Distribution and role of calmodulin in tip growing hyphae of *Saprolegnia ferax*[1]

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ABSTRACT

Since direct staining of growing hyphae with trifluoperazine (TFP) could result in the migration of protoplasm in hyphae of water mold *Saprolegnia ferax*, an improved staining method for fixation of growing hyphae was developed. When hyphae of *Saprolegnia ferax* were pre-fixed with paraformaldehyde, the TFP fluorescence showed a clear tip-high Ca^{2+}-activated calmodulin (CaM) gradient. By indirect immunohistochemical staining, we proved that total CaM also mainly concentrated in the apex of hyphae. It is noticeble that when the hyphal tip growth ceased, the high intensity of TFP fluorescence usually disappeared in non-growing tips and became evident in the newly emerged growing tips, which implied that the concentration of CaM shifted in concord with the transfer of growing point in hyphae. 2×10^{-5} mol /L CaM inhibitor TFP could reduce the tip growth rate and the percentage of growing hyphal tips to about 50%, and induce multibranching of hyphal tips. These findings strongly suggest that the high concentration of calmodulin in the hyphal tip may be involved in regulation of hyphal tip growth.

Key words: *Calmodulin, calcium, tip growth, trifluoperazine, Saprolegnia ferax.*

INTRODUCTION

Fungal hyphae extend by tip growth and have a higher concentration of Ca^{2+} in their apices than their bases, a fact which is probably related to the importent role played by calcium in establishing and maintaining apical organization, morphogenesis, and growth[1-6]. To understand the the function of Ca^{2+} in hyphal tip growth, Ca^{2+}-binding proteins must be identified and their functions determined. CaM, a ubiquitous intracellular Ca^{2+}-binding protein which functions to mediate many Ca^{2+}-regulated processes in cells, naturally has been received a lot of attention. Some mycologists suggest that CaM may be involved in hyphal tip growth as shown by the effects of the CaM inhibitors, the phenothiazines[7-9]. In the plant kingdom, tip-growing cells, such as pollen tubes and root hairs, have been proved to have high concentration of CaM in their apices, which was considered to be linked to the modulation of tip growth[10, 11]. However, in the myceteae kingdom, so far almost no one reported the distribution of CaM in the hyphal tips, excepted that of HauBer et al., who observed a non-polar distribution of CaM in hyphae stained directly with phenothianzine drugs[10]. So more data are needed to confirm and clarify the distribution and role of CaM in tip of growing hyphae.

MATERIALS AND METHODS

Culture

Saprolegnia ferax (Gruith) Thuret (ATCC No. 36051) was grown on dialysis membrane overlying an agar medium designated as OM (adjusted to pH 6.8 with KOH) by Heath and Greenwood[12]. Squares of membrane bearing hyphal tips were cut from the growing edge of the colony and allowed to recover for 30 min for further experiments[13].

Localization of CaM

(1) Immunohistochemical localization was done by the modified methods of Lin et al[11] and Suryanarayana and Thomas[14] and briefly was as follows: the growing hyphae were fixed immediately with 3% paraformaldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.4) containing 0.002% $CaCl_2$ for 6-7 h, washed in PB three times (10 min each); rinsed with 0.025 mol/L hydroxylamine for 30 min, washed; fixed onto 1% gelatin-coated slides, air-dried overnight, rinsed with lysis medium (0.59 mol/L sorbitol, 0.05 mol/L Na_2EDTA, 0.05 mol/L NaH_2PO_4, 0.00014 mol/L β-mercaptoethanol, 0.001 mol/L PMSF, pH 6.5); treated for 30 min with 2 mg/ml driselase in lysis medium, washed; permeabilized for 30 min with cold methanol (-20 ℃), washed; rinsed with 4% H_2O_2 in PB for 10 min, washed; rinsed with normal chicken serum (at 1:100 dilution) for 10 min, incubated with rabbit antiserum against pig brain calmodulin (1:50 dilution) for about 40 h at 4℃, washed; incubated with peroxidase-conjugated protein A (*Staphylococcus aureus*, 1:40 dilution) for 1 h, washed; refixed with 2.5% glutaraldehyde in PB for 10 min,washed; incubated with 0.05% 3, 3-diaminobenzidine-4 HCl in PB (pH 7.2) plus 0.01 % H_2O_2 for 2 min, washed; mounted with 50% glycerine in PB, sealed by fingernail polish and examined with a Olympus BH-2 light microscope. For control experiments, the hyphae were incubated with pre-immune serum or with antiserum against pig brain CaM which had been previously absorbed with excess antigen (100 μg/ml pig brain CaM).

(2) TFP fluorescent localization was done by a modified HauBer's method[10]: the hyphae on the membrane were mounted in liquid OM in a slide chamber and fresh OM was drawn through it

for 30 min to allow the cells to resume growth[13] unless elsewhere indicated. The growing hyphae were fixed by flowing approximately 0.3 ml 5% glutaraldehyde in 1/15 mol/L phosphate buffer (pH 7.0) through the chamber for 10 min, followed by drawing the same volume of 10^{-4} mol/L TFP in OM through it for a further 10 min, and then examined with a Nikon Labophot microscope using CF fluor objectives with phase-contrast and epi-fluorescence optics with a UV filter block (EX330-380, DM400, A420). As a control, hyphae fixed with glutaraldehyde were treated with OM instead of TFP for 10 min. All micrographs were recorded with Ilford HP5 plus (ASA 400) black and white films processed with D-76 Developer.

Effects of TFP on hyphal tip growth

Growing hyphae were flowed with TFP in OM at 0, 10^{-6}, 10^{-5}, 2×10^{-5}, 5×10^{-5} mol/L through the chamber for 30 min and then the growth rate was measured by the examination of the changes of the length of individual hyphal tips, selected randomly, 2 min apart with an ocular micrometer. The percentage of growing hyphal tips in total examined hyphal tips at various concentrations of TFP in OM was monitored by counting the numbers of growing and non-growing hyphal tips under the microscope. Morphological changes of hyphal tips were photographed with the Nikon Labophot microscope as described.

Chemicals

Rabbit antiserum against pig brain CaM and peroxidase-conjugated protein A (Staphylococcus aureus) were gifts from Professor Zhou Xi of the Department of Agronomy of Nanjing Agricultural University. Some supplies were obtained as follows: TFP (sigma), glutaraldehyde (E. Merck), driselase (Kyowa Hakko Kogyo Co., Ltd, Tokyo).

RESULTS

When growing hyphae were fixed with glutaraldehyde and then stained with 10^{-4} mol /L TFP, they showed a clear TFP fluorescence gradient from tip to base (Fig 1). The most intense fluorescence of TFP mainly existed in the hyphal tip. Beyond 30 μm of the hyphal tip the fluorscence became very weak and uniform. It was very interesting to note that if hyphae were only allowed to recover for 10 min, but not 30 min, prior to fixation and staining (see materials and methods) in order to show the very short emerging growing tip, TFP fluorescence was mainly concentrated in newly emerged growing tips, but not in non-growing tips resulted from the transfer of the hyphae to the slide (Fig 2). If hyphae were stained directly with 10^{-4}mol/L TFP without fixation, TFP immediately stopped the tip growth, and caused the redistribution, backward contraction or vacuolization of cytoplasm so that the TFP fluorescence gradient became disturbed and the apical concentration of fluorescence in hyphal tips was no longer evident (data not shown). In the control experiment, glutaraldehyde-fixed hyphae treated with OM, instead of TFP in OM, did not give any detectable autofluorescence (Fig 3).

By Immunohistochemical localization, we confirmed that the above TFP fluorescence patterns did really reflect the distribution of CaM in hyphae. When the hyphae were stained with antiserum against pig brain CaM, reaction product was found mainly in the hyphal tip, and appeared as a gradient of brownish product from tip to base (Fig 4). A similar result was also observed in the branching hyphal
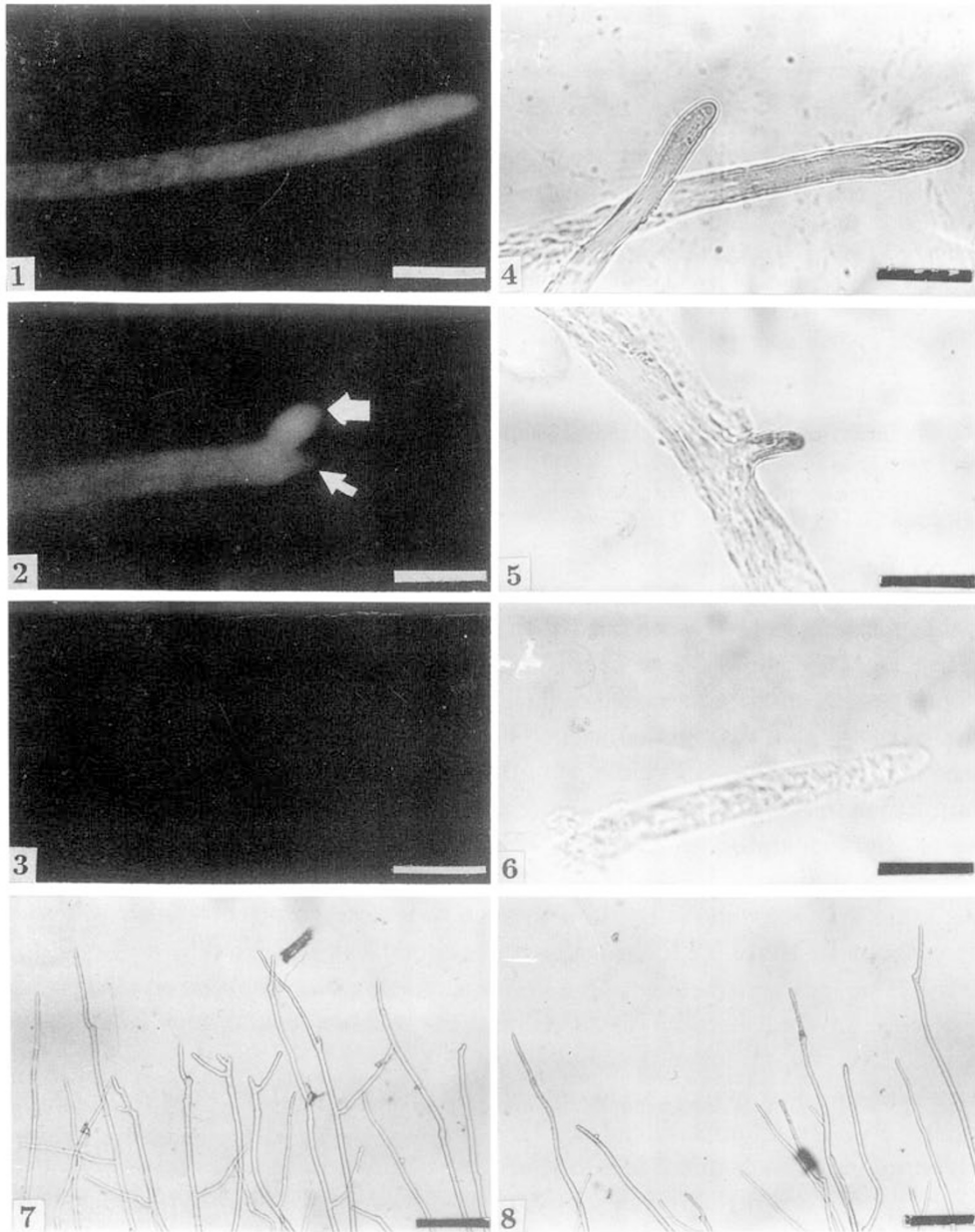
tip (Fig 5).   In the control experiment, when hyphae were incubated with pre-immune serum (or with antiserum against CaM which had been pre-absorbed with 100 μg/ml pig brain CaM), no reaction product was found (Fig 6).

The tip growth of hyphae of Saprolegnia ferax was found to be affected by TFP. The tip growth rate of individual hyphae and the percentage of growing hyphal tips were inhibited by the concentration of TFP above 10^{-6} mol/L (Tab 1). At 2×10^{-5} mol/L TFP, the growth rate of hyphae slowed down to about 50%. 5×10^{-5} mol/L TFP could immediately terminate the tip growth of hyphae. TFP also affected the morphology of hyphae and induced multibranching of hyphae. The numbers of branching tips under 2×10^{-5} mol/L TFP were greatly increased than that of the control (compare Fig 7, 8).

DISCUSSION

CaM inhibitor phenothiazines drugs such as TFP could be used as fluorescent probes for calmodulin to determine Ca^{2+}-CaM site in cells[15]. HauBer et al. (1984)[10] even reported that CaM shown by phenothiazine fluorescence did not appear as a gradient from tip to base in growing hyphae of water mold Achyla sp., quite different from tip growing cells of plant such as pollen tubes and roots hairs. However, we noticed that the materials used for the experiments made by HauBer et al were growing hyphae without pre-fixation, a condition which might lead to the possibility that the fluorescence pattern of phenothiazines reported by them did not represent the normal distribution of CaM in growing hyphal tips. Actually, as we have mentioned in the section on Results, the direct staining of hyphae with TFP could cause the redistribution and backward contraction or vacuolization of cytoplasm, leading to the disturbance of the true TFP fluorescent pattern of hyphae. An improved staining method for fixation of growing hyphae was, therefore, developed. When hyphae were pre-fixed with glutaraldehyde, migration of protoplasm did not

$\lhd$Fig 1. TFP staining pattern of growing tip of hyphae pre-fixed with glutaraldehyde, $\times 630$. bar = 20 μm.

Fig 2. TFP staining pattern of hyphae pre-fixed with glutaraldehyde but only recovered for 10 min before fixation and staining which showed that the fluorescence disappeared in the non-growing tip (small arrow) and appeared in the emerging growing tip (thick arrow) of hyphae, $\times 630$. bar = 20 μm.

Fig 3. Autofluorescence patterns of hyphae pre-fixed with glutaraldehyde, $\times 630$. bar = 20 μm.

Fig 4. Hyphal tip stained with antiserum against CaM. $\times 630$. bar= 20 μm

Fig 5. Branching hyphal tip stained with antiserum against CaM. $\times 630$. bar = 20 μm.

Fig 6. Hyphal tip stained with pre-immune serum, $\times 630$. bar = 20 μm.

Fig 7 Multibranching hyphae caused by incubating with 2×10^{-5} mol/L TFP for 30 min. $\times 50$. bar = 200 μm.

Fig 8. Normal growing hyphae without treatment of TFP.$\times 50$. bar = 200 μm

occur and TFP fluorescence appeared in a gradient pattern from tip to base in growing hyphae of water mold *Saprolegnia ferax* (Fig 1, 2). Heath[16] recently pointed out that growing hyphae are extremely labile and processes of staining and observation could easily and rapidly halt growth and thus reveal patterns which are not necessarily characteristic to normal tip growth. Therefore, it seems necessary to pre-fix growing hyphae with fixative to prevent protoplasm movement when the fluorescent probes used for cytochemical localization are toxic to hyphae.

Considering phenothiazine drugs could only give the information of Ca^{2+}-activated CaM site and may be not completely specific to CaM[17], we further determined the distribution of total CaM in tip growing hyphae by immunohistochemical method, which could be used for the localization of both inactivated and Ca^{2+}-activated CaM sites[18]. Thus, a gradient of brownish reaction product from tip to base in hyphal tips was observed (Fig 4, 5), revealing the concentration of total CaM mainly in the apex of hyphae. It is interesting to note that, for the first time, we found that the distribution of tip-high concentration of CaM was correlated with the transfer of growing point in hyphae. When the hyphal tip growth ceased, the high intensity of TFP fluorescence usually disappeared in non-growing tips and became evident in the newly emerged growing tips (Fig 2). Thus it is apparent that CaM is related to tip growth of hyphae. This conclusion is further supported by the inhibition of TFP to CaM-mediated activation through the Ca^{2+}-dependent interaction of TFP with CaM. We have demonstrated that TFP could reduce the tip growth rate and the percentage of growing tips(Tab 1). Meanwhile, TFP also induced multibranching of hyphae (Fig 7) of the Oomycetes Saprolegnia ferax, which has not been a reported before.

Tab 1. Effects of TFP on hyphal tip growth of *Saprolegnia ferax*

Treatment	n *	Growth rates (μm/min)			Percentage of growing hyphal tips		
		Mean $\pm$ SD.	Inhibition (%)		No. of total tips	No. of growing tips	Percentage of growing tips (%)
control	23	14.95 $\pm$ 1.90	0		45	39	86.7
10^{-6} mol/L TFP	25	11.36$\pm$ 2.15	24.0		30	15	50.0
10^{-5} mol/L TFP	25	10.00$\pm$ 2.49	33.1		30	12	40.0
2×10^{-5} mol/L TFP	31	7.71 $\pm$ 3.29	48.4		45	18	40.0
5×10^{-5} mol/L TFP	30	0	100.0		45	0	0

* n: The number of examined growing hyphal tips selected randomly under the microscope.

If CaM plays an importent role in hyphal tip growth,the followings are possible mechanisms of action: (1). CaM may be involved in secretory system in the extreme apex of hyphae and regulates the formation of the new cell wall. It is known

that the most apical 1-2 μm area of the tip is filled almost exclusively by a high density of Golgi body derived secretory vesicles known as wall vesicles, which bring to the cell surface raw materials (precusors, products, enzymes) needed to construct the cell wall for the maintenance of hyphal tip growth by exocytosis[19]. CaM has been implicated in numerous secretory systems[20], including cellulase secretion by *Trichoderma reesii*[21], and possibly in association with wall-hydrolysing enzymes of water mold Saprolegnia ceous[14]. (2) CaM may also control the extensibility of the actin cap in hyphal tips. It is clear that F-actin is concentrated in growing hyphal tip and forms a cap-like structure to resist the turgor pressure for supporting of hyphal tip growth[16]. In turn, the function of actin is regulated by CAM[22].

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