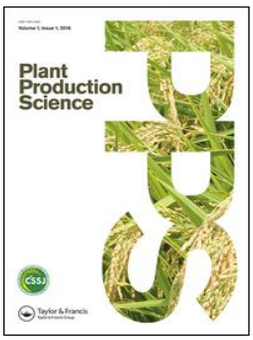

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Bacterial Impact on H_2O_2 Accumulation during the Interaction between *Xanthomonas* and Rice

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Abstract : Localization of hydrogen peroxide (H_2O_2) accumulation during the interaction between rice and *Xanthomonas oryzae* pv. *oryzae* (*X. oryzae* pv. *oryzae*) was observed by histochemical analysis and electron microscopy. The changes that occurred in an *avrXa7* mutant strain of *X. oryzae* pv. *oryzae* including the decreased production of endogenous H_2O_2, impacted on the mean level of H_2O_2 accumulation during the interaction with the plant. The results of catalase and aminotriazole treatments indicated that the changes of H_2O_2 accumulation during the interaction are induced by the impairment of endogenous H_2O_2 accumulation in *X. oryzae* pv. *oryzae*. These results suggested that bacterial pathogen is a potential source of the H_2O_2 accumulated in the interaction between rice and *X. oryzae* pv. *oryzae*.

Key words : *avrXa7*, Hydrogen peroxide, Interaction, *Xanthomonas oryzae* pv. *oryzae*.

Reactive oxygen species (ROS) accumulate transiently in plant apoplasts when attacked by a pathogen. This rapid reaction is called oxidative burst and probably functions in defense reactions directly or in signaling (Bestwick et al., 1997; Przemysław, 1997; Iwano et al., 2002; Rolke et al., 2004). During this response, hydrogen peroxide (H_2O_2) can be produced either directly or as a by superoxide dismutation. H_2O_2 can diffuse into cells and activate many of the plant defense reactions, including programmed cell death (PCD) (Apel and Hirt, 2004).

H_2O_2 released in the interaction between plant and pathogen has been considered to be of plant origin and primarily mediated by a membrane-bound NAD(P)H oxidase complex or cell wall peroxidases in plants (Torres et al., 2002), but recent evidence showed that ROS can be produced by pathogens (Li and Wang, 1999; Rolke et al., 2004; Egan et al., 2007). Although the pathogen was hypothesized to be another source of H_2O_2 production in the interaction between plant and pathogen (Deighton et al., 1999; Tenberge et al., 2002; Rolke et al., 2004), strict evidence has not been obtained to confirm this hypothesis.

Previously, we suggested the presence of endogenous H_2O_2 in phytopathogenic bacteria (Li and Wang, 1999; Li et al., 2007). We need to clarify the participation of H_2O_2 produced by the bacterial pathogen in the interaction with the plant to study the pathogenesis of phytopathogenic bacteria. *Xanthomonas oryzae* pv. *oryzae* causes bacterial leaf blight, a serious disease in rice (Leyns et al., 1984). Challenge by *X. oryzae* pv. *oryzae* provides a host interaction system for the analysis of cellular responses occurring during the hypersensitive response (HR) in rice.

The histochemical assays used to precisely localize the H_2O_2 production or accumulation during interactions between plant and bacterial pathogen, are based on the reaction of H_2O_2 with $CeCl_3$ to produce electron-dense insoluble precipitates of cerium perhydroxides, $Ce[OH]_2OOH$ and $Ce[OH]_3OOH$. This ultrastructural technique allows the precise localization of sites of H_2O_2 accumulation or production and has been used by Bestwick et al. (1995) in a study of H_2O_2 accumulation in the interaction between the plant and bacteria.

Materials and Methods

1. Bacteria

The *avrXa7* mutant (PXO0314) and a wild-type strain (PXO1865) of *X. oryzae* pv. *oryzae* were used. Both of them were kindly provided by Prof. Leach, Kansas State University. *AvrXa7* is a member of the avrBs3 avirulence gene family. The *avrXa7* gene triggers an R gene-specific plant defense reaction, which often culminates in the hypersensitive response (Büttner and Bonas, 2002). PXO0314 loses the *avrXa7* function due to partial *avrXa7* mutation as described by Cruz et al. (2000). The strains were maintained on potato sucrose agar (PSA) solid medium (1 L of boiled

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Abbreviations : H_2O_2, hydrogen peroxide; HR, hypersensitive response; ROS, Reactive oxygen species.

extract of 250 g potato tubers, 20 g of sucrose and 15 g of agar; Lee and Ronald, 2007) and cultured at 28°C for 48 hr. A single colony was cultured in Nutrient Agar (NA) liquid medium (3.0 g of beef extract, 10.0 g of agar, 8.0 g of sodium chloride, 5.0 g of peptone, 1 L of spring water; Lee and Yu, 2006) for an additional 36 hr to obtain a suspension of approximately 10^9 cells per ml. All strains were conserved in glycerol and stored at −20°C until use.

2. Virulence assay on rice plants

For the virulence analysis, 55-day-old greenhouse-grown susceptible rice plants Teyou559, Golden Century, and IIyou838 were used. All plants were grown in growth chambers kept at 28°C during the day and 25°C at night with a 14-h photoperiod and 85% humidity (Zhu et al., 2000). Rice plants were inoculated by clipping leaf tips with sterile scissors dipped in cultures of PXO1865 and PXO0314 (5×10^8 cells per ml resuspended in sterile double distilled water) (Kauffman et al., 1973). Lesion lengths were measured at 14 days after inoculation. No lesions were observed in control plants whose leaves were clipped with scissors dipped in water. In each experiment, 15 leaves were inoculated, and the mean±standard deviations of data from three independent experiments are presented. Similar results were obtained in independent experiments.

3. Electron microscopy and visualization of H_2O_2 accumulation

A histochemical method was used for H_2O_2 determination in the *X. oryzae* pv. *oryzae* strains and during the interaction with rice cultivar IIyou838 (Bestwick et al., 1997; Able et al., 2000). For the visualization of H_2O_2 accumulation in the strains, concentrated cell pellets were resuspended for 1.5 hr at 28°C in phosphate-buffered saline (PBS) containing 5 mM $CeCl_3$ (Sigma, UK). Tissue pieces (4 to 2 mm^2) were excised from inoculated leaf panels and incubated in freshly prepared 5 mM $CeCl_3$, in 50 mM Tris-maleate buffer at pH 7.5 for 1.5 hr at 28°C for visualization of H_2O_2 accumulation in the interaction system. The cerium perhydroxide deposition was monitored using transmission electron microscopy as outlined by Bestwick et al. (1997). Sections were viewed in TEM 100C (JEOL, Tokyo) at 80 kV. Four categories of cerium perhydroxide deposit density were assigned: 0, no specific deposition; 1, faint and patchy; 2, legible staining ranging from patchy to confluent; and 3, intense staining.

4. Treatment with inhibitors

Tissue segments inoculated with PXO1865 were excised and incubated for 30 min in 50 mM Tris-maleate buffer, pH 7.5, containing either 25 μg mL^{-1} bovine liver catalase (Sigma, UK), or 20 μM 3-amino-1, 2, 4-triazole (ATZ; Sigma, UK). Control tissues were incubated in Tris-maleate buffer alone. Tissues were then transferred to $CeCl_3$ solutions supplemented with inhibitors at the stated concentrations, incubated for 1 hr, and processed for transmission electron microscopy as described above.

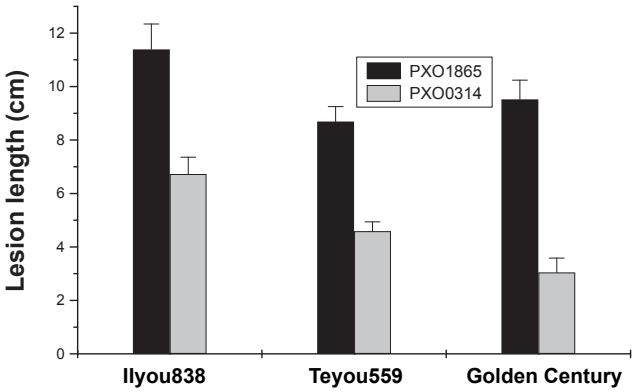

Fig. 1. *avrXa7* mutation affect *Xanthomonas oryzae* pv. *oryzae* virulence. Virulence assays were conducted by inoculating rice plants of the susceptible cultivar IIyou838, Teyou559, and Golden Century with Xanthomonas. Lesion lengths were used as a measure of virulence. PXO1865 is wild-type strain; PXO0314 is *avrXa7* mutant. On all of these rice lines, the differences between the lesions exhibited by PXO1865 and PXO0314 were highly significantly (nonparametric tests, $P<0.01$).

5. Statistical analyses

SPSS Ver. 11.5 for Windows was used for statistical analysis. The significance of differences between PXO1865 and PXO0314 was determined using nonparametric tests, including Wilcoxon Signed Ranks Test and Sign Test. The significance of differences between grouping staining into low (categories 0 or 1) and high (categories 2 and 3) classes in interactions of rice and PXO1865 or PXO0314 was determined by using x^2 analysis. Values are denoted as significant ($p<0.05$) or highly significant ($p<0.01$).

Results

1. Impact of avrXa7 mutation on virulence of *X. oryzae* pv. *oryzae*

On rice line Teyou559, Golden Century and IIyou838, the intensity of the response produced by PXO1865 was always stronger than that produced by PXO0314 (Fig. 1). At 14 days after clip-inoculation, PXO1865 exhibited 11.39±0.95 cm, 8.69±0.56 cm, and 9.52±0.72 cm lesions on Teyou559, Golden Century and IIyou838, respectively, whereas the mutant PXO0314 exhibited 6.73±0.63 cm, 4.59±0.35 cm, and 3.04±0.54 cm lesions, respectively (Fig. 1). These data indicate that the PXO0314 is less virulent than PXO1865 on rice plants (nonparametric tests, $P<0.01$).

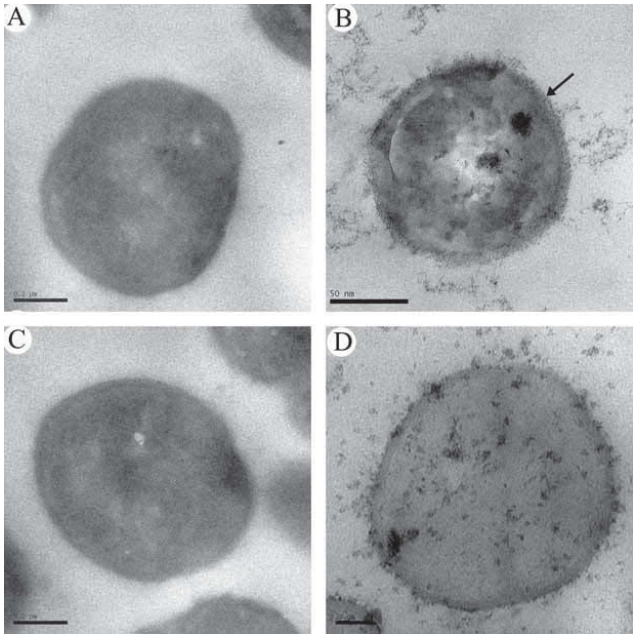

Fig. 2. Difference of endogenous H_2O_2 accumulation in *Xanthomonas oryzae* pv. *oryzae* cells. Endogenous hydrogen peroxide was localized by staining with $CeCl_3$ to form electron-dense deposits of cerium perhydroxide in *Xanthomonas oryzae* pv. *oryzae* strains. Bars are 0.2 μm in (A) and (C), 50 nm in (B) and 20 nm in (D).
(A and C) Controls are non-cerium treated cells of PXO1865 and PXO0314, respectively.
(B) Accumulation of dense deposits in the cell wall of PXO1865. Dense staining is highly localized within the wall (Category 3).
(D) No specific localization of deposits in the cell wall or other sites of PXO0314 (Category 0).

2. Difference of H_2O_2 production between mutant and wild strains of *X. oryzae* pv. *oryzae* under culture

The cellular localization of H_2O_2 in *X. oryzae* pv. *oryzae* was determined by the deposition of cerium perhydroxide. The cerium perhydroxide deposition was intensely localized in the cell wall of the wild-type (PXO1865) (Fig. 2B). In contrast, no localized deposition was detected in the cell wall of the mutant cells (PXO0314) (Fig. 2D).

3. Localization of H_2O_2 in uninoculated tissue

In noninoculated leaves, precipitates of electron-dense cerium perhydroxides, indicating the presence of H_2O_2, were located predominantly within the cell walls of xylem vessels with secondary thickening and occasionally within the walls of surrounding cells (Fig. 3A). Junctions between mesophyll cells, typically the sites of bacterial attachment to the plant cell wall, were usually free of staining (Fig. 3B).

4. Localized accumulation of H_2O_2 is detected at reaction sites

After injection, as inoculum droplets evaporated and were absorbed, bacterial cells became attached to the cell walls of rice IIyou838. PXO1865 caused stronger responses than PXO0314 within the first 5 hr of inoculation. The plasma membrane adjacent to

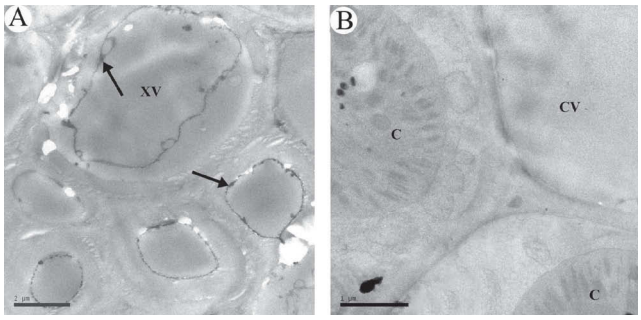

Fig. 3. Detection of H_2O_2 in uninoculated tissue.
(A) Staining with $CeCl_3$ to form electron-dense deposits of cerium perhydroxide localized to spiral thickening of the xylem vessels and parts of the associated cell wall (arrows). Bar is 2 μm.
(B) Absence of staining from a typical junction between two spongy mesophyll cells. Bar is 1 μm.
C, chloroplast: CV, central vacuole; XV, xylem vessel.

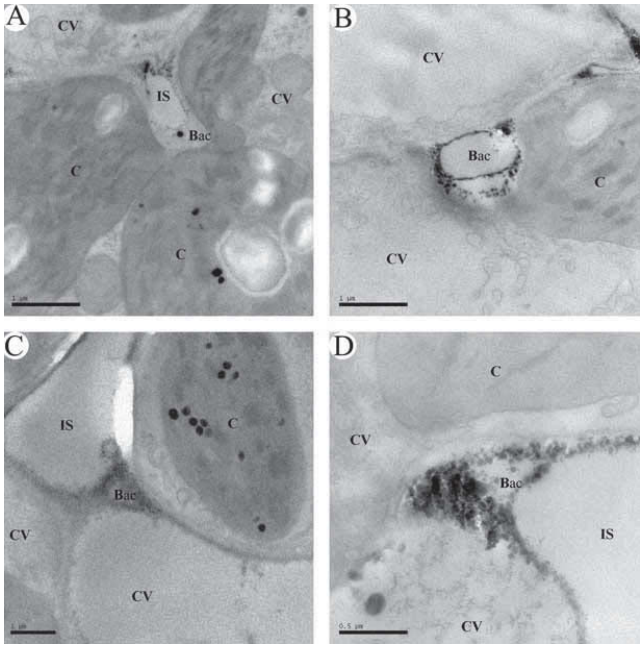

Fig. 4. Minimum intensity of staining used to quantify H_2O_2 accumulation in the cell wall as detected by the formation of cerium perhydroxides at reaction sites.
(A) Category 0, typical absence of staining.
(B) Category 1, faint and patchy staining.
(C) Category 2, dense but patchy staining.
(D) Category 3, dense and confluent staining.
(A) and (B) show tissue 16 and 8 hr after inoculation with the PXO0314, respectively; (C) and (D) show tissue 8 and 5 hr after inoculation with PXO1865, respectively.
Bac, bacterium; C, chloroplast; CV, central vacuole; IS, intercellular space. Bars are 1 μm in (A), (B) and (C) while 0.5 μm in (D).

Table 1. Quantitative assessment of staining with $CeCl_3$ to reveal H_2O_2 accumulation within the cell wall adjacent to bacteria.

Time after inoculation (hr)	Percentage of sites in each category of staining[a]							
	PXO1865[b]				PXO0314			
	0	1	2	3	0	1	2	3
3	65	27	3	5	82	15	3	0
5	13	10	7	70	61	22	9	8
8	31	10	9	50	58	12	15	15
16	51	14	20	15	74	8	13	5

[a]At least 30 sites were examined at each time from three replicate leaf samples. The minimum degrees of deposit assigned to each category of staining are as follows: 0, none; 1, faint and patchy; 2, dense but patchy; 3, confluent and dense staining. For illustrations, see Fig. 4.
[b]By 16 hr, most cells inoculated with wild-type strain had collapsed.
Statistical analysis using x^2 contingency tests showed that the differences were highly significant between the data for the PXO0314 and PXO1865 5 and 8 hr after inoculation by grouping staining into low (categories 0 or 1) and high (categories 2 and 3) classes ($P<0.001$).

Table 2. Effects of bacterial treatments on the accumulation of H_2O_2 in the cell wall adjacent to PXO1865 5 hr after inoculation.

Treatment	Percentage of sites in each category of staining in interactions between rice and bacteria[a]			
	0	1	2	3
None	10	10	5	75
Catalase	57	18	15	10
Aminotriazole	8	12	13	67

[a]Categories are as described in Table 1 and as illustrated in Fig. 4.
Statistical analysis using x^2 contingency tests showed that catalase treatment significantly reduced the frequency of sites in categories 2 and 3 compared with 0 and 1 combined ($P<0.001$), while aminotriazole did not induce significant difference in frequency of sites above ($P>0.1$).

bacteria became convoluted, and amorphous material, initiating papilla formation, was deposited between the plant plasma membrane and the cell wall. After 5 hr, vesiculation of the cytoplasm and cytoplasmic disorganization, indicative of the hypersensitive response, occurred extensively only in plant cells adjacent to PXO1865.

Preliminary observations revealed H_2O_2 accumulation in the samples treated with $CeCl_3$ 5 and 8 hr after inoculation, at reaction sites. Formation of the cerium perhydroxides was particularly striking in the plant cell walls adjacent to wild-type bacteria and was markedly localized. The most dense deposits were found adjacent to bacteria, and they extended with reducing intensity to the surrounding walls (Fig. 4A). Staining was also frequently observed in material encapsulating the bacteria. At some sites, although bacterial cells were lodged between two or three mesophyll cells, staining with $CeCl_3$ was typically absent in one cell that included chloroplasts adjacent to the cell wall, indicating differential activation of the oxidative burst dependent on the position of chloroplast (Fig. 4B).

To assess H_2O_2 accumulation objectively, we used a four-point scale to assess the appearance of deposits of cerium perhydroxides within the plant cell wall as follows: 0, no specific deposits; 1, faint and patchy; 2, dense but patchy; 3, dense and confluent deposits. The minimum degree of deposit assigned to the categories is illustrated in Fig. 4A to 4D. Fig. 4 also provides additional data on the localization of deposits within responding cells.

As shown in Fig. 4 and Table 1, accumulation of H_2O_2 occurred in response to both PXO1865 and PXO0314. The accumulation and persistence of H_2O_2 were then quantified at different times after inoculation, following assignment of a total of at least 30 reaction sites to categories. Sites were assessed for $CeCl_3$ staining, and deposits were quantified. The results presented in Table 1 clearly show that a burst of H_2O_2 accumulation was apparent about 5 hr after inoculation with the PXO1865, while it was delayed to about 8 hr after inoculation with the PXO0314. Based on the average level of H_2O_2 accumulation in at least 30 reaction sites at each time, H_2O_2 accumulation was always higher after the inoculation with PXO1865 than that with PXO0314. Analysis of data for PXO0314 and PXO1865 5 and 8 hr after inoculation, using x^2 analysis of contingency table prepared by grouping staining into low (categories 0 or 1) and high (categories 2 and 3) classes, revealed statistically significant differences between the strains

at $P<0.001$.

5. Effects of Inhibitors on H_2O_2 accumulation

To determine the specificity of $CeCl_3$ staining for H_2O_2, and the origin of the H_2O_2 generated, we conducted $CeCl_3$ staining in the presence of catalase (to decompose H_2O_2), aminotriazole (ATZ; to inhibit catalase in the plant). Sites were assessed for $CeCl_3$ staining, and deposits were quantified. The specificity of $CeCl_3$ staining for H_2O_2 was demonstrated by the striking reduction observed after treatment with catalase. By contrast, ATZ did not cause major changes (Table 2).

Discussion

The resistance (*R*) genes used in plant disease management are largely single dominant genes that direct the recognition of pathogen components encoded by avirulence (*avr*) genes; this relationship is referred to as a gene-for-gene interaction (Cruz et al., 2000). *AvrXa7* is a member of the avrBs3 avirulence gene family (Büttner and Bonas, 2002). On all of the rice plants used in this work, the virulence of strains were significantly reduced by the partial *avrXa7* mutation in *X. oryzae* pv. *oryzae* (Fig. 1). Cruz et al. (2000) reached a similar conclusion with rice line IR24. Although PXO1865 and PXO0314 are independent field strains, PXO0314 lost the *avrXa7* function caused by partial *avrXa7* mutation.

In the present study, deposits of cerium perhydroxides localized in the cell wall of *X. oryzae* pv. *oryzae*, indicative of the presence of endogenous H_2O_2, were significantly reduced in PXO0314 in comparison with that in PXO1865 (Fig. 2). These results indicated that the absence of this gene down-regulates H_2O_2 production. Although PXO1865 and PXO0314 are both field isolates, they may have a different genetic background in addition to differences in *avrXa7*. Our previous results showed that the growth rate is affected by the amount of H_2O_2 production by bacterial strains. This conclusion was confirmed by using the *ahpC* mutant strain, which has reduced H_2O_2 production (Li et al., 2007). Results showed that the growth rate of PXO1865 is higher than that of PXO0314 (unpublished data). Therefore, the higher pathogenicity of strain PXO1865 may be due to the higher level of H_2O_2 accumulation compared with PXO0314, which caused the higher growth rate of this strain. In any case, *X. oryzae* pv. *oryzae* -derived H_2O_2 plays a role in the virulence of bacteria.

Increases in cerium perhydroxide production were clearly localized at the sites of bacterial attachment to rice cell walls (Figs. 3, 4). The inhibition of perhydroxide formation by exogenous catalase confirms that H_2O_2 was the species being detected (Table 2). Interestingly, quantitative assessment confirmed that staining indicative of H_2O_2 accumulation was much more marked in the interaction between rice and PXO1865 than in the interaction between rice and PXO0314 (Table 1). Although AvrXa7, the product of *avrXa7*, is regarded as an effector protein (Büttner and Bonas, 2002) and is also suggested to be a virulence factor in *X. oryzae* pv. *oryzae* (Yang et al., 2000), our results strongly suggested that the difference of H_2O_2 accumulation in interaction systems is affected by the changes of endogenous H_2O_2 accumulation in bacteria.

The catalase inhibitor ATZ rapidly diffuses into cells, and its failure to cause increased H_2O_2 accumulation during the interaction between rice and wild-type bacteria demonstrates that reduced catalase activity in the plant does not lead to a corresponding increase in H_2O_2 concentration in the interaction system (Table 2). This result suggested that the H_2O_2 accumulation during the interaction between rice and *X. oryzae* pv. *oryzae* is impacted by the H_2O_2 produced by bacteria, and not dependent on the H_2O_2 production by plant. Therefore, the changes of H_2O_2 accumulation during the interaction are likely to be induced by the impairment of endogenous H_2O_2 accumulation in bacterial pathogen. Bacterial pathogen may be a potential source of oxidative stress during the interaction with plant. A similar view has been hypothesized in the interaction between plant and fungi (Tenberge et al., 2002; Rolke et al., 2004).

Most of the H_2O_2 was directly accumulated in the cell wall and was generally absent from the cytoplasm. The lack of staining in the cytoplasm does not reflect an inability of $CeCl_3$ to enter plant cells. In both inoculated and noninoculated rice leaves, staining of the tonoplast was occasionally encountered (unpublished data), demonstrating the potential penetration of $CeCl_3$ throughout the cytoplasm. Under similar incubation conditions, Kausch et al. (1983) reported deposition of cerium perhydroxides in peroxisomes of root parenchyma cells.

Although our findings suggested the bacterial origin of H_2O_2 production in the interaction between plant and bacterial pathogen, the role of the H_2O_2 produced by bacteria is unclear. Confirmation of the role and regulatory mechanism of H_2O_2 will require more works on the interaction between plant and bacteria. This result opens new and interesting perspectives for detailed analyses of the role of H_2O_2 in this plant-pathogen interaction.

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