Effects of Light and SH-reagent on Ultrastructural Changes in Leaf Cells Induced by AM-toxin from *Alternaria alternata* Apple Pathotype*

Norihiro SHIMOMURA**, Pyoyun PARK***, Hiroshi OTANI**, Motoichiro KODAMA** and Keisuke KOHMOTO**

**Faculty of Agriculture, Tottori University, Tottori 680, Japan
***Electron Microscopy Section, Department of Orthopaedic Surgery, Teikyo University School of Medicine, Tokyo 173, Japan

Key words: AM-toxin, light, SH-reagent, plasma membrane modification, chloroplast modification.

A distinct pathotype of *Alternaria alternata* (Fr.) Keissler (formerly, *A. mali* Roberts), the causal fungus of Alternaria leaf blotch of apple (*Malus pumila* Mill. var. *domestica* Schneid.)

Two action sites for AM-toxins in susceptible apple leaf cells have been suggested from electron microscopic and physiological studies. Plasma membrane invagination and increase in electrolyte loss caused by the toxin showed that the plasma membrane may be one of the action sites. The other site may be located in the chloroplast, because the toxin induced both vesiculation of grana lamellae and decrease in photosynthetic CO₂ fixation activity.

Recently, the process of host cell damage caused by AM-toxin was characterized by examining counteractive effects of light and SH-reagents. In light, toxin-induced necrosis was inhibited, but the loss of electrolytes and the reduction in photosynthetic CO₂ fixation were not affected. From these results, it can be concluded that light inhibits the process of dysfunctions of primary action site(s) to cell death. On the other hand, SH-reagents inhibited necrosis formation and electrolyte loss, but not reduction in photosynthetic CO₂ fixation in the toxin-treated leaves. These results indicate that necrosis comes of the plasma membrane disorders brought about by the toxin rather than the chloroplast damages. To clarify relationships among early cellular events induced by AM-toxin, we examined effects of light and an SH-reagent on the ultrastructural changes, invagination of plasma membranes and vesiculation of chloroplasts, in apple leaf cells.

Ten-day-old leaves of apple were injured slightly with a razor blade. A drop of 10⁻⁷ M AM-toxin I or distilled water was placed on the wounded site, and the leaves were incubated in a moist chamber at 26°C for 3, 6, 12 and 24 hr in the dark or light (daylight-color lamp, 630 μW/cm²). The procedures for preparing thin sections for electron microscopy were the same as those described previously by Park et al. The sections were stained with uranyl acetate and lead citrate, and observed under a JEOL 100 CX electron microscope.

In the dark, necrosis appeared at vein of young leaves of susceptible apple (cv. Red Gold) 12 hr after the toxin treatment, and then expanded to mesophyll cells in 24 hr after the treatment. At such concentration of the toxin, necrosis was not induced in moderately resistant (cv. Jonathan) and resistant (cv. Mahe 7) leaves. The earliest changes were detected as modifications of both plasma membranes and chloroplasts in susceptible cells under an electron microscope at 3 hr after the toxin treatment. The toxin caused plasma membrane invaginations at plasmodesmata, and membrane fragments and extended desmotubules were observed in invaginated sites (Plate I-1). The toxin also caused partial disorganization of chloroplasts, where grana lamella-fragments were found in stroma (Plate I-2).

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Table 1. Effect of light irradiation on plasma membrane invagination at plasmodesmata and vesiculation of grana lamellae in chloroplasts of apple leaves induced by AM-toxin I\(^a\)

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Time (hr)</th>
<th>Occurrence of invaginated plasma membrane at plasmodesmata (%)</th>
<th>Occurrence of vesiculated chloroplasts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dark</td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>Red Gold</td>
<td>Toxin</td>
<td>3</td>
<td>6.8± 4.8</td>
<td>3.1±2.4</td>
</tr>
<tr>
<td></td>
<td>Toxin</td>
<td>6</td>
<td>16.2±0.4</td>
<td>12.6±3.5</td>
</tr>
<tr>
<td></td>
<td>Toxin</td>
<td>12</td>
<td>24.5±11.4</td>
<td>21.5±8.4</td>
</tr>
<tr>
<td></td>
<td>Toxin</td>
<td>24</td>
<td>33.9±5.5</td>
<td>28.7±4.1</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>24</td>
<td>0.0± 0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Jonathan</td>
<td>Toxin</td>
<td>24</td>
<td>0.0± 0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>24</td>
<td>0.0± 0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Mahe 7</td>
<td>Toxin</td>
<td>24</td>
<td>0.0± 0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>24</td>
<td>0.0± 0.0</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

\(\text{a)}\) Apple leaves were treated with 10^{-7} M AM-toxin I, and incubated in the dark or light (630 \mu W/cm\(^2\)). At least 100 cells, and 100 plasmodesmata or chloroplasts were observed for each treatment in one experiment. Values represent the means and standard deviations of three replications.

Table 2. Effect of pretreatment of iodoacetamide on plasma membrane invagination at plasmodesmata and vesiculation of grana lamellae in chloroplasts of susceptible apple leaves induced by AM-toxin I\(^a\)

<table>
<thead>
<tr>
<th>Post-Treatment</th>
<th>Time (hr)</th>
<th>Occurrence of invaginated plasma membrane at plasmodesmata (%)</th>
<th>Occurrence of vesiculated chloroplasts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>Toxin</td>
<td>3</td>
<td>10.9± 5.5</td>
<td>2.8±2.0</td>
</tr>
<tr>
<td>Toxin</td>
<td>6</td>
<td>18.0± 6.6</td>
<td>1.4±1.4</td>
</tr>
<tr>
<td>Toxin</td>
<td>12</td>
<td>32.6±10.0</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>Toxin</td>
<td>24</td>
<td>36.1± 7.0</td>
<td>8.4±2.9</td>
</tr>
<tr>
<td>Water</td>
<td>24</td>
<td>0.0± 0.0</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

\(\text{a)}\) Apple leaves (cv. Red Gold) were pretreated with 0.1 mM iodoacetamide, and then treated with 10^{-7} M AM-toxin I. The treated leaves were incubated in the dark. At least 100 cells, and 100 plasmodesmata or chloroplasts were observed for each treatment in one experiment. Values represent the means and standard deviations of three replications.

However, no changes appeared in susceptible control and the toxin-treated resistant leaves (Plate I-3, 4).

Though necrosis was markedly inhibited by light-irradiation, invaginations of plasma membranes were observed in toxin-treated susceptible leaves even under 24 hr-illumination (Plate I-5). The number of plasmodesmata with invaginations was counted at a high magnification (×94,000). The invaginations around plasmodesmata were observed in the susceptible apple leaves treated with the toxin both in the light and dark. Light gave no significant inhibition of the invagination of plasma membranes (Table 1). On the other hand, the invaginations were not detected in the moderately resistant and resistant apple leaves treated with the toxin.

The chloroplast modifications induced by the toxin were also detected in light-irradiated susceptible leaves (Plate I-6). The modification in the toxin-treated susceptible leaves increased both in the light and dark with time (Table 1). The modification of chloroplasts was rarely observed 24 hr after toxin treatment in the moderately resistant and resistant apple leaves. These results coincide with the physiological data reported by Tabira et al.\(^7\), and confirm the previous conclusion that early events in the toxin actions in plasma membrane and chloroplasts are not affected by light. However, the mechanism in inhibition of the toxigenic necrosis by illumination has been unknown yet.

To examine the effect of an SH-reagent on toxin-induced ultrastructural changes, 0.1 mM iodoacetamide solution was infiltrated into susceptible leaves under reduced pressure for 30 min. The leaves were treated with 10^{-7} M AM-toxin and incubated in a moist chamber at 26°C in the dark.
Iodoacetamide apparently inhibited toxin-induced veinal necrosis in the susceptible leaves. The treated leaves were prepared for observation by electron microscopy. Treatment of the chemical alone did not cause any apparent ultrastructural changes in apple leaves (Plate I-7). The frequency of plasma membrane invaginations in the susceptible leaves without the chemical pretreatment reached to approximately 30% at 12 hr after the toxin treatment (Table 2). In contrast, only a few invaginations were induced by the toxin in the chemical-pretreated leaves. On the other hand, the chemical did not inhibit chloroplast modification induced by the toxin (Table 2). Thus, iodoacetamide suppressed the plasma membrane modification, but not chloroplast one (Plate I-8).

We reported previously that an SH-reagent inhibited AM-toxin-induced electrolyte loss as well as necrosis formation, but failed to reduce the toxigenic inhibition of photosynthetic CO₂ fixation⁶). We suggested that plasma membrane dysfunctions caused by the toxin may be closely correlated with necrosis⁶). The protection was detected only when leaves were treated with SH-reagent before toxin exposure, indicating that SH-containing molecules may be involved in an early recognition between AM-toxin and susceptible apple cells. This view⁶) is additionally supported by the present ultrastructural study because the SH-reagent inhibited the plasma membrane modifications but not chloroplast ones.

We reported previously that AM-toxin affects chloroplasts in host and non-host leaves, but does not induce necrosis and fungal infection on such non-host leaves, indicating that the toxin may have a non-selective effect on chloroplasts⁶). However, there is a differential toxin sensitivity in chloroplasts between susceptible apple and others⁶). In the case of susceptible apple, the effect of the toxin appears on both plasma membranes and chloroplasts at 10⁻⁵ M. On the contrary, the toxin at 10⁻⁶–10⁻⁸ M affects plasma membranes and chloroplasts of moderately resistant apple, while at the same concentration, the toxin causes chloroplast dysfunctions of resistant apple and some non-hosts without inducing plasma membrane lesion. The results imply that chloroplast dysfunctions in susceptible apple may contribute for host-specific action of AM-toxin. Therefore, further studies of the mechanism of AM-toxin actions using isolated chloroplasts remain to be done.

**Literature cited**

Explanation of plate

Plate I

1 and 2. Susceptible apple leaves treated with $10^{-7}$ M AM-toxin, and incubated for 24 hr in the dark.
1. A slightly modification of plasma membrane in susceptible apple. Vesicles and extended desmotubules were observed in the space between invaginated plasma membranes and cell walls. ($\times 34,000$)
2. Marked vesiculation of grana lamellae in the matrix of chloroplasts. Membrane fragments were present in the matrix. ($\times 18,000$)
3. Susceptible apple leaves treated with water. Leaves were incubated for 24 hr in the dark. No ultrastructural changes were observed in plasma membranes, cell walls, vacuoles, mitochondria and chloroplasts. ($\times 7,200$)
4. Resistant apple leaves treated with $10^{-7}$ M AM-toxin, and incubated for 24 hr in the dark. No ultrastructural changes were observed in the cells. ($\times 9,200$)
5 and 6. Susceptible apple leaves treated with $10^{-7}$ M AM-toxin. Leaves were incubated for 24 hr in the light (630 $\mu$W/cm$^2$).
5. Invagination of plasma membranes. Invagination of plasma membranes was induced by the toxin in light-irradiated leaves. ($\times 34,000$)
6. The vesicles derived from grana lamellae. Vesication of grana lamellae was induced by the toxin in light-irradiated leaves. ($\times 16,000$)
7. Susceptible apple leaves pretreated with 0.1 mM iodoacetamide followed by treatment with water for 24 hr. No ultrastructural changes were observed in the cells. ($\times 8,700$)
8. Susceptible apple leaves pretreated with 0.1 mM iodoacetamide, followed by treatment with $10^{-7}$ M AM-toxin for 24 hr. Vesiculation of grana lamellae but no invagination of plasma membranes was observed in the cells. ($\times 8,700$)

Abbreviations: ED, extended desmotuble; IP, invaginated plasma membrane; VG, vesiculated grana lamellae.
Plate I

1. IP
2. VG
3.
4.
5. IP
6. VG
7.
8. VG