Suppression of ATPase Activity in Pea Cells during Infection by a Compatible Race of Pseudomonas syringae pv. pisi

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In the interaction of pea plants (Pisum sativum cv. Midoriusui) and a compatible race of Pseudomonas syringae pv. pisi, which causes bacterial blight of peas, we have found that the accumulation of phytoalexin, pisatin, in pea epicotyl tissues was significantly suppressed in comparison to that caused by an incompatible race3). The accumulation of phenylalanine ammonia-lyase (PAL)-mRNA in pea tissues has also been shown to be suppressed during compatible interactions between races of P. syringae pv. pisi and pea cultivars9). Similar results were also obtained in compatible combinations of bean and P. syringae pv. phaseolicola3).

On the other hand, in the relationship between pea plants and a fungal pathogen, Mycosphaerella pinodes (Berk. et Blox.) Stone, a low molecular mass glycopeptide [<5,000 Da] (supprescin) secreted by pycnospores suppressed the early defense responses of pea plants6,7,9). Supprescin has been shown to inhibit the plasma membrane-bound ATPase (PM-ATPase) of pea in vitro10). M. pinodes also inhibits pea PM-ATPase in situ5). The results also indicated that the inhibition of PM-ATPase activity delayed the activation of pea defense responses3). That orthovanadate, an inhibitor of PM-ATPase, delays pisatin accumulation10), also points to a crucial role for PM-ATPase in the host defense response. On the basis of these results, we attempted to clarify whether PM-ATPase activity in pea was affected during the infection process by P. syringae pv. pisi.

Mature leaves were harvested from the second and third nodes of eight-week-old pea plants grown in a greenhouse and their petioles were soaked in water. After 24 hr of incubation at 20±1°C in a moistened plastic container, the epidermis from the lower leaf surface was carefully removed with a forceps and leaves were cut into ca. 5 mm square pieces. Leaf segments were floated onto a layer of 30μl of a bacterial suspension (108 cells/ml in water) so that the lower surface was always exposed to the suspension. P. syringae pv. pisi 299 A (race 1) is compatible to the cultivar Midoriusui, but incompatible to the cultivar Hurst Greenshaft. The opposite was observed in the interaction between P. syringae pv. pisi 292 (race 2) and the tested cultivars8,9).

As a control, tissues were immersed in water. After tissues were incubated for 4, 8 or 12 hr, PM-ATPase activity was assayed as described below. Approximately 15 mm segments of etiolated juvenile epicotyls were prepared from seven to eight day-old seedlings grown in dark. The segments were sliced longitudinally then inoculated with 30 μl of a bacterial suspension as described above for the leaves. Control tissues were immersed in water. Each experiment was repeated at least twice with different seedlings.

The PM-ATPase activity was determined in situ by the methods of Moore et al.4) with a minor modification. The inoculated tissues were fixed in 1 ml of fixer A [1% paraformaldehyde and 0.5% glutaraldehyde in 50 mM Tris-Mes (pH 7.2)] on ice and infiltrated under low pressure. The samples were then transferred to fixer B [4% paraformaldehyde and 0.2% glutaraldehyde in 50 mM Tris-Mes buffer (pH 7.2)] for 1 hr at 0°C in vacuo, and washed five times with 50 mM Tris-Mes buffer (pH 7.2) at 0°C. Tissues were immersed in 50 mM Tris-Mes buffer (pH 7.2) containing 4 mM Pb(NO3)2, 2.5 mM MgSO4, and 3.5 mM ATP for 1 hr at 22±1°C, then dehydrated with an ethanol series and propylene oxide. Dehydrated samples were embedded in Spurr's resin and sectioned with an ultra-microtome (OmU 2, Reichert, Austria). An 80–90 nm thick section was mounted on collodion-coated copper grids, stained with 1% uranylacetate and observed under a transmission electron microscope (JEM 100 B, JEOL Ltd., Tokyo).

In the first set of experiments, mature green leaf and juvenile epicotyl tissues of Hurst Greenshaft were inoculated either with P. syringae pv. pisi race 1 (incompatible) or race 2 (compatible) and PM-ATPase activity was determined in vivo. Inhibition of PM-ATPase activity as determined by lead phosphate precipitation in cells of leaf and epicotyl tissues was observed under an electron microscope at 4, 8 and 12 hr after inoculation with bacteria. In a compatible interaction between host Hurst Greenshaft and bacterial race 2, PM-ATPase activity was inhibited, but not abolished completely, at 4

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Fig. 1, 2. Changes in the activity of PM-ATPase in cells of leaf and epicotyl tissues of *P. sativum* cvs. Hurst Greenshaft and Midoriusui inoculated with compatible or incompatible races of *P. syringae pv. pisi*. The PM-ATPase activity was detected by lead precipitation on the plasma membrane of the cells. Transmission electron micrographs show in vivo accumulation of dark-colored lead phosphate deposits (Pb) next to the plasma membrane in 6-7 day-old epicotyls or 3-4 week-old leaves incubated with *P. syringae pv. pisi* race 1 and race 2 for 4, 8, or 12 hr. Fig. 1 (left), Hurst Greenshaft, A) epicotyl, B) leaf; showing PM-ATPase activity in an incompatible interaction with race 1 and a compatible interaction with race 2. Fig. 2 (right), Midoriusui, A) epicotyl, B) leaf; showing PM-ATPase activity in a compatible interaction with race 1 and an incompatible reaction with race 2. Bar: 2 μm. Cp, chloroplast; Pb, lead phosphate precipitation; PM, plasma membrane; R1 and R2, bacterium races 1 and 2 attached to cell wall, respectively.

or 8 hr after inoculation compared to the water controls (Fig. 1). The pattern of inhibition of PM-ATPase was similar in both leaf and epicotyl tissues compared to the water controls. However, when leaf and epicotyl tissues of host Hurst Greenshaft were challenged with race 1 (incompatible interaction), the PM-ATPase activity remained unchanged compared to water-treated tissues even in a prolonged incubation (Fig. 1).

To test whether the suppression of PM-ATPase specifically correlates to compatibility between bacterial race and host cultivar, we selected the cultivar Midoriusui which demonstrated an opposite response to race 1 (compatible) and race 2 (incompatible). In compatible interactions between Midoriusui and race 1, PM-ATPase activity was significantly suppressed in both leaf and epicotyl tissues after at least 8 hr of incubation with bacteria. On the other hand, the level of PM-ATPase observed in the incompatible interaction between Midoriusui and race 2 was essentially the same as those detected in the water-treated leaf and epicotyl controls (Fig. 2). From these results, we report here that a) in an *in vivo* compatible interaction between *P.
**P. syringae pv. pisi** and *P. sativum*, PM-ATPase is temporarily inhibited in juvenile epicotyls and mature green leaves, and b) temporal inhibition of PM-ATPase activity is race-cultivar specific.

Although bacteria remained viable, proliferated and adhered to the host cell wall in both compatible and incompatible interactions during an early stage of the interaction, the inhibition of PM-ATPase activity occurred only in compatible interactions. The duration of the incubation with bacteria had apparently no significant effect on the activity of PM-ATPase in incompatible interactions compared to that of water controls. Therefore, metabolic activity of an increasing bacterial population in the compatible interaction was not significant, according to our recent study, *P. syringae pv. pisi* requires 4–6 hr to divide in pea tissues in situ.

These results suggest that phytopathogenic bacteria and fungi may possess a common mechanism that inhibits PM-ATPase activity during early events of compatible microbe-host interactions, at least in blight disease of peas. Although the inhibition of PM-ATPase and pathogenicity to the host seems to be race-cultivar specific, further study on several other cases of plant-pathogenic bacteria interactions is imperative before any conclusion can be drawn. Interestingly, the events of the restoration of PM-ATPase activity in pea plasma membrane are essentially similar to those with fungal infection. Our results presented here, together with our previous observation of the inhibition of pisatin accumulation and PM-ATPase activity by fungal suppressor, suggest that phytopathogenic bacterium can inhibit PM-ATPase activity in a fashion similar to that of fungal suppressor. Furthermore, the plausible presence of a suppresscin-like molecule in phytopathogenic bacteria suggests that the molecular role of suppresscin in facilitating pathogenicity may be similar in fungus and bacterium.

A link between PM-ATPase activity and a signal transduction pathway was also supported by the fact that inhibition in the activity of PM-ATPase by fungal suppresscin markedly shifted the intracellular pH of the cell and decreased the ion-pumping capacity of the plasma membrane in pea cells. Plasma membrane ATPase has been previously reported to translocate protons and regulate intracellular pH. Moreover, an inhibition of polyphosphoinositide metabolism and ATPase activity by fungal suppresscin in pea plasma membrane also suggest that phospholipids, essential components for the maintenance and regulation of ATPase, were also affected by suppresscin molecules. These results collectively suggest the possible involvement of PM-ATPase in the host defense response during early molecular communication between host-pathogen interaction.

Because of the indication of a bacterial suppresscin-like molecule, we are currently focusing on isolating an active fraction from *P. syringae pv. pisi* that confers suppresscin-like activity. The isolation of a bacterial suppressor and further study on the signal transduction pathway that triggers the suppression of PM-ATPase activity is imperative for a better understanding of the mechanism underlying the early plant defense response against microbial attack.

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**Literature cited**

和 文 摘 要

下間奈津美・林 昌治・Kamal A. MALIK・一瀬勇規・白石友紀・山田哲治：エンドウツつる枯れ病細菌の感染によるエンドウ原形質膜 ATPase の in vivo における阻害

エンドウ褐絹病菌が胞子発芽液中に分泌する低分子糖ペプチド（supprescin）は、宿主エンドウの原形質膜 ATPase 活性を阻害することにより初期の防御応答を抑制している。植物病原細菌が病原系状菌の supprescin と類似の機能を有する生体分子を保有するか検討を加えた。暗黒下で生育させた幼エンドウ

植物体の上胚軸あるいは成熟葉にエンドウつる枯れ病細菌を接種し、in vivo における原形質膜 ATPase 活性をリン酸鉱の沈着によって組織化学的に解析した結果、親和性関係においてのみ原形質膜 ATPase の活性が一時的に阻害された。レース・品種の異なる組み合わせにおいても原形質膜 ATPase の阻害は親和性レースを接種した組織においてのみ観察され、非親和性レースの接種では観察されなかった。植物病原系状菌の supprescin に類似の機能を有する植物病原細菌のサプレッサー分子の存在の可能性、さらに病原細菌の病原性における作用機作について論ずる。

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