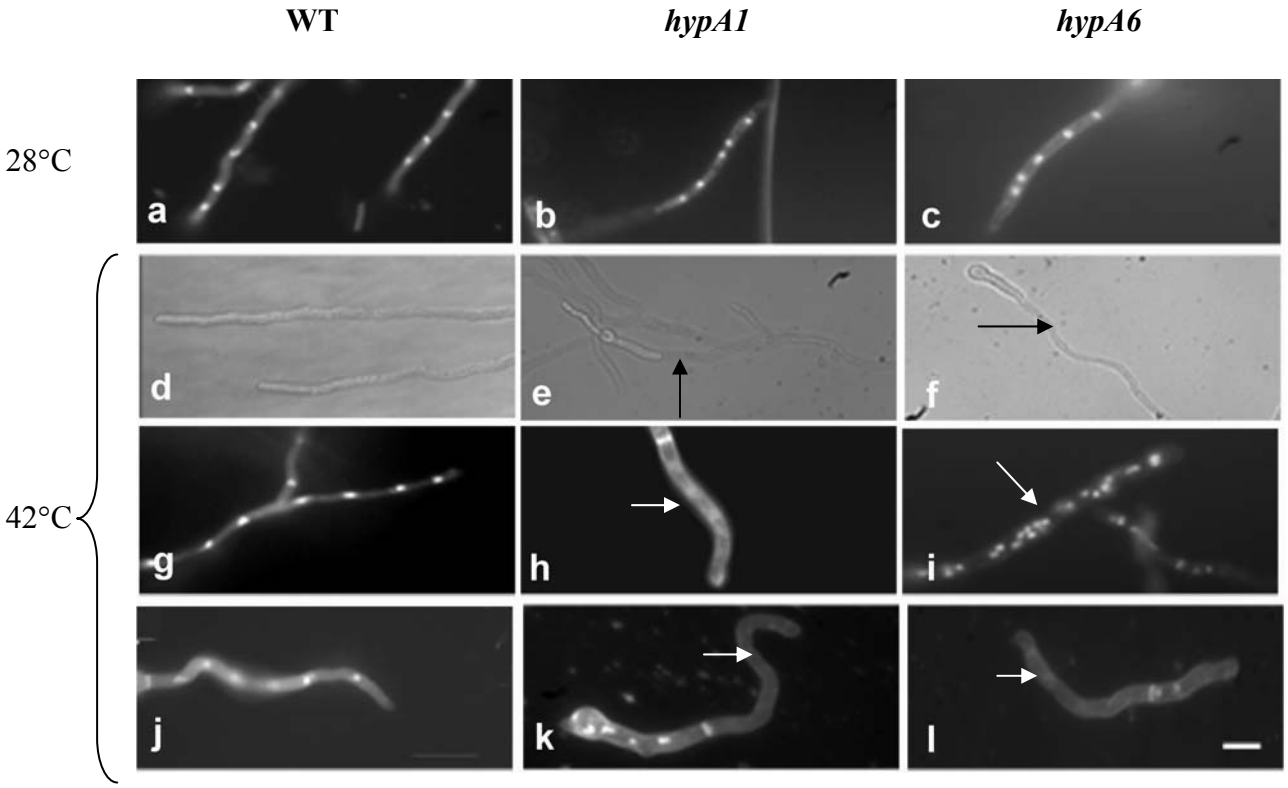


Figure 3.3 Phenotypes *of A. nidulans hypA* mutants shifted from permissive to restrictive temperature. Cells were grown at 28°C for 14 hr, then shifted to 42°C for 3 hr. Using DIC microscopy, the tip cells of both *hypA1* (e) and *hypA6* (f) hyphae ceased growing (arrows), while subapical cells swelled. These tip cells strained with Hoechst 33258 exhibited either degrading of nuclei (arrows in h, i,) or are empty of nuclei (arrows in k, l), indicating a death of tip cells in the upshifted temperature. Bar: 10 μm.

others had lost their nuclei already, confirming the death of cells. These results were consistent with that loss of *hypA* function resulting in the cessation of tip growth and the resumption of growth in basal cells (Kaminskyj and Hamer, 1998; Kaminskyj, 2000), suggesting *hypA* gene play important role in normal hyphal growth.

3.1.3.2 *hypA1* and *hypA6* exhibit similar phenotypes in downshift experiment

When restrictive temperature grown *hypA1* cells are shifted to permissive temperature, they re-initiate wildtype growth by branching, a readily-quantifiable manifestation of their ability to polarize. To compare *hypA1* and *hypA6* strains for their ability to branch after restrictive temperature growth, *hypA1* and *hypA6* strains were grown for 14 h at 42°C, and then shifted to 28°C. Samples were taken for microscopy at 30 min intervals. In addition to the *hypA1* and *hypA6* strains studied by Kaminskyj and Hamer (1998) and Harris *et al.* (1999), whose nutritional auxotrophies were pyridoxin and para-aminobenzoic acid, respectively, a *hypA1 pabaA6* strain, ASK39, was also used in this analysis, to preclude possible quantitative phenotypic effects due to nutrition (e. g., Serlupi-Crescenz *et al*., 1983). At each time point about 200 germlings were scored for the number of tips (**Table 3.1.2**). The average number of tips per germling was significantly increased within 1 h ~ 2 h after downshift. By 2.5 h after downshift, the average number of new polarized sites from the restrictive-grown part of the germlings ranged from 1.76±0.07 to 2.75±0.08. These newly formed branches were predominantly at the ends of the germling with number ranging from 1 to up to 5 (**Figure 3.4**). There was no significant difference between *hypA1* and either *hypA6* strain in average number of newly formed polarized sites per germling ($p=0.581$, ANOVA).

Table 3.1.2 Average number of tips per germling of *hypA* mutant strains in the downshifting experiment

Strains	Time after downshift to 28°C (h)						P value*
	0 A±SE	0.5 A±SE	1 A±SE	1.5 A±SE	2 A±SE	2.5 A±SE	
ASK30	1.16±0.04	1.30±0.04	1.41±0.04	1.99±0.06	2.32±0.06	2.64±0.10	0.581
(*hypA1; pyroA4*)	(n=244)	(n=256)	(n=273)	(n=214)	(n=217)	(n=226)	
ASK80	1.23±0.04	1.32±0.04	1.54±0.04	1.72±0.04	2.34±0.07	2.75±0.08	
(*hypA6, pabaA6; pyroA4*)	(n=235)	(n=221)	(n=209)	(n=226)	(n=200)	(n=200)	
ASK39	1.04±0.03	1.00±0.05	1.17±0.05	1.69±0.06	1.61±0.06	1.76±0.07	
(*hypA1, pabaA6*)	(n=202)	(n=206)	(n=210)	(n=203)	(n=212)	(n=232)	

* Statistical comparison by ANOVA

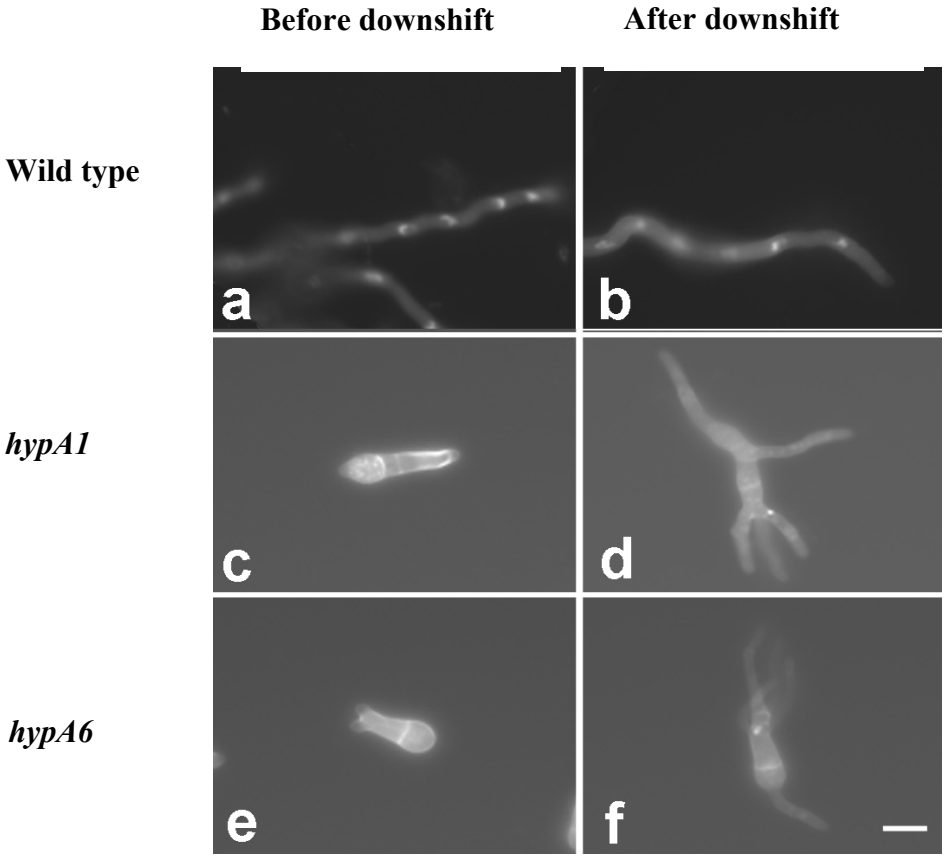


Figure 3.4 Epifluorescence microscopy showing similar branching patterns of *hypA1* and *hypA6* mutant cells in the downshifting temperature. Spores were incubated at 42°C for 14 h, and then shifted to 28°C. Samples were fixed and stained with Calcofluor and Hoechst 33258. After downshift to permissive temperature, germlings of *hypA1* and *hypA6* temperature sensitive mutants displayed similar timing of branching and branching patterns. Bar: 10 μm.

3.1.4 Endomembrane system analysis with dye FM4-64

The polarized hyphal growth is a function of polarized exocytosis at the growing tip. The wall forming vesicles are derived from the endomembrane secretory system (Grove *et al*., 1968; 1970) in the subapical and near apical regions of growing hyphal tips, and culminate at the hyphal apex forming the Spitzenkörper in higher filamentous fungi, which determines the hyphal shape and growth direction. Among fungal orthologues, *A. nidulans hypA* has a highly related sequence in *S. cerevisiae*, *TRS120*, which is involved in the regulation of the TRAPP II complex that mediates the Golgi transit (Sacher *et al*., 1998). At 42°C, a temperature sensitive mutant of *TRS120* has abnormal Golgi equivalents like *A. nidulans hypA1* (Kaminskyj and Boire, submitted). In order to compare the endomembrane arrays in *hypA1*and *hypA6* cells, the membrane-selective dye, FM4-64, was used to stain the endomembranes of wild type, as well as *hypA1* and *hypA6* strains at 28°C and 42°C.

In the FM4-64 stained hyphae grown at 28°C for 14 hr, the endomembrane arrays were similar in wild type, *hypA1* and *hypA6* cells. A round zone of fluorescence in the center of the hyphal apex was distinguished from the other fluorescent structures in the hypha (**Figure 3.5**), corresponding to the region of the putative Spitzenkörper in higher fungi (López-Franco and Bracker, 1996; Fischer-Parton *et al*., 2000; Bartnicki-Garcia, 2003). In the apex, other endomembranes also became stained. Most of them were spherical or tubular. These structures were more abundant in the apical than 20 μm further back to the basal area. They were concentrated in the central cytoplasm and

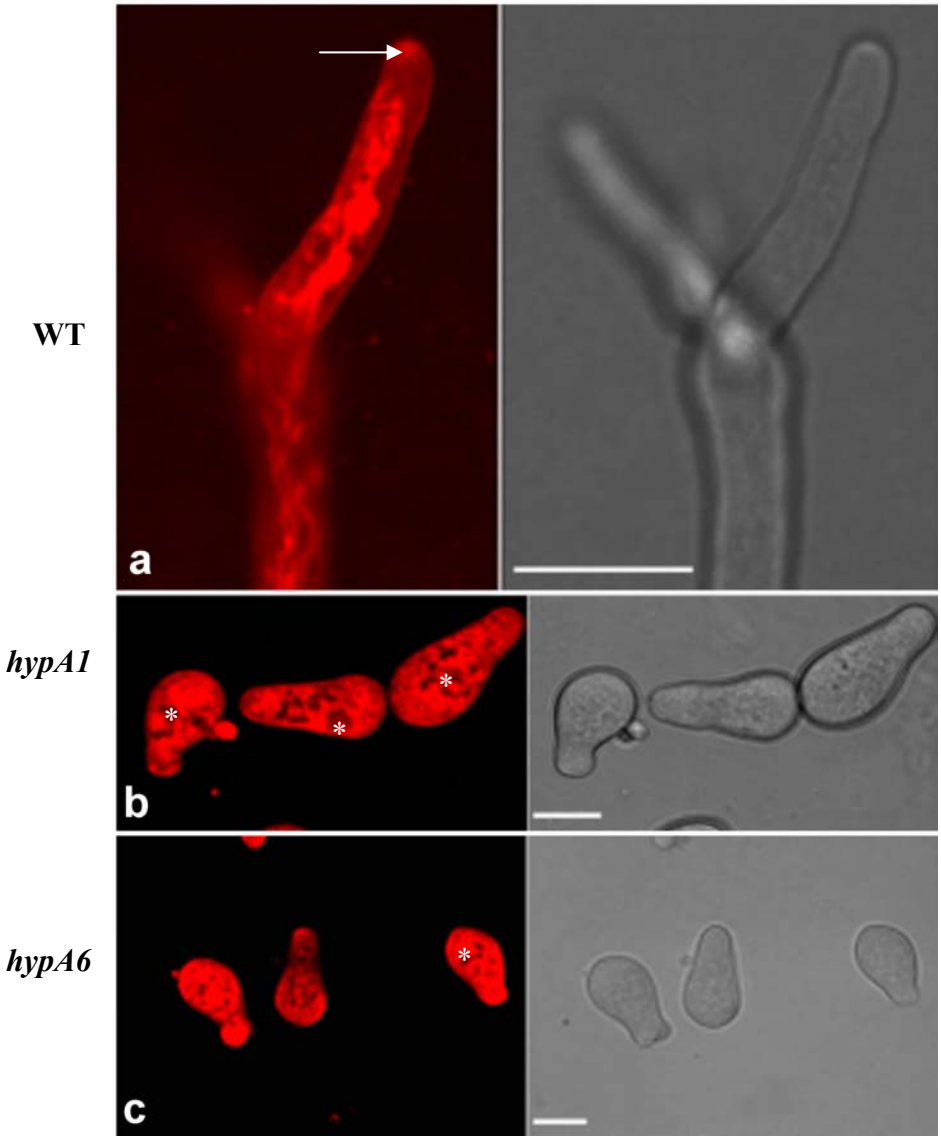


Figure 3.5 The endomembrane arrays in *hypA* mutant cells stained with FM4-64 *in vivo*. Cells were grown at 42°C for 14 hr, immersed in 25 μM FM4-64 and examined by LSM 510 META confocal microscope. Endomembranes in wild type *A. nidulans* hyphae were polarized and culminate in a Spitzenkörper (a, arrow). In both *hypA1* (b) and *hypA6* (c) cells grown at 42°C, endomembrane structures were distributed all over the cells without polarization. The regions of dye exclusion in the cell correspond to the size and location of nuclei (stars). No obvious Spitzenkörper was observed. Bar: 10 μm.

polarized towards the hyphal apex with some of them pointed close to the putative Spitzenkörper region. The highly polarised endomembrane arrays continued into the subapical area in the hyphae.

The abundant, highly polarised endomembrane arrays were not seen in germlings of *hypA1* or *hypA6* strains grown at 42°C. Fourteen hours old germlings incubated at 42°C had bright fluorescence-stained round structures dispersed without polarised organization in a more diffuse background staining of the cytoplasm, some of which distributed along the plasma membrane. No structure like the putative Spitzenkörper was observed at the tip area of germlings. In addition, there were some globose regions of dye exclusion in ~2-3 μm diameter within the cell which corresponded to the size and location of nuclei. The FM4-64 stained endomembrane arrays were similar in both *hypA1* and *hypA6* strains at 42°C.

When the *hypA* mutant germlings grown at 42°C were downshifted to 28°C, 3 hr later the well organised and polarised endomembrane arrays in both *hypA1* and *hypA6* strains recovered and the putative Spitzenkörper reappeared at the hyphal apex (**Figure 3.6**) along the resumption of polarized hyphal growth. The coincidence of the polarised endomembrane arrays and the Spitzenkörper, and the recovery of *hypA* function at permissive temperature indicated that maybe *hypA* function to regulate the polarized arrays of endomembrane system for exocytosis in hyphal morphogensis.

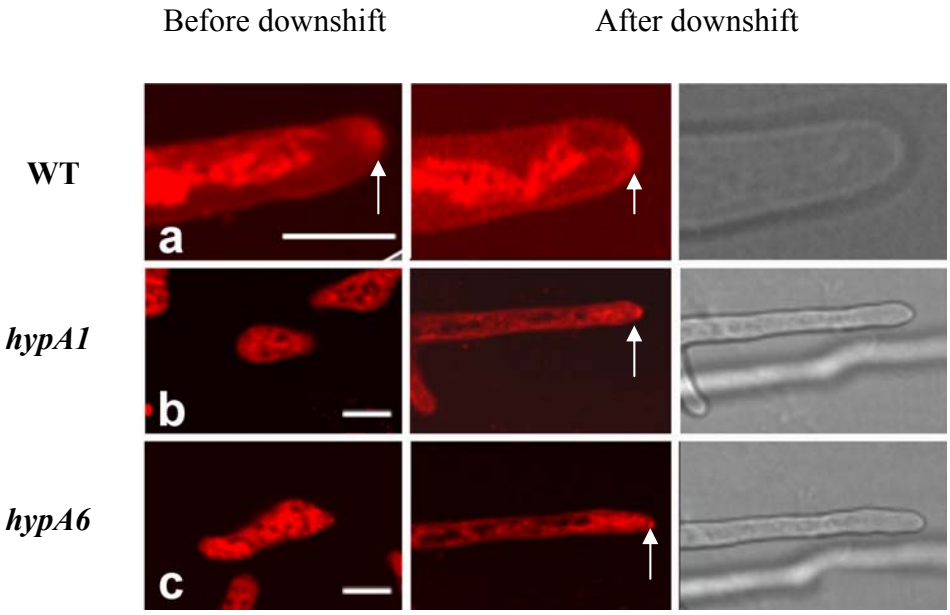


Figure 3.6 Endomembrane arrays in the downshifted *hypA1* and *hypA6* mutant cells. Conidiospores were grown on solid medium at 42°C for 14 hr. Germlings were downshifted to 28°C and incubated for 3 hr, stained with 25 μM FM4-64 and examined under confocal microscope. In the downshifted *hypA1* (b) and *hypA6* (c) cells, the polarized endomembrane arrays along the hyphae were recovered and the Spitzenkörper was reappeared at the hyphal tip (arrows). Bar: 10 μm

3.1.5 The cytoplasmic microtubule cytoskeleton of *hypA1* and *hypA6* strains

Aspergillus nidulans cytoplasmic microtubules are longitudinally arranged, and typically several are found in any one cross section using light microscopy (Han *et al*., 2001). When *A. nidulans* hyphae are prepared by freeze-substitution and serially cross sectioned, TEM shows that up to half of the microtubules visualized by epifluorescence are bundles of two or three (R. Roberson, personal communication). Nevertheless, the total number of microtubules at any position with respect to the tip (apart from the extreme apex) is typically five to fifteen. Although cytoplasmic microtubules have a less critical role than F-actin in hyphal tip growth and morphogenesis, disrupting the microtubule cytoskeleton in *Neurospora crassa* using benomyl resulted in apical branching (Riquelme *et al*., 1998).

The *hypA1* and/or *hypA6* restrictive phenotypes were related to the establishment of polarity, and cytoplasmic microtubules are important for nuclear distribution in *A. nidulans* (Morris, 1976). At 42°C, *hypA1* and *hypA6* have compact nuclei and abnormal nuclear distribution. Thus, it was worth investigating whether cytoplasmic microtubules were abnormally distributed, or abundant in *hypA1* and *hypA6* strains at 42°C.

3.1.5.1 Cytoplasmic microtubule arrangement in restrictive phenotypes of *hypA1* and *hypA6* strains

The *hypA1* and *hypA6* cytoplasmic microtubule arrays were examined in germlings at restrictive temperature and in hyphae after downshift to permissive temperature. *hypA1* and *hypA6* strains with GFP-tagged cytoplasmic microtubules were created by mating with a wildtype strain that had GFP-tagged alpha-tubulin.

The wildtype GFP-tagged alpha-tubulin strain had two or more cytoplasmic microtubules when imaged with confocal microscopy, and as expected all were roughly parallel to hyphal axis. In contrast, both *hypA1* and *hypA6* GFP-microtubule strains had reduced cytoplasmic microtubule arrays, but wild type arrangement at 42°C (**Figure 3.7**). In each case, there was typically only one cytoplasmic microtubule at any longitudinal position in the cell. But 3 h after the restrictive grown *hypA1* and / or *hypA6* cells were downshifted to permissive temperature, the cytoplasmic microtubules were similar to those in wild type cells in numbers (**Figure 3.8**). These results indicated that both hypA1 and hypA6 have normal microtubule arrangement at 42°C and the phenotype of *hypA1* and *hypA6* mutants at restrictive temperature were not due to the depolymerization of cytoplasmic microtubules, which was consistent with the benomyl experiment where the disruption of cytoplasmic microtubules did not cause high branching in *hypA1* and / or *hypA6* during the germination at restrictive temperature.

3.1.5.2 Mitosis in *hypA* temperature sensitive alleles is normal at 42°C

Microtubules form spindles that are involved in separating the chromosomes into two daughter nuclei in mitosis and meiosis. In a series of time course images under confocal microscope, the GFP-tagged alpha tubulin revealed a expected change of a set of microtubules during the mitosis in a *hypA6* allele cell at 42°C (**Figure 3.9**). At the beginning when the first image was taken, 8 small granules (mitotic spindles) scattered in the cell were brightly stained. The granules first grew in length and width. Some of them looked like short bars, and others were V-shaped. Four minutes later respect to the time the first picture was taken, they grew into long and straight threadlike structures with less

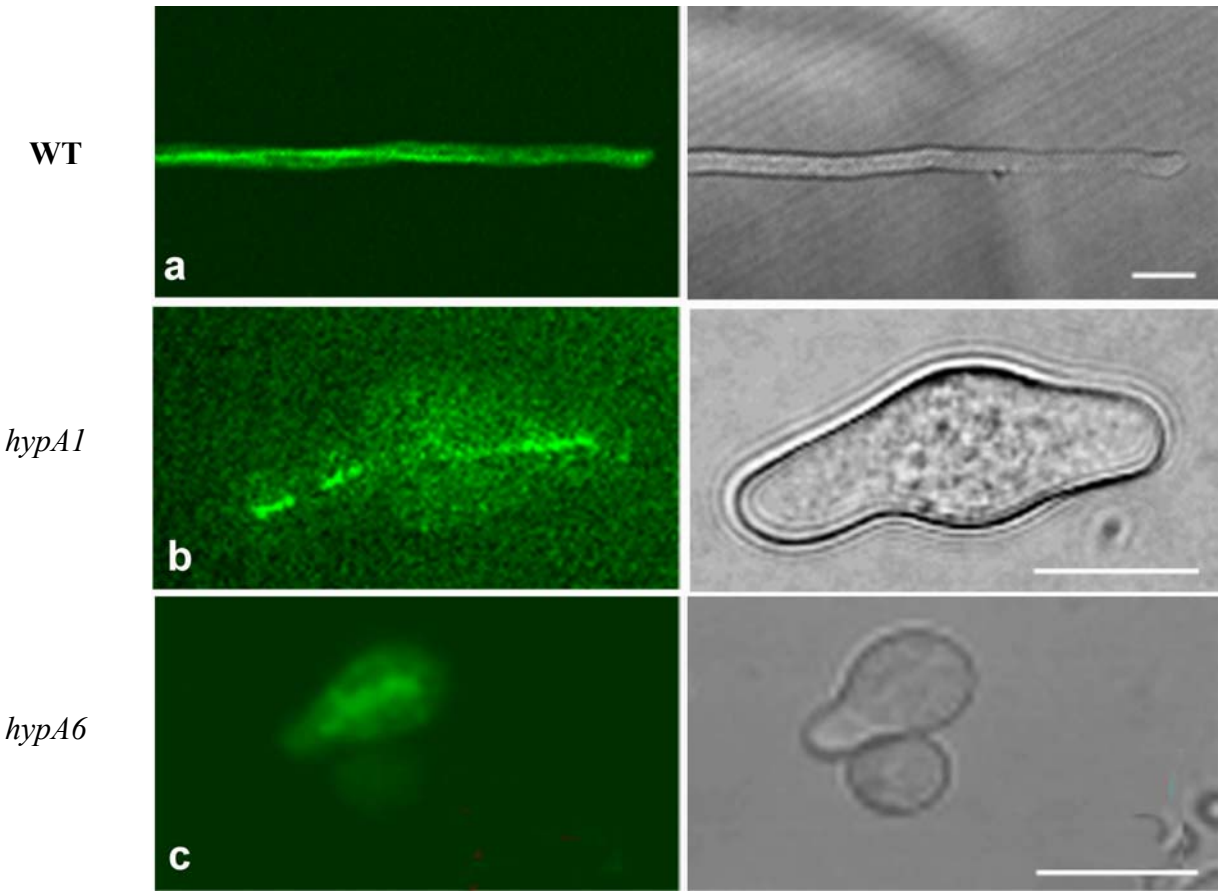


Figure 3.7 The GFP-tagged microtubule arrays in *hypA* mutant cells. Wild type GFP-*tubA*, and *hypA1* GFP-*tubA* and *hypA6* GFP-*tubA* strains were grown on solid CM glycerol at 42°C for 14 hr and examined with confocal microscopy. The GFP-tagged microtubules extended parallel to each other along the hyphae towards the tip in wild type cells. At least 2 microtubules can be recognized (a). Similarly in both *hypA1* (b) and *hypA6* (c) mutant cells, the microtubules also arrayed along the cell axis but were fewer than those in wild type, usually only one cytoplasmic microtubule can be observed in a cell. Bar: 10 μm.

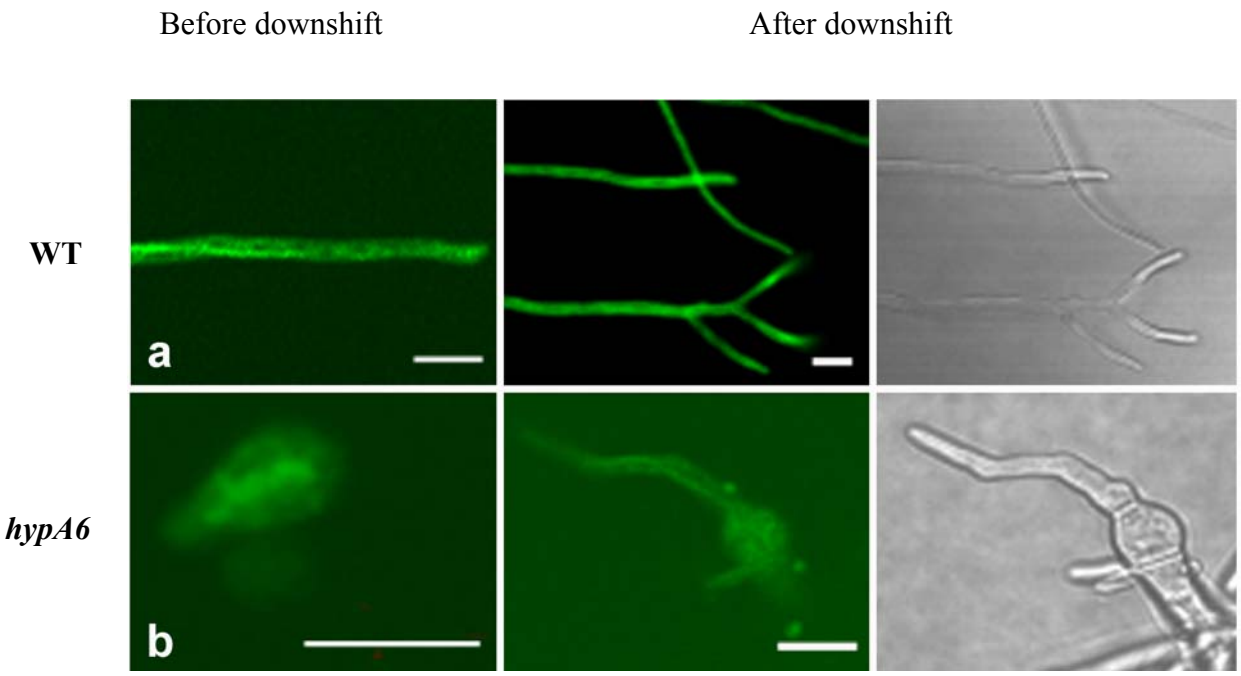


Figure 3.8 Microtubule arrays in the downshifted hyphae of *hypA* mutant cells. After the GFP-*tubA* spores were incubated at 42°C for 14 hr, the germlings were downshifted to 28°C for 3 hr and examined for the microtubule arrangement with confocal microscope. After 3 hr incubation at permissive temperature, more than one microtubule was found along the hyphal long axis in *hypA6* mutant cell (b). Bar: 10 μm

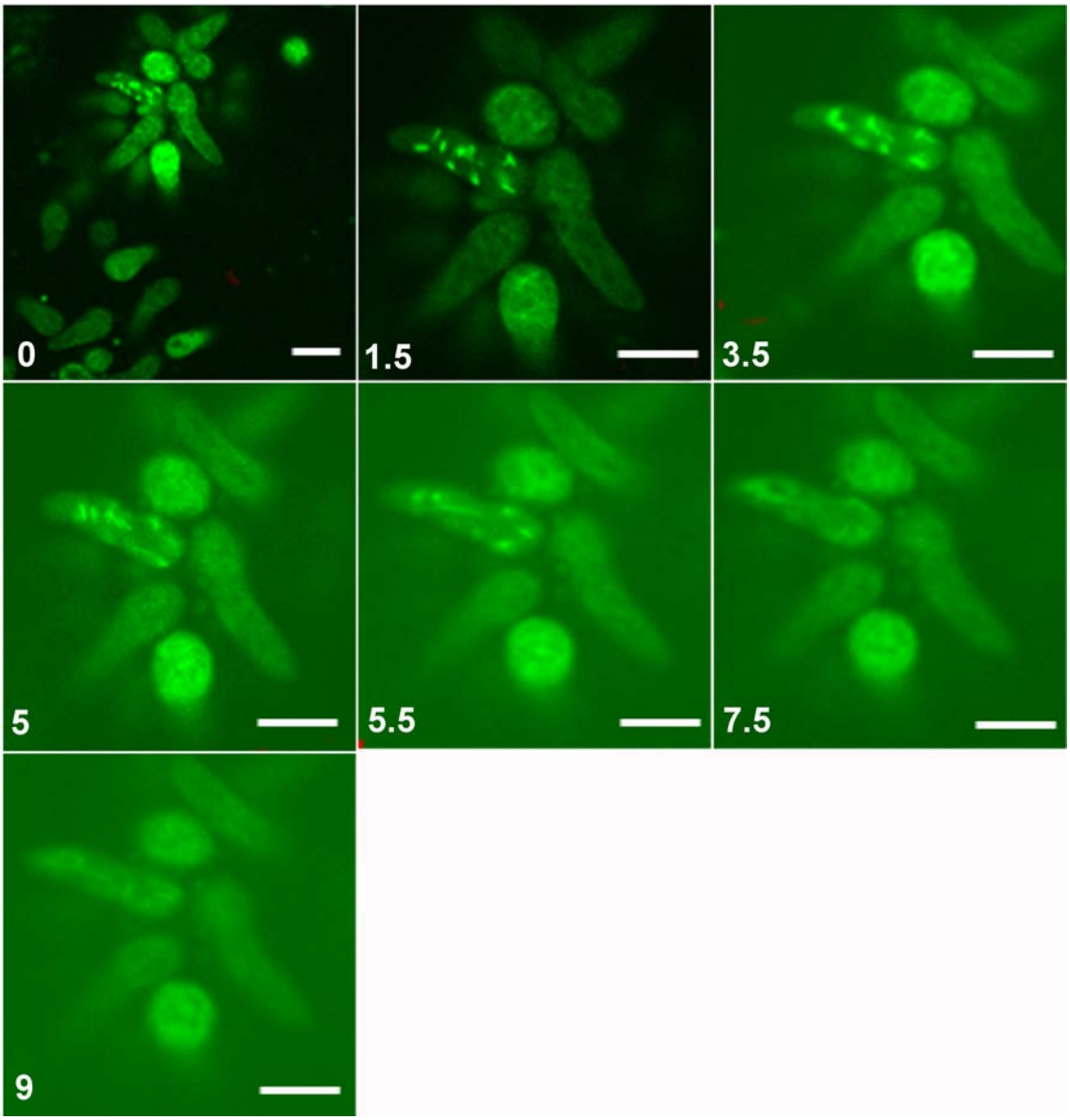


Figure 3.9 Time-lapse confocal micrographs of mitosis in *A. nidulans hypA6* mutant cells visualized by GFP-α tubulin. The *hypA6* spores with GFP-tagged α-tubulin were grown 14 hr at 42°C on solid CM glycerol. Numbers indicate minutes elapsed since the first picture was taken. The fluorescence of GFP-α tubulin changed gradually from compact and high intense in the first picture (0 min) into less stained (1.5 min, 3.5 min, 5 min, and 5.5 min), and finally fused into the cytoplasmic background (7.5 min, 9 min). The time of this process is correspondent with the time of mitosis in wild type *A. nidulans* cells described by Bergen and Morris (1983). Bar: 10 μm.

intensity in staining. The process continued until about 6-8 minutes later when the granules disappeared and the fibres merged into the cytoplasmic background. The germling imaged here was at the stage of fourth mitosis, from 8 to 16 nuclei. Besides, similar mitotic kinesis at the stage over 8 nuclei was also observed in some other *hypA6* and *hypA1* cells at 42°C.

The time period of this process is consistent with that of the mitosis during the cell cycle in wild type *A. ndiulans* calculated by Bergen and Morris (1983). The dynamic change of GFP-tagged alpha-tubulin in the cell revealed a normal mitosis in *hypA1* and *hypA6* mutants at restrictive temperature, indicating *hypA* temperature sensitive mutants have normal nuclear division and the *hypA1* and *hypA6* defects do not affect spindle formation and dynamics.

3.1.6 Effect of benomyl on *hypA* restrictive phenotypes at 42°C

At 28°C, when the 6 h old germlings were challenged with benomyl for 3 h, the germlings of both wild type and *hypA* temperature sensitive mutants were similar to those of wild type grown at 42°C with benomyl. Germlings were short and highly branched, but their apices were not significantly swollen (**Figure 3.10**). In contrast, the benomyl treated germlings of *hypA1* and / or *hypA6* strains at 42°C displayed the restrictive phenotype as those without benomyl with a same delayed germination up to 9 h. The germlings were wide, short, and had only one or two tips. No significant apical branching was observed (**Figure 3.10**).

The effects of benomyl on the hyphal tip growth of *hypA* temperature sensitive strains at 42°C were also investigated. *hypA1*and *hypA6* wild type hyphae grown at 28°C for 14 h were upshifted to 42°C and challenged with benomyl for 3 h. The phenotype was

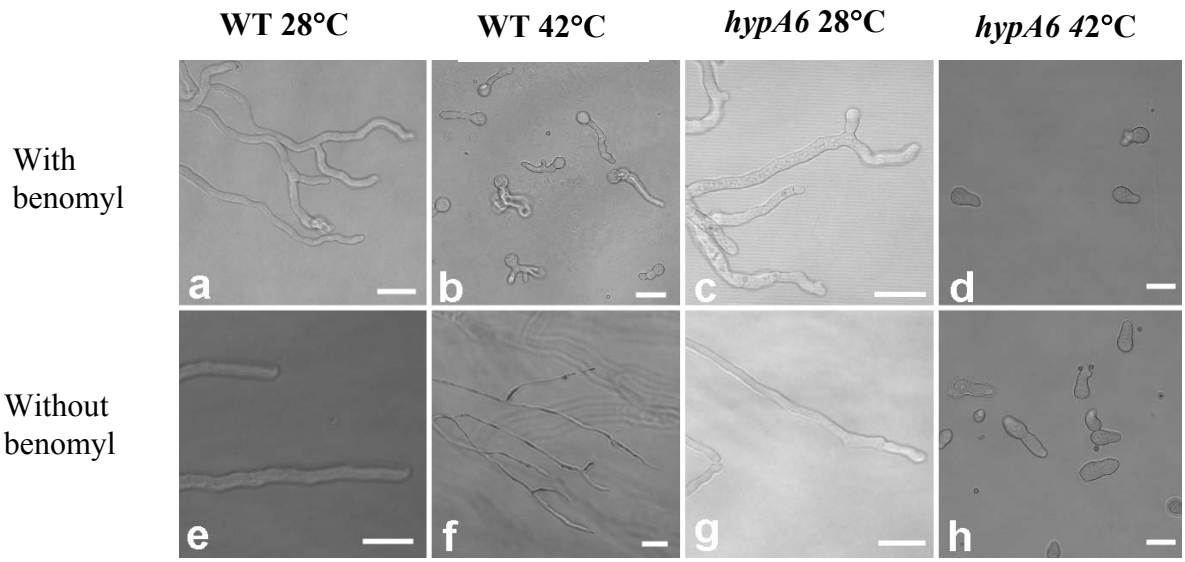


Figure 3.10 Phenotypes of wild type and *hypA6* mutant in the presence of benomyl. Wild type and *hypA6* spores were grown on solid medium at 28°C and 42°C for 9 hr, then incubated with 0.5 µl benomyl (10 mg/ml) on the agar about 2 mm away from the edge of the inoculation for another 3 hr, and germlings were then examined under DIC microscope. The tip growth of wild type germlings treated with benomyl decreased with high branching at both 28°C and 42°C (a, b). While unlike the *hypA6* spores treated with benomyl at 28°C (c), the germlings of *hypA6* treated with benomyl at 42°C (d) did not show obvious increase in branching, but displayed similar spatial pattern of germ-tube emergence as those without benomyl treatment at 42°C (h). Bar: 20 µm.

similar to that of *hypA* mutant in the temperature upshifting experiment, where the hyphal tip cells died 3 h after upshifted to restrictive temperature, and the basal cells enlarged in width without high branching. No significant curly hypha was found compared with that of wild type or *hypA* mutant hyphae incubated with benomyl at 28°C (**Figure 3.11**). The benomyl experiments above indicated that at permissive temperature, the disruption of microtubule polymerazition caused direction changes in *A. nidulans* growing hyphae, resulting curly hyphal, which is consistent with the observation on *N. cressa* (Riquelme *et al*., 1998). However benomyl had less obvious effects on *hypA* temperature sensitive mutants at restrictive temperature.

3.2. Genetic analysis

Since *hypA* function is not fully understood, the study of its alleles can help better understand it. The critical comparison of the two *hypA* temperature sensitive alleles inevitably comes to the comparison of their gene sequences.

The *hypA1* and *hypA6* alleles were amplified using *Pfx* polymerase, and sequenced directly from at least two independent PCR products without cloning. The nucleotide sequences were aligned and compared using BLAST (www.ebi.ac.uk) with that of wild type *A. nidulans* in GenBank (AF001273). A single nucleotide mutation in *hypA1* was shown previously to cause a non-conservative amino acid substitution from *gly*-329 to *arg*-329, G329R, in the predicted protein, which is responsible for the *hypA1* phenotype at restrictive temperature. In this study, the same single base pair lesion as that in *hypA1* was also found in *hypA6*, causing G329R (**Figure 3.12**).

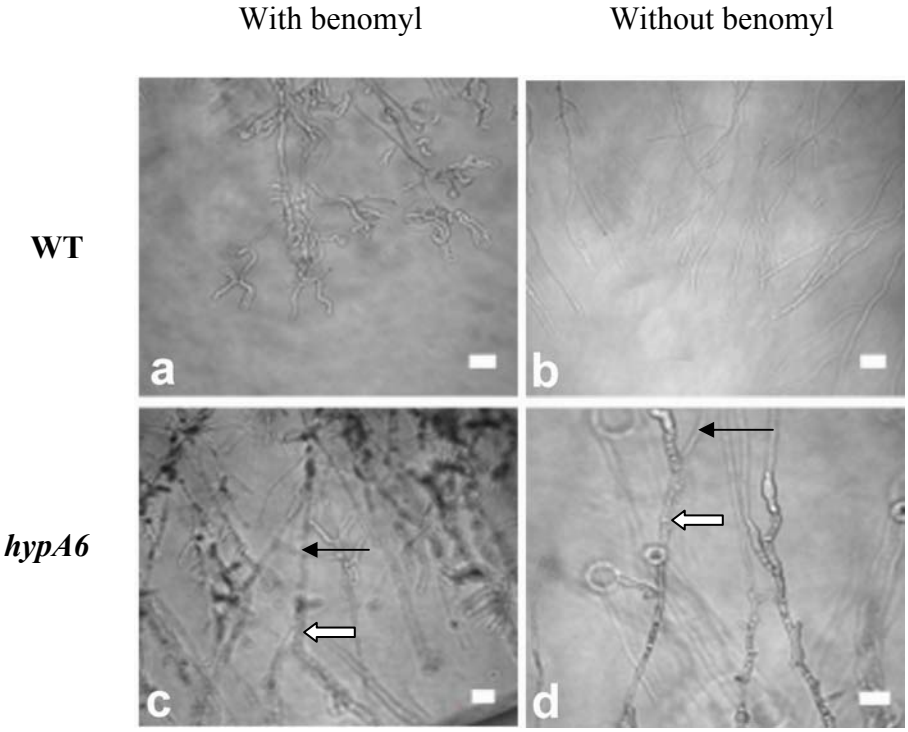
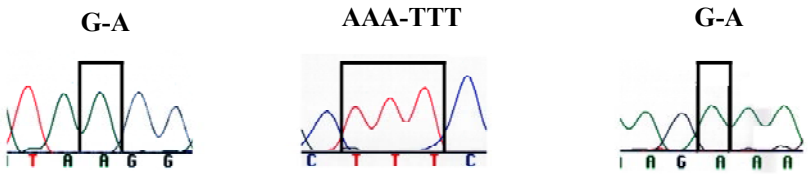


Figure 3.11 Hyphal phenotypes of upshifted wild type and *hypA6* mutant treated with benomyl. Conidia were inoculated on solid medium for 14 hr at 28°C, then upshifted to 42°C and incubated for 3 hr with benomyl put about 2 mm away from the edge of leading hyphae. Under DIC microscope, the wild type hyphae became highly branched and curling (a). But the tip cells of *hypA6* exhibited a phenotype (c) similar to the upshifted hyphae without benomyl treatment (d), the tip cells died (arrows) and the basal cells swelled (block arrows). Bar: 20 μm.



A4 *hypA*+	gtgata**G**GGaca……	agc**AAA**cctatattt…..	acaaag**G**AAgga
A28 *hypA*+	gtgata**G**GGaca…….....	agc**TTT**cctatattt. …..	acaaag**A**AAgga
hypA6	gtgata**A**GGaca……...	agc**TTT**cctatattt…..	acaaag**A**AAgga
hypA1	gtgata**A**GGaca……	agc**TTT**cctatattt. ….	acaaag**A**AAgga
	G329R	**K885F**	**E932K**

Figure 3.12 Genetic lesions in *hypA1* and *hypA6* alleles respective to *hypA* sequence from wild type strains A4 and A28. The gene sequences show that *hypA1* and *hypA6* have same lesions that cause three non-conservative amino acid changes in the predicted *hypA* protein, including G329R, K885F and E932K. The *hypA* sequences from wild type strains A4 and A28 differ in that *hypA* from A28 contains the same nucleotide replacements as *hypA1* and *hypA6* that cause K885F and E932K.

Besides, the *hypA6* sequence revealed another four nucleotide changes respective to the wild type *hypA* in the GenBank, resulting in another two non-conservative amino acid changes in the predicted *hypA* protein, K885F and E932K. Since *hypA1* was not completely sequenced before, for comparison, the sequencing of the whole open reading frame of *hypA1* was performed in this study. Surprisingly, it was found that the two lesions causing K885F and E932K also existed in *hypA1* sequence. Thus, the two *hypA* temperature sensitive alleles, *hypA1* and *hypA6* have identical mutation sites in their structure genes, which is consistent with their indistinguishable restrictive phenotypes at 42°C.

The *hypA* in the wild type strain A28, from which *hypA1* and *hypA6* were mutagenized, were sequenced in regions that contain the mutations found in both *hypA1* and *hypA6*. The result revealed that the *hypA* from wild type strain A28 has identical nucleotide substitutions causing the same amino acid changes, K885F and E932K, as in both *hypA1* and *hypA6*. This is different from the *hypA* sequence in GenBank, which was from the wild type *A. nidulans* strain A4.

3.3 Phenotypic complementation of *hypA6* mutant

The relationship between the amino acid substitution, G329R, in the predicted *hypA* protein and the *hypA6* phenotype was investigated by transforming the *hypA6* strain ASY1 with a ~ 1 kbp *hypA* fragment spanning the G329R lesion site amplified from the wild type strain A28. The wild type *hypA* DNA fragment was amplified using *Pfx* polymerase and gel purified. This fragment was able to repair both *hypA1* and *hypA6* alleles. About 35 wild type colonies were found in totally 10 μg transforming DNA at

42°C. The transformants were checked again for the complementation by wild type phenotypes in coverslip culture at 42°C. The complementation with the wild type *hypA* fragments spanning either K885F or E932K failed. The phenotypic complementation of *hypA6* indicated that G329R contributes to the phenotype of both *hypA* temperature sensitive alleles.

4. DISCUSSION

In the research of this thesis, the study of *hypA* gene function in *Aspergillus nidulans* contains two aspects: the phenotypic and genetic comparison of *hypA1* and *hypA6* mutants, and the fine structural characterization in mutant cells. The results in this study show that the *hypA1* and *hypA6* alleles are identical, that they may function through the endomembrane system, and that cytoplasmic microtubule array changes associated with the *hypA1* and *hypA6* restrictive phenotype are consequences of the altered growth pattern. Nuclear migration may be driven in part by an actin-myosin system in the absence of co-distributed cytoplasmic microtubules.

4.1 *hypA1* and *hypA6* have similar restrictive phenotypes at 42°C

The *hypA* locus was first identified as mutant that resembled wild type at 28°C, but had abnormal hyphae at 42°C (Kaminskyj and Hamer, 1998). The *hypA* locus has two temperature sensitive alleles, *hypA1* and *hypA6*, which were each created by 4-NQO mutagenesis and selected in different studies. In previously published work, the description of the *hypA1* and *hypA6* phenotypes differs. Kaminskyj and Hamer (1998) reported that all the *hyp* mutants, including *hypA1*, could complete their asexual life cycle at restrictive temperature 42°C. In contrast, Harris *et al*. (1999) reported that the growth of *hypA6* at 42°C was arrested a day after incubation. In

order to clarify this contradiction and better understand the *hypA* gene product, the *hypA1* and *hypA6* alleles were compared genetically and morphologically in this research.

At 42°C, both *hypA1* and *hypA6* spores produce short and wide hyphae after a delay of 9 h in germination. Germlings have compact nuclei that are not aligned with the longitudinal axis, and have misplaced septa. The delayed germination indicates a defect in establishing polarity, and the wide, short hyphae indicate the defect in maintaining polarized tip growth at restrictive temperature. When *hypA1* and *hypA6* hyphae are shifted from 28°C to 42°C, the tip cells in both strains cease growth and die within 3 h after the incubation. However, the basal cells begin to swell and eventually branch, indicating a resumption of growth. The branching from the basal cells allows *hypA1* and *hypA6* strains complete their asexual life cycle and finally sporulate at restrictive temperature, which is consistent with Kaminskyj and Hamer (1998) that the *hypA1* defect is not lethal.

After 24 hr incubation at 42°C, the cell membrane of some germlings in both *hypA1* and *hypA6* strains separates from cell wall, a sign of cell dying, although basal cells swell and branch which allows *hypA* temperature sensitive mutants to complete their asexual life cycle and sporulate at restrictive temperature. The cells counted for the numbers of tips and septa in each germling after the incubation at 42°C for 24 hr include both living cells and those are dying and dead already. This may be one of the reasons that the average numbers of tips and septa per germling are different in *hypA1* and *hypA6* although the statistic analysis did not show significant difference. The *hypA1* and *hypA6* strains used in this comparison, the ASK30 and ASH80, have

different nutritional background. Since some nutritional auxotrophic mutants, including *pabaA6*, have effects on phenotypes even though the supplements are fully offered (Serlupi-Crescenzi *et al*., 1983; Kaminskyj, 2001), the other reason for the average number differences of tips and septa per germling between *hypA1* and *hypA6* may be due to *pabaA6*.

The germling branching after a 42°C to 28°C shifting tests the ability of *hypA1* and *hypA6* strains to establish potential sites of polarity at restrictive temperature. Branching began at similar times after downshift for *hypA1* and *hypA6* strains, and similar numbers and positions of branches were produced.

All the phenotypic comparison results suggest that *hypA1* and *hypA6* mutants have similar phenotypes at restrictive temperature, a defect in establishing and maintaining hyphal polar growth, and both strains can complete their asexual life cycle at 42°C.

4.2 *hypA1* and *hypA6* have identical mutation sites

The similar restrictive phenotypes of *hypA1* and *hypA6* alleles at 42°C is explained by their gene sequences: both *hypA1* and *hypA6* sequences are identical.

Compared with the *hypA* sequence in the GenBank (AF001273), a single base pair replacement was discovered in both *hypA1* and *hypA6*, causing a non-conservative amino acid substitution, G329R, in the predicted *hypA* protein. Another two non-conservative amino acid substitutions, K885F and E932K were also found. This was surprising on discovery since 4-NQO mutation typically causes single base pair changes, and since it seemed unlikely that *hypA6* could have two

additional non-conservative amino acid changes that were phenotypically silent. This was clarified by sequencing the region encompassing K885F and E932K in the *hypA1* strain ASK30 and in wild type strain A28. Identical changes of K885 and E932K with respect to the *hypA* sequence in Genbank were present in all these strains. Thus, the two *hypA* temperature sensitive alleles, *hypA1* and *hypA6*, have identical mutation sites, which is consistent with their identical restrictive phenotypes at 42°C. And the chemical mutagensis 4-NQO caused the same single base pair replacement in both *hypA1* and *hypA6* alleles, resulting G329R. Meanwhile, their genetic parent, the wild type A28 strain, has undergone genetic changes causing K885F and E932K with respect to the wild type strain used to create the Brody *et al*. (1991) library. Since these two mutations do not cause phenotypes in A28, they indicate parts of the *hypA* product that are not involved in temperature modulated function.

The *hypA1* lesion was confirmed by repairing the restrictive phenotype using a PCR fragment spanning the lesion site, amplified from wild type A28 *hypA* (Shi *et al*., submitted). This same region could repair the *hypA6* restrictive phenotype. As expected, given the sequence identify in this region from A28, a fragment spanning K885F and E932K did not repair the *hypA1* or *hypA6* defect.

The transformation rates can increase up to 250-fold (Gems *et al*., 1991) when linear or circular DNA is co-transformed with ARp1, derived from plasmid pILJ16 containing an AMA1 fragment, a sequence similar to a *S. pombe* centromere that increase transformation frequency in an unknown way (Osmani *et al*., 1987). Usually, plasmids are not stable in recipient nuclei, and do not integrate into the genome (Gems *et al*., 1991). But in some cases (Tilburn *et al*., 1983; Miller *et al*., 1985), the

transforming DNA became integrated into the genome, and often at homologous sites. Only homologous recombination events regenerate a selectable wild type allele and complement a mutation by expression of the wild type function (Timberlake and Marshall, 1989). In previous research on *hypA*, the *hypA1* phentoype was complemented by transforming a PCR fragment of *hypA* from wild type A28 spanning the lesion G329R. The phenotypically complemented strain was sequenced and found a integrity of the transforming DNA at the homologous site. So in my experiment, the phenotypic complementation of the *hypA* mutants at 42°C is considered resulting from the homologous replacement of the mutation with the wild type *hypA* fragment.

4.3 *hypA* has function in endomembrane arrangement

Cell wall expansion is the ultimate visible manifestation of the cellular machinery that brings polarized growth. In order for the wall in a hyphal tip or a bud to grow, the cell wall biosynthetic enzymes must be deposited at just that location. In fungi these enzymes are synthesized in ER, packaged into vesicles in Golgi equivalent and transported to the cell surface (Bracker *et al*., 1976; Bartnicki-Garcia and Bracker, 1984). The vesicles accumulate at the apex to form the Spitzenkörper whose position determines the growth and shape of hyphae. Fusion of the vesicles with the surface provides new membrane for expansion and enzymes for wall synthesis. Thus polarized growth is an example of polarized exocytosis of endomembrane-derived vesicles.

Since the *hypA* homologue in *S. cerevisiae, TRS120*, regulates Golgi transit, I investigated the effect of *hypA* on endomembrane arrays in *A. nidulans*. In wild type *A. nidulans*, the components in the endomembrane system are arranged in polarized distribution towards the hyphal tip, so that the fusion of the vesicles with the cell membrane and the deposition of the cell wall building materials are precisely controlled. But in *hypA1* and *hypA6* mutant cells at 42°C, the polarized arrangement of the components in the endomembrane system disappears, as does the Spitzenkörper from the hyphal tip. The disappearance of Spitzenkörper from the hyphal tip may allow insertion of the vesicles into the cell membrane randomly, resulting cells enlarged in width and having thicker cell wall as showed in Kaminskyj and Hamer (1998) and Kaminskyj and Boire (submitted). So the *hypA* function results in the loss of polarity of endomembrane arrays.

4.4 *hypA1* and *hypA6* mutants have normal cytoplasmic microtubule arrangement and mitosis at 42°C

Cytoplasmic microtubules have usually been thought to participate as a cytoskeleton and/or by mediating subcellular organization and intracellular transport as they are present throughout the subapical cytoplasm. Many reports suggest that microtubules transport different orgalleles in different species, which raise the possibility that microtubules function in association with other cytockeletal components, actin filaments and intermediate filaments (Gow, 1989). But how do cytoplasmic microtubules distribute in cells? Experiments using chemical fixed TEM failed to find any cytoplasmic microtubules in cells (Kaminskyj and Boire,

submitted), which could be due to their relative rarity, or to instability during fixation that would have been slowed due to the thick cell wall. In this study, a GFP-tagged alpha tubulin strain was used to investigate *in vivo* microtubule localizations, and this can offer us with more information on hyphal growth or nuclear distribution in *hypA1* and *hypA6* temperature sensitive mutants.

In *A. nidulans*, microtubules are important for nuclear distritution (Morris, 1976) and hyphae in wild type *A. nidulans* have several long cytoplasmic microtubules that extend nearly to the tip. Some interact with Spitzenkörper region, but relatively loosely (Kaminskyj and Heath, 1996) compared to *Allomyces* (Roberson and Vargas, 1994; Vargas *et al*., 1993). The cytoplasmic microtubules displayed expected wild type pattern along the hyphal axis at 28°C. But at 42°C in *hypA1* and *hypA6* cells, the cytoplasmic microtubule population is severely reduced, usually only one at any longitudinal position. The increased *hypA* width in the restrictive phenotype is not associated with an increase in cytoplasmic microtubule population, which is consistent with the TEM observation by Kaminskyj and Boire (submitted). Despite the reduced cytoplasmic microtubule population, its distribution is parallel with hyphal axis at 42°C, suggesting that the restrictive microtubule distribution is a response to reduced growth. The disappearance of the FM4-64 stained Spitzenkörper from the hyphal tip of *hypA1* and *hypA6* at 42°C maybe also result from the reduced cytoplasmic mirotubule population. The normal distritution of cytoplasmic microtubules in *hypA1* and *hypA6* mutant cells is consistent with the benomyl experiment, where the depolymerization of microtubules has no significant effect on their phenotypes at 42°C.

Cytoplasmic microtubules are important for nuclear distribution in *A. nidulans* (Morris, 1976). But most nuclei in *hypA1* and *hypA6* mutant cells at 42°C are not found along the hyphal axis, where the microtubule is formed. However, the nuclei distribution is even throughout cells, which suggests there must also have some actin-based component contributing to nuclear distribution in *hypA1* and *hypA6* mutant cells at restrictive temperature.

Similar to cytoplasmic microtubules at 42°C, the formation of spindles in *hypA1* and *hypA6* strains during mitosis is not obviously affected. The observation of the spindle morphological changes were similar to that describted by Robinow and Caten (1969) using electron microscopy, who found that the fibres were mitotic spindles by using acid fuchsin and HCl-Giemsa to stain the spindles and chromosomes, respectively. The granules were formed in the regions where the spindle pole bodies (SPB) should be located. The time required for mitotic progression is consistent with that of the mitosis during the cell cycle in wild type *A. nidulans* calculated by Bergen and Morris (1983).

Consistent with Robinow and Caten (1969), although the division of all the nuclei is synchronous within a single cell, however, the synchrony is not perfect and a sequence in the microtubule morphologic change existed in the cell, where in my experiment includes the different shape of the granules, a indicating of separation of the SPB (Robinow and Caten, 1969; Prigozhina *et al*., 2001), and the time when spindles appeared were different.

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