Induction of Deformed Pollen Tube Tips and Their Morphological Characteristics in Self-incompatible Japanese Pear

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Summary

Aberrations in pollen tube tips of Japanese pear, Pyrus serotina, in vivo studies following pollen-incompatible and -compatible pollinations; and in vitro studies on the effects of chemical additives to the growth medium were investigated.

Incompatible and compatible pollen/stigma-style combinations resulted in pollen tubes with deformed tips which were mainly detected as a swelling. The incompatible combinations developed approximately twice as many swollen tips. Their surface structure revealed irregular features such as concave and unusual furrows, whereas a smooth surface was observed in normally growing tips.

In in vitro trials, ABA, ovarian extract (OE), polyethylene glycol (PEG), H$_3$BO$_3$ and fusicoccin (FC) increased the number of abnormal pollen tube tips. The tip surfaces of these tubes were considerably irregular, except in the case of H$_3$BO$_3$, which seemed to be similar to those deformed tips observed in incompatible styles. Boric acid caused regular fine furrows along the tube axis but CaCl$_2$ had no effect on the deformed tip formation; the tube surface structure was relatively smooth. Thus, the growth inhibitors tend to induce irregularities on pollen tube surface similar to those observed on pollen tubes growing in an incompatible style, whereas growth promoters did not induce such symptoms. The similarities in tip morphology may indicate that cell wall synthesis in pollen tubes growing in incompatible styles is altered by inhibitors or water stress following a specific recognition reaction on the stigmatic surface and/or in the transmitting tissue. This alteration causes cessation of tube growth.

Introduction

In several plants with gametophytic self-incompatibility systems, deformed tips of pollen tubes such as swelling, thickening of tube wall and rupturing have been observed in an incompatible style (5, 9, 16, 18, 21). The tips are also deformed by interspecific pollination in Lilium; the morphology is characterized as branching or bulbing (2). Although it is unclear whether the morphological change is directly correlated with self-incompatibility reaction or not, gross deformation of incompatible tube tips, especially tip swelling in Japanese pears, was reported earlier in this century (1, 24).

Self-incompatibility is considered to involve recognition and rejection reactions (14). The former may be established as the function of glycoproteins produced in stigma and/or style (3, 4, 6, 13), but little is known on the latter reaction. Although some glycoproteins associated with S-genotypes directly inhibit the incompatible pollen tube growth in vitro (11, 25), inhibitors with low molecular weights, which are widespread in plant tissues, or physiological stress, such as water stress, may also play a role in the rejection reaction. Subsequent to the specific recognition reaction on the stigma or in the style, the action of an inhibitor on pollen tubes may be a non-specific reaction.

The present paper reports on the morphological characteristics of incompatible pollen tube tips and those of pollen germinated on agar media containing several substances which are important in plant growth and development. Based on our findings,
the relationship of tip deformation to self-incompatibility and self-incompatible rejection reaction from a morphological standpoint are discussed.

Materials and Methods

1. Observation of pollen tubes in styles

Japanese pears, Pyrus serotina Rehd. culta Rehd., cvs. Chojuro (S2S3) and Nijisseiki (S2S4) were selected in the orchard of Mie University. Flowers of ‘Chojuro’ were emasculated immediately before anthesis and self-pollinated (incompatible pollination) or cross-pollinated with pollen of ‘Nijisseiki’ (compatible pollination). The flowers were then covered with waxed paper bags to prevent additional pollination. The pollinated flowers were sampled at 12-hr intervals and fixed with F.A.A. (formalin : acetic acid : 70% ethanol = 5 : 5 : 90 v/v).

To observe the overall features of deformed pollen tube tips in a style by the squash method, styles were initially hydrolyzed in 8N NaOH for 8 hr, washed in running tap water, and immersed in 0.1% aniline blue dissolved in 0.1N K3PO4 for 24 hr. The stained styles were mounted with glycerin on a glass slide, crushed with cover glass, and observed under the fluorescent microscope (Olympus, BH) (9). The overall profiles of deformed pollen tube tips were clearly observed by this method. Comparable samples were prepared by the usual paraffin section method for finer, detailed morphological examination.

The stained styles were dehydrated with ethanol-butanol, embedded in paraffin and cut 10 μm of cross sections. The sections were stained for 2 hr with 0.1% aniline blue dissolved in 0.1N K3PO4 and observed under the fluorescent microscope. The number of pollen tubes with abnormally larger areas in the stylar sections was recorded at 0.3, 2, 4 and 6 mm from stigmatic surface (Fig. 1-A). Shrunken tubes were not counted because the basal parts (near the pollen grains) of the normally elongated pollen tubes frequently shrank.

For morphological observations of the tube surface, pollinated styles were collected from flowers 48 hr after pollination. These were split longitudinally into two. Each section was prefixed with glutaraldehyde (4% solution in 0.1M cacodylate buffer, pH 7.2) for 24 hr, postfixed with osmic acid (1% solution in distilled water) for 1~2 hr, and dehydrated through a graded ethanol series (30~100%). Samples dipped in isoamyl acetate for 2 hr were dried in a critical point dryer, mounted on a sample supporter, coated with gold for 8 min in a vacuum evaporator, and observed under SEM.

2. Observation of pollen tubes on agar media containing various substances

Anthers of ‘Chojuro’ were gathered from flowers
before they dehisced. Upon dehiscence, pollen was collected, dried, and stored at 4°C in a desiccator. H3BO3, CaCl2 or polyethylene glycol (PEG M.W. = 4,000) purchased from Nakarai Co. Ltd. Osaka, Japan, was dissolved in distilled water and 100 mg liter⁻¹, 200 mg liter⁻¹ or 190 mg ml⁻¹ solution was prepared, respectively. Fusicoccin (FC, Sigma Co. Ltd.), melted in small amount of ethanol, was made to 12.5 mg liter⁻¹ with water. Abscisic acid (ABA, Sigma Co. Ltd.) was dissolved in 0.1N NaOH, adjusted pH at 5.8 with 0.1N HCl and diluted to 200 mg liter⁻¹. This concentration of ABA was selected because at less than 200 mg liter⁻¹ the hormone did not function as an inhibitor to pollen tube growth. An ovarian extract (OE) was made by homogenizing 1 part of mature ovarian tissues of ‘Chojuro’ with 5 parts of water in a glass homogenizer. The homogenate was centrifuged at 20,000g for 10 min at 4°C and the supernatant used as the OE.

A basic medium consisted of 10 ml of 1% agar containing 10% sucrose and 0.01% H3BO3 in a 9 cm petri dish. A 10-μl aliquot of test solution was placed in a 5 mm diam. well made with a cork borer in the center of the medium. After the solution was absorbed into the gel, pollen grains were transferred onto the medium by using the edge of cover glass as described previously (8). After culturing for 1 day at 25°C in the dark, germinating pollen was stained with 0.5% cotton blue dye dissolved in a solution of lactic acid : phenol : glycerol : distilled water = 1 : 1 : 1 : 1 (v/v). The percentages of pollen tubes with deformed tips and the pollen tube length in the treatment and control medium were recorded at 0, 5, 10, 15 and 20 mm from the well.

Germinating pollen on agar medium was collected with a pair of tweezers, fixed as described above and subjected to the observation for SEM. Unless indicated otherwise, pollen tubes were gathered after a 24 hr incubation period at 10 mm from the central well.

**Results**

Deformed tips, especially swollen tips, were clearly observed in the crushed styles under the fluorescent microscope (Fig. 1-C). The deformed tips were found not only in incompatible combination but also in the compatible one. However, the frequency of abnormal tubes in an incompatible style was about twice that of compatible combinations (Table 1). Although the deformed tips were present throughout the transmitting tissues, many of them were observed at upper parts (near the stigma). The growth of large number of pollen tubes in Japanese pears is known to be arrested at this portion (7).

The fine structure of the surface of the deformed tip was concave or exhibited unusual furrows which were detected not only at the tip but also at some distance from the tip (Fig. 2-A, B). In compatible styles, the normally growing tubes possessed smooth surface and their tips appeared to be oval (Fig. 2-C). The styles of ‘Chojuro’ are 6~7mm long and 1 mm wide. Their transmitting tissues are classified as a fixed type in which the pollen tubes elongate between intercellular spaces. Because of technical difficulties, no more than 10 deformed tips were detected in the styles under the SEM.

Although CaCl2 and H3BO3 both promoted the elongation of pollen tubes about 8% more than the

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#### Table 1. Percentages of pollen tubes having swollen tips in the styles after self- or cross-pollination.

<table>
<thead>
<tr>
<th>Cut position (mm) from stigmatic surface</th>
<th>Self-pollination</th>
<th>Cross-pollination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 Hr</td>
<td>24 Hr</td>
</tr>
<tr>
<td>0.3</td>
<td>2.2</td>
<td>3.7</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>2.2</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Each value in the table exhibits percentage of pollen tubes with abnormally larger areas in the paraffin sections and was obtained from 15 or more styles. 0: No swollen tip was observed, —: No pollen tube was observed.
control, the inorganic substances educed different morphological responses by the deformed tip (Fig. 3-A). H₃BO₃ strongly accelerated tip deformation but CaCl₂ did not. The deformed tips induced by H₃BO₃, however, seemed to have a regular, spherical shape.

FC, OE and PEG strongly inhibited pollen germination and pollen tube growth in vitro (Fig. 3-B, C). These inhibitors caused excessive tip deformation (Fig. 4). ABA mildly inhibited pollen tube growth and increased the number of deformed tips in the zone around the well. The concentration of ABA used may have been too low to express its full inhibitory potential.

Inhibitors (ABA, PEG, FC and OE) and promoters (H₃BO₃ and CaCl₂) of pollen tube growth caused distinctive effects on fine structures...
of tube surface. Although the various structures were observed under SEM, they seemed to be characterized as several types. The typical characteristics of the surface are summarized in Table 2. In the control medium without H$_3$BO$_3$, majority of the tubes (70%) possessed smooth surface or surface with only localized furrows (Fig. 4-A). These features were similar to those cultured in the presence of CaCl$_2$ (Fig. 4-B). The distinctive effect of H$_3$BO$_3$ was the formation of furrows along the pollen tube axis; 85% of the tubes exhibited furrows (Fig. 4-C). The direction of these furrows differed from those observed on tube surface with deformed tips in vivo or in vitro. The furrows caused by H$_3$BO$_3$ appeared to be formed parallel to the tube axis, whereas those by inhibitors or in an incompatible style formed considerably irregular and often at right angle to the tube axis. The use of the growth inhibitors, ABA, FC and PEG, resulted in the formation of hollows near the tip zone (Fig. 4-D, E). OE mainly caused concave or convex at the tip zone (Fig. 4-F). Although severe
deformation in whole tips or splitting of the tips was sometimes observed, the overall features of surface deformed by inhibitors seemed to be similar to those observed in incompatible styles.

**Discussion**

The number of swollen tips in the style section may be much higher than recorded in Table 1 because; a) the paraffin sections were prepared only at distances of 0.3, 2, 4 and 6 mm below the stigmatic surface, and b) pollen tubes were presumed to have normal tips if the tips were not visible in the paraffin section.

Aberrations of tips in compatible pollinations may be attributed to the S2-allele present in the 'Chojuro' and 'Nijisseiki'. Hence, they were incompatible at one locus. Our findings confirm the relationship between deformed pollen tube tips and self-incompatibility in Japanese pears because the incompatible pollinations, in this case, produced twice the number of aberrations as predicted.

In gametophytically controlled self-incompatibility systems, the growth of incompatible pollen tubes are generally arrested in a style several hr after pollination; the synthesis of tube wall and/or membrane is disturbed by self-incompatibility mechanism. This inhibitory process on pollen tube growth is not well understood because what substance and when it evokes the process is uncertain. That is, 1) when does the pollen tube perceive the information from the stigma and/or style that the pollen is incompatible (time of recognition reaction)? 2) when does the actual inhibition of tube wall and/or membrane formation take place (time of rejection reaction)? 3) does the rejection reaction accompany the recognition reaction or follow the completion of the latter? and/or 4) what substances play a role in recognition of incompatible mating or in inhibition of tube growth? In the present study, pollen tubes having deformed tips with abnormally irregular surface furrows, both concave and convex, were often observed. The abnormality was not confined to the tip but at considerable distances from the tip. Judging from these observations, incompatible pollen tubes form unusual cell wall structures before ceasing growth, indicating that the recognition reaction is induced at fairly early stages of tube development; the rejection reaction sets in gradually before cessation of tube growth. However, it is not clear if both reactions are progressing simultaneously.

In our previous experiments (10), the growth of incompatible pollen tubes in *Lilium* was retarded even though the pollinated styles were immersed into hot water 3 hr after pollination in order to overcome self-incompatibility. Apparently the incompatibility mechanism was operative by this time. This was the stage that incompatible pollen tubes grew, at most, one-fourth of the final length of the tubes in untreated styles and first contacted with transmitting cells of the style. This inhibition process in *Lilium* may correspond to the case of Japanese pears based on the morphological characteristics of pollen tubes that we observed.

CaCl₂ and H₃BO₃, promoters of the pollen tube

<table>
<thead>
<tr>
<th>Substances tested</th>
<th>No. of pollen tubes observed</th>
<th>Characteristics of tube surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smooth</td>
<td>Furrows (whole tubes)</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>38</td>
<td>18</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>34</td>
<td>—</td>
</tr>
<tr>
<td>ABA</td>
<td>47</td>
<td>5</td>
</tr>
<tr>
<td>PEG</td>
<td>42</td>
<td>6</td>
</tr>
<tr>
<td>OE</td>
<td>32</td>
<td>—</td>
</tr>
<tr>
<td>FC</td>
<td>27</td>
<td>—</td>
</tr>
<tr>
<td>Cont.1</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Cont.2</td>
<td>33</td>
<td>15</td>
</tr>
</tbody>
</table>

Each value in the table exhibits the number of pollen tubes observed. 1: Attachment of unknown materials or severe abnormality in shape. Cont.1: Presence of 100 mg liter⁻¹ H₃BO₃ in the basic medium. Cont. 2: Absence of H₃BO₃ in the basic medium. Pollen tubes were sampled 24 hr after culture at 10 mm from the well into which substances were injected. Details are described in the text.
growth, did not have a strong effect on Japanese pear pollen. The basic medium contained 100 mg-liter\(^{-1}\) H\(_3\)BO\(_3\), additional effect of H\(_3\)BO\(_3\) for promotion of tube growth was not clear. However, H\(_3\)BO\(_3\) affected the fine structure of pollen tube surface. Although the addition of CaCl\(_2\) into the medium had almost no effect, the H\(_3\)BO\(_3\) caused fine furrows to form parallel to the tube axis. These furrows were different from those found on incompatible tube surface which were considerably irregular and often at right angle to the tube axis. The direction of furrows may determine whether or not pollen tube continues to elongate. Calcium ion is thought to stimulate the fusion of golgi vesicles, components of tube wall produced by golgi apparatus, at the tip of elongating pollen tube (17), whereas boron seems to enhance carbohydrate metabolism in pollen tubes (19, 23). The fine structure of tube surfaces found in Ca-treated Japanese pears indicate that Ca promotes the pollen tube growth in ways different from B.

Little is known about the structural composition and the soluble constituents of the stigma and transmitting tissue of the pear and how they affect pollen germination and growth. To avoid this complexity, a simple agar medium containing H\(_3\)BO\(_3\) and sucrose was used in this study. Thus, tip aberrations observed on agar medium may not always correspond to those seen in the style. The inhibitors used in this study are believed to have physiologically important roles in metabolism of pollen tube growth. OE of Japanese pear, containing water soluble inhibitors, acted differently on the growth of incompatible and compatible pollen tubes (8); FC, which is the major toxin produced by Fusicoccum, has hormonal functions in plants similar to those of gibberellins and auxins, and anti-ABA on stomatal opening or breaking of seed dormancy (15). PEG is well known as a water-stress inducer against the plants (12). Furthermore, pollen hydration seems to be regulated by sporophytic self-incompatibility mechanism in Brassica (20). OE, PEG and FC strongly inhibited both the pollen tube growth and germination but ABA did not. However, structural features of the tips treated with ABA were similar to those treated with other inhibitors. Although marked deformations of tube tips, such as cracks, deep concave furrow and rough surface were often induced by the above inhibitors, and/or ABA, in both in vivo and in vitro studies, the appearances of the structures seemed to be fairly similar.

If the substance acting on recognition reaction is different from that on rejection reaction, recognition may be mediated by S-proteins as shown in many recent reports (3, 4, 6, 13). However, actual inhibition after specific recognition reaction may not always require a specific inhibitor. This hypothesis is partly supported by the formation of callose deposits in cells of the stigmatic surface. This deposition of callose is the first visible step in the self-incompatible rejection reaction of Brassica. The rejection reaction is induced not only by the incompatible pollen grains but also by all sporophytic tissues of a sporophytic self-incompatible plant (22). Based on the morphological similarity of deformed tips induced in incompatible styles and also on agar medium containing inhibitors, the possibility remains that the probable inhibitor(s) acts on incompatible pollen tubes after specific recognition reaction. These inhibitory substance(s) and other stress-inducers are seemingly abundant in plant tissues.

**Acknowledgement**

We thank to Prof. H. Kunoh of Mie University for his kind advice in SEM operation.

**Literature Cited**


自家不和合性ニホンナシにおける先端異常花粉管の
誘発とその形態的特性

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摘 要

ニホンナシの和合ならびに不和合受粉において、花柱にみられる異常な先端をもった花粉管（主として
肥大）の発生を調査するとともに、数種の物質を加え
た工夫培地上での発芽花粉における花粉管の先端異常
の発生を検討した。

不和合花柱内での肥大した先端をもった花粉管数は、
和合花柱内の約2倍であった。また、異常花粉管の表
面は凹凸や不規則な渦が見られたが、正常な花粉管で
は比較的滑らかであった。

花粉管生長促進物質である CaCl₂は花粉管の先端異
常の発生には影響せず、花粉管の先端の表面構造は滑
らかであったが、H₃BO₃は先端肥大を促進し、その表
面には縦方向の渦が多数見られた。しかし、後者で見
られた肥大した先端は比較的円形に近い規則的なもの
であり、和や不和合花粉管のものとは異なって規則的
であった。

花粉管生長抑制物質である子房抽出液、フシコシン、
ABA および水ストレスを与える培養液で生育した花
粉管の先端異常の発生を抑制し、それらの表面構
造は不和合受粉で多く見られる花粉管の異常と類似し
ていた。

以上の形態的特性より、不和合の拒絶反応（特異的
な認識反応後に生じ、実際に花粉管生長を抑制すると
考えられる反応）については、植物体内に広く分布する抑制
物質やストレスが関与している可能性を示唆された。

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