Full Length Research Paper

The role of microtubules in the growth of pollen tube tip

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Pollen grains were sown on agar germination media containing increasing concentrations of the following disruptors, oryzalin and colchicines. Germination tube length was examined and results were evaluated. Treatment with oryzalin caused a slight but significant reduction in pollen germination while colchicines did not affect germination. All the microtubules (MTs) disruptors caused a decline in pollen tube length. The concentration that caused a significant reduction in tube length was 100 µM for oryzalin and 500 µM for colchicines. The effectiveness of these inhibitors varied. At the highest tested concentrations, oryzalin was more effective than colchicines in reducing tube length. In addition, MT disruptors induced tube curving. Curving was caused by disruption of the microtubules in the tip. We concluded that microtubules were involved in tip growth of pollen tubes. Therefore, the functional MT configuration is essential for the pollen tube to exert the penetration force.

Key words: Pollen germination, pollen tube growth, inhibitor treatments, gelling agent.

INTRODUCTION

The plant cytoskeleton

The cytoskeleton is involved in many dynamic processes such as cell division and cell morphogenesis. The plant cytoskeleton consists of microtubules, microfilaments and intermediate filaments.

Composition and formation of microtubules in the cells

Microtubule formation is a process of polymerization: The first stage of formation is called nucleation. The process requires tubulin, Mg²⁺ and GTP. This stage is relatively slow until the microtubule is initially formed. Then, the second phase, called elongation proceeds much more rapidly.

Tip growing cells

In plants and fungi, there are several different examples of cells that exhibit polarized growth, including pollen tubes, root hairs, fern-moss protonema, algal rhizoids, and fungal hyphae. The uniaxial extension permits the cell to explore the local environment. The pollen tube is usually well provisioned and its function is directed towards sexual reproduction and its explorations are focused on finding of the ovule and delivering the sperm so that fertilization can occur (Lord, 2000).

Configuration of microtubules in the tip growing cells

In root hairs and pollen tubes, MTs are longitudinally oriented and sometimes adopt slight helical distribution (Geitmann and Emons, 2000), microtubules in root hairs extend close to the tip of the cell where they become randomly oriented, whereas, in pollen tubes, they are absent from the apical domain. In the tip-growing Medicago trunculata root hairs, cortical MTs are present in all developmental stages while endoplasmic MTs are a specific feature of growing hairs in which they form a three-dimensional array throughout the sub-apical cytoplasmic dense region (Björn et al., 2002).

The role of microtubules in the growth of the tip growing cells

As far as pollen tube is concerned, it was shown that its elongation is dependent on the operation of the secretion
pathway which includes:

1. Synthesis of proteins and polysaccharides in the endoplasmic reticulum.
2. Their modification in the dyctiosomes.
3. Their transport in vesicles to the plasma membrane to which they fuse.

Microtubules (MTs) and microfilaments (MFs) are involved in the transport of secretory vesicles to the apical plasma or in the orientation of the organelles of the secretory pathway towards the tube apex with the help of microtubule motors kinesine and dynein. Furthermore, the role of MTs in the transport of the vegetative nucleus (VN) and in the generative cell (GC) has been demonstrated as the movement of the VN and GC from the basal parts into the tip region of the tube was significantly slowed down in the absence of MTs (Raudaskoski, 2002).

**Interference with MT functioning by drug application**

Anti-microtubule drugs have contributed to the understanding of the functions of the microtubule cytoskeleton in morphogenetic programmes of oriented cell division and differentiation; thus, they remain powerful probes of microtubule function. The most commonly used plant microtubule depolymerization compounds are colchicine, and several synthetic herbicides belonging to three different chemicals classes, the dinitroanilines, phosphoric amides, and N-phenyl carbamates. Taxol, a secondary plant product, is the only drug found to promote the polymerization of plant microtubules (Morejohn, 1991).

**Colchicine**

Colchicine blocks the polymerization of microtubules while depolymerisation goes on. The plant alkaloid colchicine exerts characteristic antimitotic activity by binding specifically to the dimeric protein tubulin, an important constituent of the mitotic spindle. Colchicine binding to tubulin results in inhibition of microtubule assembly both *in vitro* and *in vivo*. The binding is slow, non-covalent, poorly reversible and occurs with a stoichiometry of one mole of colchicine per mole of the tubulin dimer. Both tubulin and colchicines undergo characteristic conformational changes upon interaction, which are often used as probes to monitor the progress of the reaction (Bose, 1997).

**Oryzalin**

Oryzalin, a dinitroaniline herbicide, was previously reported to bind to plant tubulin with a moderate strength interaction that appeared inconsistent with nanomolar concentrations of drug that causes the loss of microtubules, inhibits mitosis, and produces herbicidal effects in plants.

Oryzalin binds rapidly, reversibly and with affinity to the plant tubulin dimer to form a tubulin-oryzalin complex such that, at concentrations sub-stoichiometric to tubulin, it copolymerizes with unliganded tubulin and slows further assembly (Morejohn, 1993). Also, it has been indicated that sub-stoichiometric concentrations of the tubulin-oryzalin complex bind either ends of the microtubules or cause their depolymerization. Possibly, oryzalin impedes the formation of new microtubules because of the reduction of the tubulin content.

In the elongating pollen tubes of *Picea abies*, oryzalin causes linear decrease in elongation. It disrupts MTs preferentially at the tip and thus, induces tip swelling. High concentrations of oryzalin decrease germination while low concentrations induce tip swelling.

**Amiprophosphos-methyl (AMP)**

AMP is a phosphoric amide herbicide which inhibits the *in vitro* polymerization of isolated plant tubulin. It was shown that oryzalin and AMP caused a complete loss of microtubules from the tip back towards the tube mid-point but live microtubules intact from the mid-point back to the grain in elongating conifer pollen tubes.

The hypothesis we tested showed that microtubules are involved in the regulation of tip growth. Pollen grains were germinated in media containing microtubule disruptors and were analyzed for germination and tube length. Disruption of microtubules inhibits tube elongation and induces tube curving. Curving is caused by disruption of the microtubules in the tip.

**OBJECTIVE OF THE STUDY**

In this study, we tested the hypothesis that the functional MT configuration is essential for the pollen tube to exert the penetration force. Pollen grains were grown *in vitro* on germination media containing agarose in the presence or absence of colchicine and oryzalin. Germination tube length was examined and results were evaluated. We wanted to find out whether the microtubule is important for the tip growth of pollen tube and that was why we applied the inhibitors colchicines, oryzalin and AMP to see if by inhibiting MT growth they will inhibit tip growth.

**MATERIALS AND METHODS**

**Pollen tube culture**

Pollen grains from *Lilium* and *Papaver rhoeas* were collected, dried
and stored at -20°C in a freezer. Pollen grains were germinated in liquid medium [stock solution (20x): 0.01% H₂BO₃; 0.043% Ca (NO₃)₂·4H₂O; 0.01% KNO₃; 0.02% MgSO₄·7H₂O in 50 ml of water] containing 5% sucrose for Lilium. P. rheas was germinated in Brewbaker and Kwack medium [stock solution (10x): 0.29 g MES; 0.099 g H₂BO₃; 0.0147 g solid medium CaCl₂]. It was ten times concentrated (0.29 g MES; 0.099 g H₂BO₃; 0.0147 g CaCl₂).

Semi-solid medium was prepared by adding 0.2% of a gelling agent to the PCM or BK, heating it in the microwave oven and subsequently cooling it to 42°C in the water. Pollen grains were added and the mixture was spread immediately on microscope slides. Pollen was incubated for 1 h 30 min for Lilium and for 2 h for P. rheas in a humid chamber. Each sample was prepared twice.

Inhibitor treatments

Oryzalin, colchicine was prepared in stock solutions containing DMSO, methanol and acetone respectively. Control experiments contained the amount of solvent corresponding to the highest concentration used in drug containing samples. Drugs were incorporated in the germination medium at the same time as pollen was added.

Colchicine was used in a series of concentrations between 50 mM and 50 μM from a stock solution of 1 M in water. For the first experiment, we used concentrations between 2 mM and 16 μM in semi-solid medium made of 0.2% of agarose and PCM with Lilium. In the second experiment, we used concentrations between 50 mM and 50 μM for colchicines. Oryzalin was used in a series of concentrations between 10 mM and 1 μM from a stock solution.

RESULTS AND DISCUSSION

Growing pollen in liquid and semi-solid medium

Sample preparation was not easy for high concentrations of agarose, however, since it tended to gellify rapidly. Therefore, we tested other gelling agents such as noble agar, agarose, microbiological agar and DNA agar. The choice of a gelling agent that does not gellify quickly in the high concentrations such as 3, 4 and 5% is important for the relevance of the results, and as such when we compared four different gelling agents at 1%: noble agar, agarose, microbiological agar and DNA agar, we saw that microbiological agar and DNA agar gellified quickly while agarose and noble agar did not.

Effect of different concentrations of the gelling agent on pollen tube germination

Six increasing concentrations of noble agar were used to test the effect of increasing rigidity of the medium on germination on Papaver (Figure 1). The results showed that increasing rigidity of the medium decreased the percentage of germination.

Effect of the optimal concentrations of the depolymerising drugs colchicine, oryzalin and AMP

Colchicine

We know already that the range of effective concentrations of colchicine is between 50 mM and 50 μM and as such, we tested this range of concentrations.
Table 1. Effect of the optimal concentrations of the colchicines.

<table>
<thead>
<tr>
<th>Concentrations of colchicine</th>
<th>2 mM</th>
<th>400 µM</th>
<th>16 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of germination (%)</td>
<td>10</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
</table>

Experiment 2

<table>
<thead>
<tr>
<th>Concentration of colchicine</th>
<th>50 mM</th>
<th>500 µM</th>
<th>50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of pollen tube</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Effect of oryzalin on the *lilium* in the semi-solid medium.

<table>
<thead>
<tr>
<th>Concentration of oryzalin</th>
<th>10 mM</th>
<th>1 mM</th>
<th>100 µM</th>
<th>10 µM</th>
<th>1 µM</th>
<th>0.1% DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of germination (%)</td>
<td>11</td>
<td>10</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Length of pollen tube</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 2. Effect of the optimal concentrations of oryzalin without temperature respectively on pollen germination and pollen tube length.

We noticed that the percentage of germination remained approximately the same (Experiment 1), while we noticed a decline of pollen tube length (Experiment 2). Results are summarized in Table 1.

Oryzalin

It is known that oryzalin is effective in a range of concentrations between 200 and 100 µM in decreasing pollen germination (Table 2). We expected that there would be no growth in the 10 and 1 mM because these are letal concentrations on *Lilium* and as a result, we made the hypothesis of sensibility of oryzalin to the temperature of the Marie bath in order to inhibits its effect on the germination and that was why we grew pollen tubes in liquid medium by first putting pollen tubes on the slide on a dry slide, adding PCM on them and then mixing the preparation.

Results at 1 mM, 100 and 10 µM respectively, was appreciatively equal to the control therefore, it was possibly that the temperature deactivated oryzalin (Figure 2).

AMP

We tested the effect of increasing concentrations of AMP with control containing acetone at 1%, because 1% corresponds to the acetone concentration in the sample with the highest AMP contents (Table 3). Like oryzalin, AMP induced a decrease in the percentage of germination.
Table 3. Effect of AMP on the germination of pollen tube on the liquid medium.

<table>
<thead>
<tr>
<th>AMP</th>
<th>10 mM</th>
<th>1 mM</th>
<th>10 µM</th>
<th>1 µM</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of germination %</td>
<td>23</td>
<td>15</td>
<td>18</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>pollen tube length</td>
<td>5</td>
<td>1.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**Effect of oryzalin depending on the concentration of agarose**

We used oryzalin in a concentration of 100 µM with increasing concentrations of agarose 0.2, 1, 2, 3, 4 and 5% because we saw that increasing concentrations of agarose disturbs germination and we wanted to verify the effect of oryzalin on germination depending on the increasing concentrations of agarose hence, we compared germination in slides with oryzalin (shown in red) and others without it (shown in blue), this means that we dissolved methanol as a stock solution of 200 mM (Figure 3). We concluded that the effect of oryzalin depending on agarose is remarkable since percentage of germination decreases.

This study tested our hypothesis that microtubules are involved in the regulation of tip growth. Pollen grains were germinated in media containing microtubule disruptors and analyzed for germination and tube length. Disruption of microtubules significantly inhibited tube elongation and induced tube curving. Curving was caused by disruption of the microtubules in the tip. Treatment with oryzalin caused a slight but significant reduction in pollen germination while colchicines did not affect germination. All the MT disruptors caused a decline in pollen tube length.

With more skilful manipulation of the gelling agent, effect of inhibitors can be shown clearly. The visualisation of microtubules through indirect immunofluorescence microscopy (IIF) is direct, while oryzalin binds to microtubules based on how it acts on them. Further applications of stabilizing agents such as taxol will allow us to compare polymerizing with the depolymerising effect and to come up with conclusions about the dynamism of microtubules in the pollen tubes.

Our hypothesis that microtubules are involved in tip growth of pollen tubes is worth future investigation.

**Conclusion**

Colchicines, oryzalin and AMP by inhibiting MT growth inhibited tip growth. Therefore, the microtubule is important for the tip growth of pollen tube.

**REFERENCES**

Morejohn (1993), Rapid and reversible high affinity binding of the
dinitroaniline herbicide oryzalin to tubulin from zea-mays, Plant physiol., 102(3): 725-740.