Fungal Genetics and Biology 48 (2011) 512–518

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

An investigation of the effects of Ca^{2+} channel inhibitors on branching and chemotropism in the oomycete *Achlya bisexualis*: Support for a role for Ca^{2+} in apical dominance

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ARTICLE INFO

Article history:
Received 11 October 2010
Accepted 28 October 2010
Available online 2 November 2010

Keywords:
Branching
Chemotropism
Ca^{2+} channel inhibitors
Verapamil
Gd^{3+}
La^{3+}
Oomycete
Achlya bisexualis
Hypha
Apical dominance

ABSTRACT

In an attempt to better understand branching and chemotropism, we describe the effects of Ca^{2+} channel inhibitors on these processes in *Achlya bisexualis*, using a branch induction technique and whole plate assays. Branching appears to be a two step process with the initial formation of a bump from which a branch emerges. Verapamil increased numbers of branches in whole plate assays and decreased the distance from the first branch to the tip. In induction assays verapamil increased the number of bumps formed, although in some hyphae it inhibited the transition from an initial bump to a branch. When a branch formed it did not affect the time taken to branch. It had no effect on chemotropism. Lanthanum (La^{3+}) and gadolinium (Gd^{3+}) also increased branching in whole plate assays but their effect was much less marked and they had no effect on bump/branch number in induction assays. Gd^{3+} decreased the time taken to branch. Both La^{3+} and Gd^{3+} increased chemotropism. These data suggest firstly that the respective inhibitors may affect different parts of the branching process and secondly that Ca^{2+} influx through channels may not be a requirement for branching, indeed such movements may suppress branching. This would fit with elevated Ca^{2+} at the tip playing a role in apical dominance.

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1. Introduction

Oomycete and fungal mycelia are hyphal collectives which colonise their substrata by tip growth and branching. These processes enable the continual searching for, and acquisition of, nutrients. In their quest for nutrients, hyphae can put out new hyphal tips that can then extend by the process of tip growth (the process of branching), or can reorient their direction of growth (the process of chemotropism) toward a particular nutrient source. The highly polarised nature of hyphae means that studies of tip growth, chemotropism and branching are likely to provide useful insights into the mechanisms that underlie the generation, maintenance and dynamics of cell polarity. While tip growth has been extensively studied, the process of branching and chemotropism are less well understood.

Branching can take several forms and be influenced by a number of factors, both internal and external. The most common form of branching is lateral branching, in which a branch forms on the distal wall of the hypha. These lateral branches may be associated with a septum and it is thought that the septum and associated proteins may provide a spatial cue for the branching process. A lateral branch can also appear in a seemingly random position, although again, there are likely to be spatial cues that determine where the branch will occur. The exact nature of these mechanisms is currently unknown, although localised increases in Ca^{2+} have been suggested as a possibility. Calcium has also been suggested as a possible factor in determining apical dominance suppressing the formation of branches close to the hyphal tip.

Data indicate that that branching in oomycetes and fungi may be similar processes (Harris, 2008). While more is known about branching in fungi the oomycetes are a good system to study random lateral branching in as they do not have septa, indeed many of the earlier studies on branching concentrated on the oomycete *Achlya ambisexualis* (Thomas, 1970; Barksdale, 1970; Mullins, 1979; Harold and Harold, 1986). There is a rich body of data describing tip growth in this species, furthermore the relatively large diameter of its hyphae also means that they are particularly amenable to physiological studies.

In order to carry out physiological studies on the sequential mechanisms that underlie branching, accurate determination of the site and timing of branching is required. As this has proven to be a difficult task probably the best experimental approach is

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doi:10.1016/j.fgb.2010.10.008

to use a branch induction technique. One method is the use of localised UV irradiations which, in *Saprolegnia ferax*, invoke increases in cytoplasmic Ca^{2+} at sites where branches then subsequently appear (Grinberg and Heath, 1997). This has led to the suggestion that an increase in Ca^{2+} is necessary for the accumulation of branching initiation factors. An alternate approach is to use micropipettes for the localised application of amino acids which avoids any damage to hyphae by UV irradiation (Schreurs et al., 1989). Using this technique Schreurs et al. (1989) found that branching was affected neither by changes in external Ca^{2+} nor by the inclusion of the Ca^{2+} channel blocker La^{3+}. This argues against an increase in Ca^{2+} playing a role in branching. This observation fits with a possible role of Ca^{2+} in determining apical dominance; where a tip-high gradient of Ca^{2+} is thought to repress the formation of new branches in the vicinity of the tip. Clearly more work is needed to clarify any role that Ca^{2+} may play in branching.

Chemotropism has been suggested to be related to branching in that hyphal tips reorient towards a supply of nutrients in a similar way to branches that form in response to localised application of amino acids (Schreurs et al., 1989). This is supported by observations on *Achlya bisexualis* which show a chemotropic response to mixtures of amino acids (Manavathu and des Thomas, 1985; Musgrave et al., 1977), while *Phytophthora is also known to* grow chemotropically toward host plant roots, extracts or exudates (Tyler, 2002). Little is known of the mechanisms that underlie the process in oomycetes although tropic responses in fungal species such as *Candida albicans* and *Aspergillus niger* are thought to involve Ca^{2+} signalling, GTPases and microtubules (Brand and Gow, 2009).

In view of the uncertainties surrounding the role of Ca^{2+} in the induction of branching and in chemotropism we have modified the technique of (Schreurs et al., 1989) and used it to study the effect of Ca^{2+} channel inhibitors on branching and chemotropism. Plasma membrane Ca^{2+} fluxes can be inhibited by a number of ion channel inhibitors; these include the inorganic lanthanides, La^{3+} and Gd^{3+}, which inhibit stretch activated ion channels and the organic phenylalkylamine verapamil, an inhibitor of L-type Ca^{2+} channels (Hille, 2001). Our data indicate that these inhibitors affect different aspects of lateral branching that Ca^{2+} influx through channels may suppress branching.

2. Materials and methods

2.1. Organism

Hyphae of the oomycete *A. bisexualis* (a female strain isolated in New Zealand from *Xenopus laevis* dung and available from the University of Canterbury culture collection) were grown as described previously (Yu et al., 2004), with the exception that $DMA_{0.06}$ medium (Schreurs et al., 1989) was used rather than PYG medium. This was necessary to control the amino acid concentrations that hyphae were exposed to, PYG being an undefined medium. $DMA_{0.06}$ medium contains KPIPES (piperazine-N-N′-bis[2-ethane-sulphonic acid]), 1.0 mM; KH_2PO_4 and K_2HPO_4, 0.5 mM each; glucose, 10 mM; $MgC1_2$, 1.0 mM; $CaC1_2$, 0.5 mM; H_3BO_3, 11 μM; $MnSO_4$, 1.8 μM; $CoSO_4{\cdot}7H_2O$, 0.7 mM; NaMoO, $2H_2O$ and $ZnSO_4{\cdot}7H_2O$, 0.4 mM each; $CuSO_4{\cdot}5H_2O$, 0.3 μM; glutamic acid, 0.027 mM; methionine, 5 μM; isoleucine, leucine, threonine, valine, and lysine, 4 μM each; glycine, arginine, phenylalanine, tyrosine, and serine, 2 μM each; histidine, 1 μM and tryptophan, 0.5 μM. The pH was adjusted to 6.5 with KOH. For experimental purposes, mycelial mats growing on cellophane overlying $DMA_{0.06}$ media were excised 1 cm behind the growing margin and placed hyphal side down in welled slides. The cellophane was then gently removed and hyphae were overlaid with five drops of 2% $DMA_{0.06}$ agar. The agar was left to solidify for 3 min after which six drops of liquid $DMA_{0.06}$ was added on top of the agar. Hyphae were left for a minimum of 1 h to resume growth prior to branch induction.

2.2. Micropipette fabrication

Micropipettes were pulled from borosilicate glass capillaries (outer wall 1 mm × inner wall 0.58 mm with an inner filament) (Clark Electro-medical Instruments, Reading UK) using a Narashige PC-10 pipette puller. To ensure that the amount of induction medium applied in each trial was relatively constant, only pipettes that had a bubble number (Lew et al., 1990) of 24 (tested using a 60 cm^3 syringe) were used.

2.3. Branch induction

Branches were induced using an induction medium comprising 1 mM phenylalanine made in liquid $DMA_{0.06}$ as described in Jackson et al. (2001). The induction medium was placed in a micropipette which was then inserted into a water filled pressure micro-injector and put under gentle pressure. This was then placed 10 μm away from the hypha about 50 μm back from the growing tip using micromanipulators. This point was taken as the start of the induction experiment. Control experiments suggested that it was the induction media itself and not the simple presence of the micropipette that induced branching.

Inhibitor experiments were carried out as described for the branching induction trials except that $GdCl_3$, $LaCl_3$ or verapamil were also included in the induction medium at the required concentration. At the concentrations tested, none of the inhibitors had a significant effect on the rate of tip growth of the hypha, mean values (±SD) were 7.5 ± 3.1 μm min^{-1} (n = 22) (control), 7.8 ± 2.2 μm min^{-1} (n = 12) (Gd^{3+}), 7.7 ± 3.4 μm min^{-1} (n = 11) (La^{3+}) and 5.4 ± 2.2 μm min^{-1} (n = 12) (verapamil). Images were collected using a Bio-Rad MRC1024 confocal microscope (Bio-Rad, Mississauga, Ontario, Canada).

2.4. Whole plate inhibitor experiments

In addition to the branch induction inhibitor experiments, the effects of verapamil, Gd^{3+} and La^{3+} on numbers of branches formed per unit length of hypha on whole plates were determined. One cm^3 plugs taken from stock plates were inoculated onto Petri dishes made up with $DMA_{3.2}$ agar medium (Kropf et al., 1984) to which the inhibitor (at concentrations ranging from 5 μM up to 1 mM) had been added. $DMA_{3.2}$ medium contains KPIPES (piperazine-N-N′-bis[2-ethane-sulphonic acid]), 1.0 mM; KH_2PO_4 and K_2HPO_4, 0.5 mM each; glucose, 10 mM; $MgC1_2$, 1.0 mM; $CaC1_2$, 0.5 mM; H_3BO_3, 11 μM; $MnSO_4$, 1.8 μM; $CoSO_4{\cdot}7H_2O$, 0.7 mM; $NaMoO{\cdot}2H_2O$ and $ZnSO_4{\cdot}7H_2O$, 0.4 mM each; $CuSO_4{\cdot}5H_2O$, 0.3 μM; glutamic acid, 1.36 mM; methionine, 0.22 mM; isoleucine, leucine, threonine, valine, and lysine, 0.20 mM each; glycine, arginine, phenylalanine, tyrosine, and serine, 0.10 mM each; histidine, 0.05 mM and tryptophan, 0.02 mM. The pH was adjusted to 6.5 with KOH. $DMA_{3.2}$ medium was used as hyphae form more branches when grown on this than compared to the $DMA_{0.06}$ medium used in the branch induction assays. After 48 h growth, plates were observed under an Olympus BH-2 microscope. Fifty to one hundred hyphae were selected and the numbers of branches formed over 1000 μm lengths back from the tip were counted.

2.5. Ca^{2+} imaging

For Ca^{2+} imaging, hyphae were initially grown in a welled microscope slide for at least 1 h in $DMA_{0.06}$ medium containing 1.25% sorbitol. As *A. bisexualis* is unable to turgor regulate (Lew et al., 2004) the sorbitol reduces turgor, making injection of Ca^{2+}

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