74. The Mitochondrion: The Prime Site for a Host-Selective 
Toxin (ACR-Toxin I) produced by Alternaria 
alternata Pathogenic to Rough Lemon

By Keisuke KOHMOTO,* Tetsuyuki KOHGUCHI,* Yukari KONDOH,*
Hiroshi OTANI,** Syoyo NISHIMURA,** Shin-ichi NAKATSUKA,***
and Toshio GOTO****

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Host specificity of plant pathogens is one of the most intriguing problems 
in the field of plant pathology. Host-selective toxins (HST) from fungal patho-
gens are known as initiation factors of pathogenesis and primary determinants 
for host-parasite specificity, and thus are very useful for the study of the 
underlying mechanism of host specificity in parasitism as reliable substitutes 
for the pathogens that produce them.10)

A distinct pathotype of Alternaria alternata (Fries) Keissler with selective 
pathogenicity to rough lemon (Citrus jambhiri Lush.), which was found in 1976 
in Florida by Whiteside,12) affected rough lemon but not Dancy tangerine, 
Emperor mandarin (C. reticulata Blanco) and nonhost plants. The pathotype 
produced multiple HSTs (ACR-toxin) with the same specificity as the producing 
fungus.51 Nakatsuka et al.9) recently isolated the major HST in pure state 
and determined its chemical structure to be 6-(2,4,8-trihydroxy-3,7,9-trimethyl-
undeca-5,9-dienyl)-4-hydroxy-5,6-dihydro-2-pyrone (ACR-toxin I). Independent-
ly, Gardner's group1),2) also has completed the characterization of the major 
toxin as the same structure (ACRL-toxin I). Furthermore, they have charac-
terized other five, less toxic α-pyrone analogues,7) ACR-toxin caused veinal 
necrosis and water soaking of susceptible leaves. Electron microscope observa-
tions indicated that mitochondria may have the initial site of toxin action.61 
However, physiological evidence has lacked yet, since there was a frustrating 
difficulty to isolate intact mitochondria from citrus leaves by common methods; 
the loss of intactness mostly appeared to be due to the liberation of injurious 
phenolics and oils against mitochondria from the tissues during homogenizing 
and purifying processes. We wish to present here effects of ACR-toxin I on the 
function of in situ and isolated citrus mitochondria. An abstract of the study 
has been read.41

Materials and methods. ACR-toxin I was purified by the method described 
previously,6) except TLC procedure subsequently was employed after CLC. In vivo 
mitochondrial activity in toxin-treated citrus leaves was examined by Hacken-
brock's method using 2-deoxyglucose(2DOG).3) Procedures for electron micro-
scope observation were given in the previous paper.6) Mitochondria were isolated

* Laboratory of Plant Pathology, Faculty of Agriculture, Tottori University, 
Tottori 680.
** Laboratory of Plant Pathology, Faculty of Agriculture, Nagoya University, 
Nagoya 464.
*** Laboratory of Organic Chemistry, Faculty of Agriculture, Nagoya University, 
Nagoya 464.
from young rough lemon leaves. To solve the difficulty mentioned above, large amounts of Amberlite XAD-7 and PEG 4000 were added to a common isolation buffer, according to the kind advice by Dr. J. M. Gardner of Florida University. The details of XAD procedure will be described elsewhere. O$_2$-Uptake was measured polarographically at 25°C using a Gilson Oxygraph (model 5/6).

**Results and discussion.** ACR-toxin was highly toxic to susceptible rough lemon leaves: When small drops of toxin solution (10 ng/ml) were applied to leaves, water congestion and veinal necrosis were induced within 24 hr after treatment. The relation between toxin concentrations and necrotic leaf area was depicted as a sigmoidal line (Fig. 1). No effects were evident on Dancy tangerine leaves at 100 µg/ml.

![Fig. 1. Relationship between the concentration of ACR-toxin I and the necrotic leaf area of rough lemon. Leaves were scratched at the center of the lower epidermal surface with the broken end of a small glass capillary tube. A small drop (25 µl) of toxin solution or distilled water as a control, was placed on wounded site. The leaves were incubated in a moist chamber at 26°C. Necrotic area of the leaves was measured 48 hr after toxin treatment.](image)

Leaf tissues were pre- or post-treated with 2.5 mM 2DOG at the interval of 1 hr before or after toxin treatment. Mitochondrial types *in situ* under electron microscope were classified into three types of Malone's category$^8$) and into another, toxin-affected mitochondrial type.$^6$) Type I mitochondria had a small amount of matrix which densely stained, and relatively few cristae. The inner compartment (IC) was relatively large in comparison with the outer compartment (OC). Type II mitochondria had more amount of densely stained matrix and more cristae than type I. The IC was smaller and the OC was larger than type I. Type III had a large amount of densely stained matrix, and many inflated and interconnecting cristae. Of the three types, type III had the smallest IC and the largest OC. Type IV was the swollen mitochondria with leached matrix, and with reduced, sometimes vesiculated cristae, which were apparently and characteristically caused by ACR-toxin.$^6$) Table I shows that mitochondria in cells of toxin-treated rough lemon leaves failed to contract when tissues were exposed to 2DOG, whereas mitochondria in cells of toxin-treated Dancy tangerine leaves and of untreated both citrus leaves had the ability to contract in the presence of 2DOG. This indicates that ACR-toxin I presumably affects *in situ* both state 3- and state 4-respiration in susceptible, but not in resistant, mitochondria. Our result on ACR-toxin seemed comparable to that of HMT-toxin action to T-cytoplasma corn mitochondria.$^8$)
Table I. Effect of ACR-toxin I on mitochondria in citrus leaf tissues pre- and post-treated with 2-deoxyglucose

<table>
<thead>
<tr>
<th>Plant</th>
<th>Treatment*</th>
<th>Mitochondrial type** (%)</th>
<th>Contraction***</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pre-</td>
<td>Post-</td>
<td></td>
</tr>
<tr>
<td>Rough lemon</td>
<td>Toxin 2DOG</td>
<td>24.2 23.2 3.2 39.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2DOG</td>
<td>14.9 62.9 17.2 5.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DW 2DOG</td>
<td>3.2 19.2 77.6 0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DW Toxin</td>
<td>18.3 54.6 16.4 10.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DW DW</td>
<td>35.5 36.0 28.5 0</td>
<td>-</td>
</tr>
<tr>
<td>Dancy tangerine</td>
<td>Toxin 2DOG</td>
<td>4.8 20.7 74.5 0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2DOG</td>
<td>8.4 48.9 42.7 0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DW 2DOG</td>
<td>12.3 52.1 35.6 0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DW Toxin</td>
<td>13.7 69.1 17.2 0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DW DW</td>
<td>15.0 62.7 22.3 0</td>
<td>-</td>
</tr>
</tbody>
</table>

* Leaf tissues were previously vacuum-infiltrated with chemical solution; subsequently, the tissues were posttreated with small drops of solutions at scratched sites 1 hr after pretreatment. Toxin: 1 µg/ml ACR-toxin I; 2DOG: 2.5 mM 2-deoxyglucose in pH 7.4 cacodylate buffer. ** For each treatment, more than 600 mitochondria were investigated under electron microscope 4.5 hr after posttreatment, and classified into 4 types as described in the text. *** Contraction was determined by significant increases in percentage of type III in the presence of 2DOG as compared with that of DW control. +, contraction occurred; -, no contraction occurred.

Fig. 2. Traces of rough lemon mitochondria oxidizing NADH and malate. The assay medium in a final volume of 1.5 ml of the reaction vessel contained 0.4 M sucrose, 20 mM Hepes-Tris, 10 mM KCl, 4 mM MgCl₂, and 0.1% (W/V) BSA at pH 7.2. Mitochondria were allowed to oxidized exogenous addition of either 1 mM NADH or 10 mM malate plus 10 mM glutamate. A limiting amount of ADP (100 µM) was used established state 3 rates before and after toxin treatment. Toxin stands for ACR-toxin I was added at final concentration of 1 µg/ml where indicated. Numbers along the traces indicate O₂-uptake in nmol/min·mg protein.
We could prepare active mitochondria with repeated cycles of state 3 and state 4 from young lemon leaves (Fig. 2). The isolated mitochondria were affected with 1 μg/ml ACR-toxin I. When exogenous NADH was the respiratory substrate supplied, O₂-uptake was significantly stimulated by the toxin just like a classic uncoupler, 2,4-dinitrophenol. In contrast, the malate oxidation was almost completely inhibited by the toxin. An additional supply of exogenous NADH to the vessel restored to take up oxygen but the function of respiratory control already lost in the mitochondria. On the other hand, mitochondria from resistant Dancy tangerine and Emperor mandarin leaves were not affected at all by the toxin. The responses of rough lemon mitochondria to ACR-toxin I seem to be similar to those of mitochondria from T-cytoplasm corn to HMT-toxin except that NADH oxidation in T-mitochondria was still coupled to ADP utilization during complete inhibition of malate oxidation by HMT-toxin.²¹

ACR-toxin caused a rapid loss of electrolytes from susceptible tissues.² Gardener et al.² recently have reported the inhibition by 30 ng/ml ACRL-toxin I of ¹⁴C-proline incorporation into ethanol-insoluble fraction of susceptible leaf tissues. The incorporation was determined for 2 or 3 hr after 2-hr-preincubation with toxin²; however, ACR-toxin-affected mitochondria in situ were detectable as early as 1 hr after toxin exposure and almost all mitochondria became altered by 3 hr after treatment.⁶ Moreover, ACR-toxin momentarily had effects on isolated mitochondria. The rapid reduction in mitochondrial source of energy may account for such other dysfunctions in susceptible cells. Although the present data are still fragmentary and much intensive efforts must be paid on the mode-of-action study of ACR-toxin, it is most likely at present to conclude that mitochondria in rough lemon leaves should be the target organelles carrying the primary action site for ACR-toxin I.

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References

2) ———: Phytochemistry (in press).