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Studies on Calcium Release and H_2O_2 Level Produced by the Elicitor Induced Plant Cell by Fluorescence Probing

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Abstract Using fluorescence probing technology, we studied the mechanism and interrelations of calcium release and H_2O_2 production in situ in living tissues of tobacco and cotton plants which were induced by pathogen elicitor, salicylic acid (SA) and pectinase respectively. Results showed that (1) pathogen elicitors could induced H_2O_2 response in epidermis cells regardless of environmental calcium, but in mesophyll protoplast, H_2O_2 response could only be induced at calcium condition. Similarly, SA and pectinase induced H_2O_2 response could only be observed at calcium condition; (2) pathogen elicitors could induce calcium response in both epidermis cells and protoplasts regardless of environmental calcium, while calcium response couldn't be induced at non-calcium condition by SA and pectinase; (3) H_2O_2 response and calcium response in protoplast were faster than that in the whole cell. These results indicated that pathogen elicitors can induce the release of cell wall calcium and the cell wall calcium release is independent to pectinase. And it is concluded that free calcium influx is necessary for the oxidative burst and cell wall calcium has an irreplaceable role in defense signal transduction.

Keywords Calcium · Cotton · Hydrogen peroxide · Pathogen elicitor

Abbreviations

conc	concentration
DCFHDA	2′,7′-dichlorofluorescin diacetate
HR	hypersensitive reaction
IPTG	isopropy-β-d-thiogalactose
LMTA	Low melting-temperature agarose
SA	salicylic acid
PMSF	phenylmethyl sulfonylfluoride
ROS	reactive oxygen species
RT	room temperature

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Introduction

Oxidative burst, a rapid process of reactive oxygen species (ROS) release, is ubiquitous in plant defense and signal transduction [1, 2]. Plants usually keep levels of ROS under tight control by the production of scavenging enzymes and non-enzymatic antioxidants [1, 3]. During hypersensitive responses (HR) to pathogen attack and mechanical stress, ROS plays an important role in killing invading (avirulent) pathogens and in eliminating damaged cells (necrosis). Furthermore, it can also serve as signals that lead to the activation of other defense mechanisms [4]. It is supposed, that rapid and prolonged accumulation of hydrogen peroxide (major component of ROS) is due to membrane-bound peroxidases [5], and that calcium ions under certain conditions are capable to stimulate formation of ROS in plant cells [5, 6].

Calcium, a common signal molecular, is closely related to oxidative burst. An increasement in free calcium concentration ($[Ca^{2+}]$) is one of the general events that relay the attack signal to the internal cellular machinery to mount a biological response [7, 8]. Rather than total amount of calcium, the dynamic changes of Ca^{2+} play a decisive part in signal transduction. So, most of the reviews on calcium signaling in plants are concentrated on the regulation of changes in cytosolic $[Ca^{2+}]$ and their effects on the functioning of plant cells. The vacuole and the cell

wall are considered both as sources and buffering compartments of calcium mobilized to or expelled from the cytosol. The cell wall calcium mainly bounds to the pectin and is considered to strengthen the cell wall. However, there are no direct sufficient proofs of the cell wall calcium to show that it plays role in the formation of ROS induced by the pathogen elicitor.

The objective of this research was to further clarify if cell wall calcium is involved in generation of ROS during plant defense against pathogen attack. To achieve this goal, a method for visualisation cytosolic Ca^{2+} distribution was applied to living plant tissue. With fluorescence probe DCFHDA (for detecting H_2O_2), Fluo-3, Fura-2 and Fura-2AM (for detecting free calcium), we studied the change level of H_2O_2 and calcium in situ in the leaf cells of cotton, peanut and tobacco after the induction of elicitors from plant pathogens *Verticillium dahliae Kleb* and *Pseudomonas solanacearu*.

Materials and Methods

Plant Materials and Its Treatment

Different materials used in this paper were listed in Table 1. Seeds were marinated in 95% ethanol for 10 min and 0.1% $HgCl_2$ for 15 min, and then germinated in culture dish at 30 °C, at 80% relative humidity in growth chamber without light for 36 h. When embryo came out, culture condition was changed to 10/14 h light/dark cycle at 25 °C. And fully expanded true leaves were harvested 12–15 d after sowing.

Abaxial epidermis and mesophyll cell protoplasts were prepared according to Dodds's book [9]. Abaxial epidermis was kept in MES-KOH buffer (5 mmol/L MES, 0.5 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 10 μmol/L KH_2PO_4, Mannitol 0.45 mol/L, pH7.2). Protoplasts were kept in liquid medium (KH_2PO_4 27.2 mg/L, KNO_3 101.0 mg/L, $CaCl_2{\cdot}2H_2O$ 1480.0 mg/L, $MgSO_4{\cdot}2H_2O$ 246.0 mg/L, KI 0.16 mg/L, $CuSO_4{\cdot}5H_2O$ 0.025 mg/L, sucrose 0.5 mol/L; pH5.8). The number of protoplasts were estimated with a hemocytometer and adjusted to 10^5/mL. The viability of protoplasts was estimated by fluorescein diacetate (FDA) with slightly changes [10]. FDA (stored in acetone, 0.2 mg/mL) was added to protoplast solution to final concentration of 0.01%. After standing at RT for 10 min, protoplast was collected by centrifugation at 500 rpm for 3 min and washed with fresh buffer for two times. At last, protoplasts were examined for fluorescence using Olympus BH-2-RFCA microscopy.

Table 1 plant materials used in the paper

Name	Type	Character
EM 18	Cotton cultivar, leaf	Fungal wilt sensitive
ZM 12	Cotton cultivar, leaf and protoplast	Fungal wilt resistant
EH 4	Peanut, leaf	bacterial wilt sensitive
ZH 2	Peanut, leaf	bacterial wilt resistant
Tobacco	Tobacco, leaf and protoplast	bacterial wilt resistant

Elicitor Preparation

Verticillium dahliae Kleb was used for derivation of fungal elicitor. It was cultured in potato-sucrose medium at 25 °C for 3 d. Mycelium was harvested, resuspended in phosphate buffer (pH8.5), and homogenized at 20000 rpm for 5 min. After centrifugation at 2000×g for 20 min, the precipitation was collected, resuspended in ddH_2O, and then sterilized at 121 °C for 1 h. At last, filtrate of the suspension was used as fungal elicitor.

Pseudomonas solanacearum E. F. Smith P7 was used for derivation of bacterial elicitor. Strain was culture at 30 °C in broth medium, IPTG (1 mmol/L) was added when OD_{600} was about 0.6. After overnight induction, strain was collected, resuspended in phosphate buffer containing 1 mmol/L PMSF and then treated by sonication. After centrifugation at 10000 ×g for 30 min, the precipitation was resuspended in sterilized water and hydrolyzed at 121 °C for 2 h. Followed by centrifugation at 10000×g for 10 min, the supernatant was collected and used as bacterial elicitor.

Total sugar contents of all elicitors were estimated by Handel's method [11].

Fluorescence Probe Loading

Plant materials were loaded with probe before detection and protoplast was added with probe to 5 μmol/L. After slightly shaking, it was kept in darkness for 30 min. Then, protoplast was washed with fresh medium for three times and incubated at RT for 3 h; Epidermis was incubated with probe at 4 °C with slightly shaking for 2 h, then washed with fresh MES-KOH buffer and kept in RT for 3 h.

In Situ Detection of H_2O_2 and Calcium in Normal Cell

H_2O_2 and calcium were detected by Fluorescent probe DCFH-DA and Fluo-3 respectively. Epidermis loaded with probe was spread on microscope slide. Mixed with two drops of MES-KOH, the epidermis was fixed by cover slip. Then, it was examined by microscopy to observe guard cells. The pictures of the guard cells were taken for consideration. After that, mercury lamp of microscopy was turned on and elicitor was added to the sugar concentration of 50 μg/ml. During this process, operation should be carefully enough to keep the position of guard cell in eyeshot. Then cell fluorescence was monitored, and

fluorescence pictures were captured. Each experiment was repeated for 5 times.

Low melting-temperature agarose (LMTA) was used to fix protoplasts. LMTA and probe loaded protoplasts (1:99) were mixed and melted at 35 °C, 100 μl of this suspension was added to microwell plate. After solidification, elicitors were added and fluorescence pictures were taken as previously described. The whole experiment should be done in 3 h to avoid cell death.

In Situ Detection of H_2O_2 and Calcium Ions in Calcium Chelated Cells

Plant materials from ZM12 were treated with 5 mmol/L EGTA for 5 min first, and then incubated for 10 min. The other operations were conducted the same as the step for probe loading, and then subjected to H_2O_2 or calcium detection as previously described.

Results

H_2O_2 Production in Stimulated Plant Tissues at Calcium Condition

Figures 1 and 2 showed the fluorescence of guard cells in DCFHDA loaded epidermis after induction. Fluorescence intensity reflects the production level of H_2O_2. One point has to be mentioned that, no fluorescence occurred in the inside cell wall of guard cells.

In presence of calcium ions, fungal and bacterial elicitors could induce large amount of H_2O_2 in very short time in the guard cells of ZM12 (Fig. 1, a→e) and tobacco ((Fig. 1, o→q) respectively, but they couldn't induce oxidative burst in the bacterial wilt sensitive peanut cultivar EH 4 and fungal wilt sensitive cotton cultivar EM 18 (not show in the figure), which means the elicitors could induce oxidative burst in pathogen-resistant plants. Figure 1 showed that the initiative time and strength of oxidative burst were related to elicitor concentration. When the fungal elicitor was just added into the plate, slight fluorescence could be observed in guard cell, but no fluorescence in epidermis. After 3 min, the fluorescent intensity in guard cell increased significantly. 5 min later, the fluorescence continued to increase and already extended to the cell periphery, and the surrounding epidermis cell became fluorescent too. Figure 2 further described that the mobilization of H_2O_2 in epidermis relied on guard cell. Guard cells near nervure showed fluorescence first, then further guard cells started to produce H_2O_2, and this indicated that fluorescence distribution seemed to relate to vascular bundles in epidermis. Water-soluble substances were first translocated by conducting tissue to intercellular space and then spread between cells by diffusion effects. But the diffusion speed in intercellular space was much slower than the translocation speed in conducting tissue. As a result, the guard cells near nervure contacted with elicitor first and H_2O_2 was generated as well. It was notable that no fluorescence appeared in moat (medial wall of guard cell).

In calcium condition, SA (Fig. 1, f→h), methyl jasmonate (Fig. 1, d→k) and pectinase (Fig. 1, l→n) can also induce the production of H_2O_2. The induction of SA and methy jasmonate was very quick. Fluorescent pictures of 3 min induction showed that strong oxidative burst had already started in guard cells. Also, fluorescent intensity did not increase later. And pectinase induction was very slow. Enough H_2O_2 was generated after 5–10 min induction.

Similar observations could be seen in protoplast. The fungal and bacterial elicitors could induce oxidative burst in cotton (Fig. 3, a→c) and tobacco (Fig. 3, e→h) mesophyll protoplasts, respectively. Protoplast had faster oxidative burst speed than epidermis, which might be resulted from faster diffusion speed for the elicitors in LMTA and more sensitive of protoplasts to environmental changes and inducers. Obviously, the elicitor needs to overcome the obstacle of intercellular space and cell wall in the plant epidermis. What's more, materials from EM 18, EH 4 and ZH 2 didn't show obviously change in fluorescent intensity after elicitor's induction (pictures were not shown).

H_2O_2 Production in Stimulated Plant Tissues at Non-calcium Condition

The Fig. 3 also showed that mesophyll protoplast didn't start oxidative burst when calcium ions in the media was chelated by EGTA (Fig. 3, d and e). The results indicate that oxidative burst depends on free Ca^{2+} in media. But as shown in Fig. 4, production of H_2O_2 in epidermis guard cells from ZM12 in both media, MES buffer without calcium ions and the buffer with calcium ions chelating agent EGTA, was not inhibited under induction of the elicitor from pathogen *V. dahliae*. The fluorescent intensity increased obviously after 3–5 min induction (Fig. 4, f and k; g and l) and fluorescence continued to increase thereafter.

Figure 4 also showed the fluorescence change of ZM 12 epidermis (loaded with DCFH-DA in MES buffer without calcium ions) after the induction of SA and pectinase. The vague profile of guard cell and stronger background fluorescence showed in Fig. 4 (d, i and e, j) were due to fixation of plant materials with more low melting-temperature agarose, but as shown in N and O (Fig. 4), obvious fluorescence increasement indicated that both salicylic acid and pectinase could stimulate production of H_2O_2 in the media without free calcium ions.

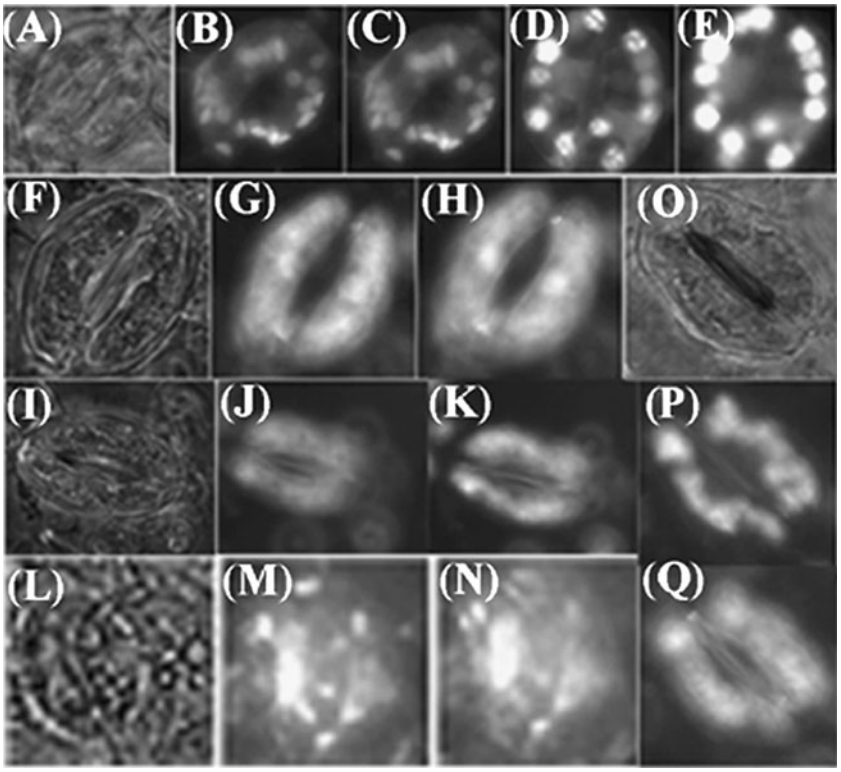

Fig. 1 The fluorescence change in DCFH-DA loaded plant leaf abaxial epidermis when stimulated by inducers in presence of calcium ions. **a**, **f**, **d**, **l** and **o**(O) were light photomicrographs of guard cells in leaf abaxial epidermis with just added inducers. **a**, **f**, **d** and **l** were from ZM 12, **o** from tobacco. The others were fluorescence micrographs of the same epidermis. In them, the photos from the same cotton cells were lined horizontally, but (**o**), (**p**) and (**q**) from the same tobacco cell were lined vertically. **b**, **d**, **g**, **j** and **p** were at 3 min after inducing, **c**, **e**, **h**, **k** and **q** were at 5 min after inducing, **m** was at 0 min, **n** at 20 min after induction. The inducers were (1) the elicitors from *V.dahliae* which were used in (**a**), (**b**) and (**c**) with conc. of 10 μg/mL and in (**d**) and (**e**) with 50 μg/mL each, (2) salicylic acid(0.36 mmol/L) in (**f**),(**g**) and (**h**), (3)methalester jasminic (0.3 μmol/L) in ((**i**),(**j**) and (**k**), (4) pectinase(4%) in (**l**),(**m**) and (**n**), and (5)the elicitor(50 μg/mL) from *P. solanacearum* P7 in (**o**),(**p**) and (**q**). The media for them were MES buffer. Excitation wavelength were 490 nm

The Influence of Elicitor, Pectinase and SA on Influx of Calcium Ions

According to above results, extracellular calcium influx is prerequisite for oxidative burst of protoplasts without cell wall, but didn't affect that in epidermic cells, so it was suspected that the calcium store in cell walls could play a part in starting oxidative burst of plants after attack from pathogens. To find the answer, we compared the effects of calcium ions in media on intracellular calcium ions.

When the epidermis of ZM12 loaded with Fluo-3AM was induced by elicitor, pectin and H_2O_2 in the media with free calcium ions, the strong calcium signals appeared in 2 min after induction (Fig. 5). Fluorescence along the cell periphery indicated an uneven distribution and the strongest fluorescence was in wall near membrane of guard cells.

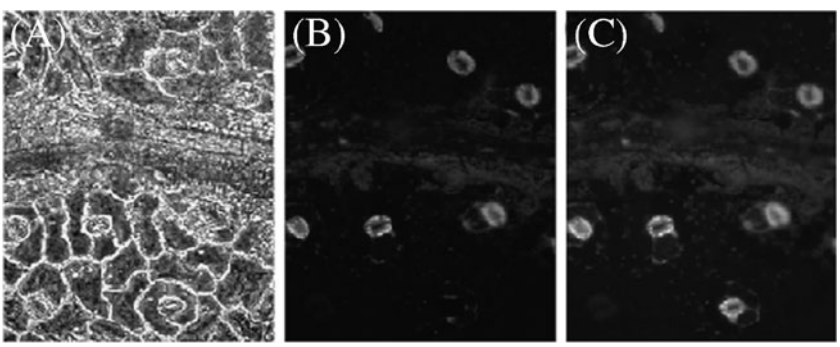

Fig. 2 The distribution of DCFH-DA loaded fluorescence in cotton leaf abaxial epidermis when induced by the elicitor from *V.dahliae*. **a** A light photomicrograph of ZM 12 epidermis with just added elicitor. **b** and **c** were fluorescence micrographs of the same epidermis after adding the elicitor(50 μg/mL). Excitation wavelength were 490 nm

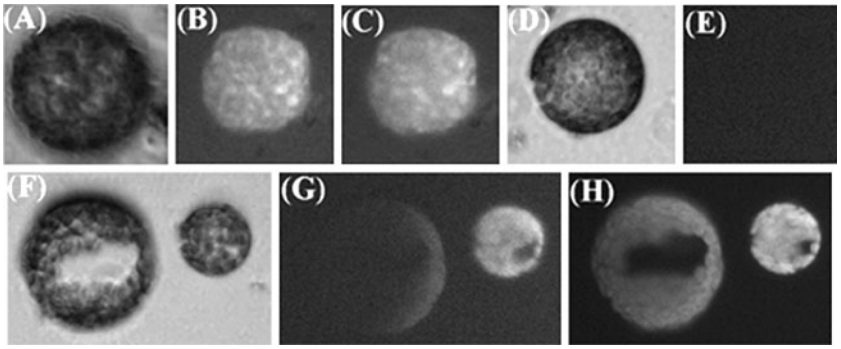

Fig. 3 The fluorescence change in DCFH-DA loaded protoplast from mesophyll cells when induced by elicitors. **a**, **d** and **f** were light photomicrograph of DCFH-DA loaded protoplast from mesophyll cells with just added the elicitors(50 μg/mL), **a** and **d** were from cotton ZM 12, **f** from tobacco. The others were fluorescence micrographs of the same cells, in which the photos from the same cells were lined horizontally. **b** and **g** were at 3 min after adding *V. dahlia* elicitor (50 μg/mL) or *P. solanacearum* elicitor (50 μg/mL) each, (**c**),(**e**) and (**h**) at 5 min after inducing. EGTA was added in DCFH-DA-loading and inducing media for (**d**) and (**e**) up to final concentration of 5 mmol/L. Excitation wavelength was 490 nm

Fluo-3AM is a membrane-permeable derivative of the ratiometric calcium indicator that used to measure the intracellular calcium concentrations by fluorescence. The fluorescence in Fig. 5 indicated that (1) elicitors can stimulate the cell wall to absorb calcium from environment or release calcium from calcium stores; (2) esterase in cell wall can hydrolyze Fluo-3AM into Fluo-3, and Fluo-3 combined with calcium in cell wall; (3) guard cell medial wall had a higher concentration of calcium stores than outer wall. Although enzymatic hybridization reduced Fluo-3AM in cytoplasm of guard cell and epidermis cell, the intracellular fluorescence can be observed in Fig. 5. These results showed that this detection technology could be used for whole cell calcium observation in plant.

Experiments with protoplasts showed the similar results. At calcium condition, mesophyll protoplast of ZM12 started to obviously increase its fluorescence under the induction of fungal elicitor after 1 min. Pictures were taken

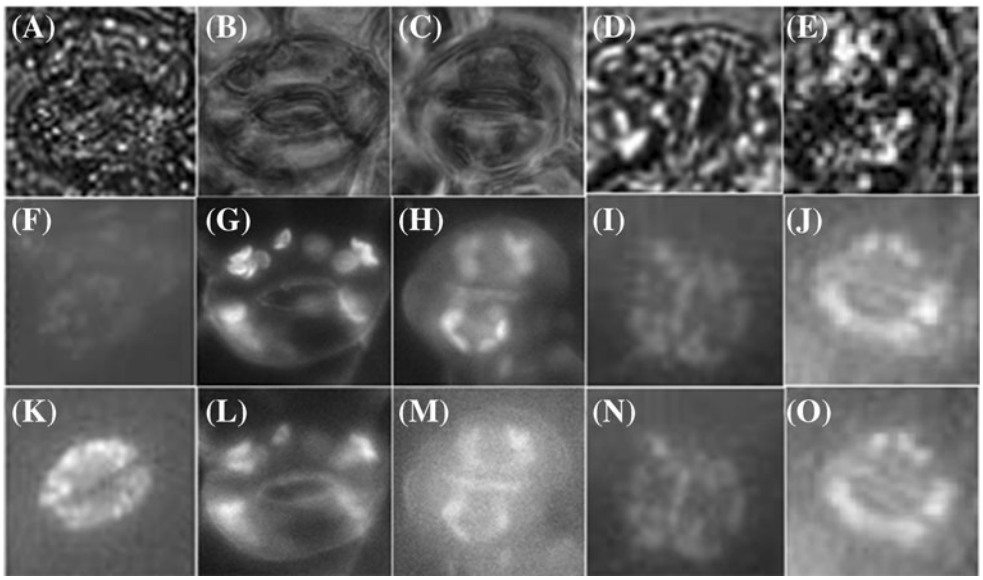

Fig. 4 The fluorescence in DCFH-DA loaded leaf abaxial epidermis from cotton when stimulated by inducers in absence of calcium ions. **a**, **b**, **c**, **d** and **e** were light photomicrographs of leaf epidermis from cotton cultivar ZM12 with just added inducers, while other photos were fluorescence micrographs of the same cells. In them, the photos from the same cells were lined vertically. The leaf epidermis in 1, 3 and 5 column were fixed by LMTA and that in 2 and 3 column were fixed by coverslip. Then epidermis in column 3 was vacuumed to remove air. EGTA was added in DCFH-DA-loading media for (**b**, **g**, **l**) and (**c**, **h**, **m**) up to final conc. of 5 mmol/L to chelating calcium ions, and there were not calcium ions were in the other inducing medium. In the fluorescence micrographs of the same epidermis, (**f**) was at 0 min and (**k**) at 7 min after adding *V. dahliae* elicitors(50 μg/mL), (**g**) and (**h**) were at 3 min, (**l**) and (**m**) at 7 min after adding the *V. dahliae* elicitors, (**i**) and (**j**) were at 0 min after adding salicylic acid (0.36 mmol/L) and pectinase(4%) each, (**n**) and (**o**) were at 7 min. Excitation wavelength was 490 nm

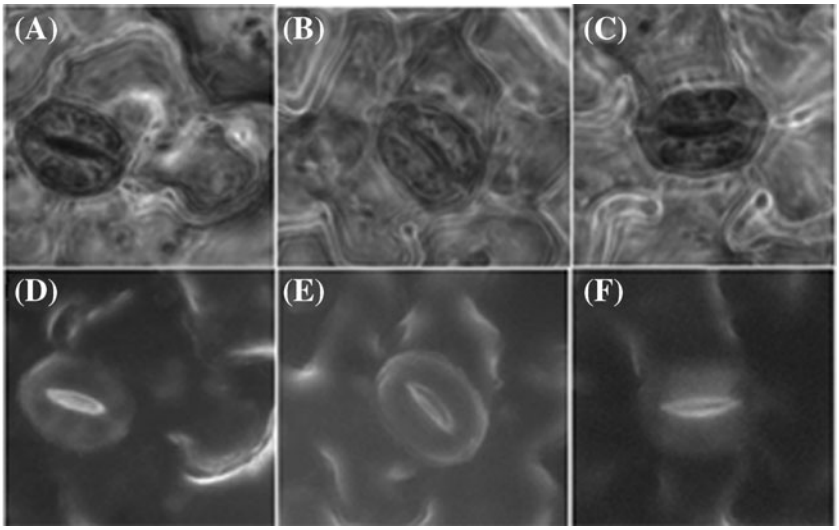

Fig. 5 The fluorescence in Fluo-3 loaded cotton abaxial epidermis when induced by elicitors, SA and H_2O_2. **a**, **b** and **c** were light photomicrographs of abaxial epidermis just treated with fungal elicitor, SA and H_2O_2 each. **e**, **d** and **f** were fluorescence micrographs of the same cells at 2 min after adding *V.dahlia* elicitor (50 μg/mL), SA(1 mmol/L) and H_2O_2 (5 mmol/L). The media were MES buffer. Excitation wavelength was 490 nm

every 5 s and listed in Fig. 6. As shown in Fig. 6, fluorescence was appeared first in parts of one protoplast, and then it became stronger and spread to the other protoplast gradually. Because calcium can't freely diffuse inner and between cells [12], the spread of fluorescence between cells could reveal that communication was carried out between them. Figure 6 also showed that elicitors could still induce the increasement of calcium ions in protoplast cytoplasm when EGTA was added. EGTA couldn't cross membrane, so it was inferred that pathogen elicitors can cause the mobilization and release of calcium in cytoplasmic calcium stores when there was no calcium in cell wall. As the release of calcium in protoplast was not affected by EGTA, the pathogen elicitors induced release of calcium in epidermis at non-calcium condition was omitted in this article. At non-calcium condition, the pathogen elicitor should lead to calcium signaling in guard cells, and this can corroborated from the elicitor induced oxidative burst at non-calcium condition.

At non-calcium condition, SA and pectinase couldn't induce calcium release in epidermis. Figure 7 showed the fluorescence of guard cells (loaded with Fura-2 or Fura-2AM) after the induction of SA or pectinase. Fura-2 is water soluble and its fluorescence intensity increased 3 times after being saturated with calcium. It could enter the space in plant cell wall but couldn't across the membrane, so Fura-2 probe could be used for observing changes of calcium ions in cell wall and Fluo-2/AM for intracellular calcium. SA and pectinase could not cause fluorescence change in cell and cell wall, which means SA and pectinase hydrolyte don't induce calcium release in calcium store of intracellular and cell wall. The fluorescence in background of Fig. 7 showed that the existed calcium is cell and cell wall. This calcium releasing could be due to the mechanical stress during the epidermis fixing.

Discussion

Oxidative burst was found to relate to hypersensitive response of plants to pathogens [13] and it is essentially incompatible interaction between plant and it's pathogen [12, 14]. So the main research materials in the paper were wilt-resistant cotton cultivar and the pathogen which induces wilt.

Oxidative burst is a rapid process of ROS release, and the major component of ROS is H_2O_2. There are two views in explaining the present of plentiful H_2O_2 in plant defense

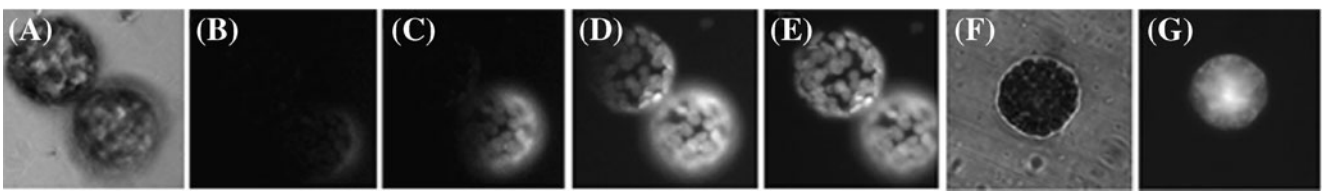

Fig. 6 The fluorescence in Fluo-3 loaded cotton protoplast when induced by elicitor from *V. dahliae*. **a** and **f** were light photomicrograph of the protoplasts from mesophyll cells of cotton cultivar ZM 12 in MES buffer and MES buffer adding EGTA each. The others were fluorescence micrographs of the same protoplasts at 0 s, 5 s, 10 s and 15 s (from (**b**) to (**e**)) and at (**g**) after elicitor induction. Excitation wavelength was 490 nm

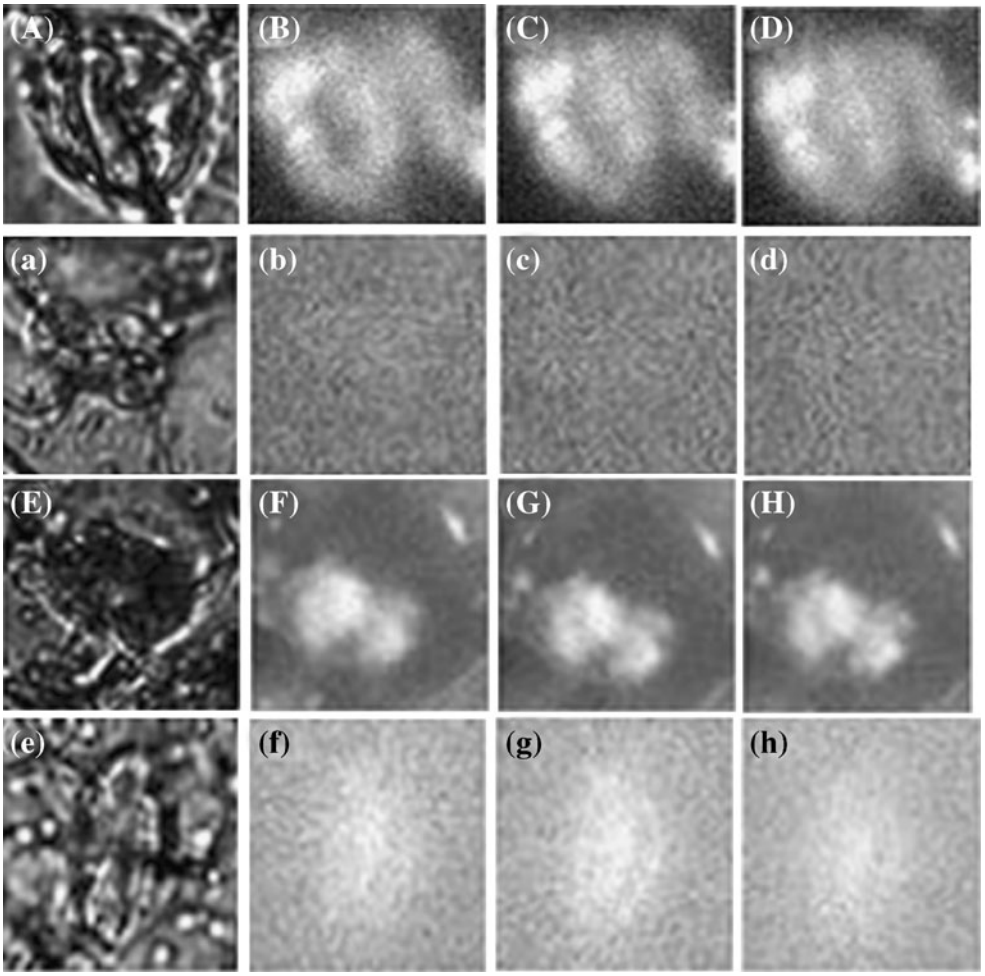

Fig. 7 The fluorescence in Fura-2 or Fura-2AM loaded cotton epidermis when induced by SA and pectinase in absence of calcium ions. **a**, *a*, **e** and *e* were light photomicrographs of Fura2 loaded (**a**, **e**) or Fura-2AM (*a*,*e*) epidermis from cotton cultivar ZM 12. The others were fluorescence micrographs of the same cells at 0 min, 5 min and 12 min after adding SA(1 mmol/L) (from **b** (*b*) to **d**(*d*)) or pectinase (4%) (from **f**(*f*) to **h**(*h*)). Media were MES buffer without calcium ions. Excitation wavelength was 380 nm

reaction. One view is that oxidative burst is catalyzed by NADPH oxidase in plasma membrane (catalytic equation was listed below). And the resulted H_2O_2 could induce further response as defense signals [15–17]; the other view is that both oxidative burst and peroxidase activity increasing will attribute to cell wall-related events[18]. H_2O_2 is the main component of oxidative burst. It is generated by pH dependent cell wall peroxidase or extracellular peroxidase [19].

$$\mathrm{NADPH} + \mathrm{O_2} \rightarrow \mathrm{O_2^-} + \mathrm{NADP^+} + \mathrm{H^+}$$
$$2\,\mathrm{O_2^-} + 2\,\mathrm{H^+} \rightarrow 2\,\mathrm{H_2O_2} + \mathrm{O_2}$$

Fluorescence in situ determination of the H_2O_2 or specific inhibition is an effective way to solve this argument. Lee confirmed that H_2O_2 wasn't present in cell wall of the stoma guard cell in tomato during 10 min induction by oligogalacturonides and chitosan [20]. Data on the NADH-dependent H_2O_2 production from Samuilov was indicative of generation of reactive oxygen species in the chloroplasts, mitochondria, and nuclear region of guard cells [21]. Similar results were obtained when epidermis and mesophyll protoplasts of cotton and peanut induced by fungal and bacterial elicitors in our study. Fluorescence, characterization of H_2O_2 generation, were presented in periphery of the cytoplasm first, and the cell wall of stoma guard cell showed no fluorescence (Figs. 1, 2, 3 and 4) at early stage. However, some people think that lack of esterase is the cause of no fluorescence in cell wall at early stage. But through Fluo-3AM indication, we proved the existence of esterase in cell wall. So the only reasonable explanation to no fluorescence in cell wall at early stage is that H_2O_2 is membrane-related events, not cell wall-related events. While, plenty of generated H_2O_2 would spread to cell wall and react with DCFH later. So, high intensity fluorescence was observed after 30 min induction with fungal elicitor.

A large number of literatures have confirmed that calcium increase and ROS in oxidative burst are closely related. The main points can be summaries as:

(1) Extracellular calcium influx is a prerequisite for oxidative burst. Evidence is that calcium influx inhibitor could prevent oxidative burst, and addition of calcium could initiate oxidative burst at inhibitor

free condition, and calcium transients often return to near basal levels just before any H_2O_2 synthesi [5].

(2) Oxidative burst triggers the influx of calcium as intermediate messengers [22]. Kawano further pointed out that the occurrence of early ROS is independent on intracellular calcium but the independent oxidative burst is very short. While the intracellular calcium dependent oxidative burst could last for a few minutes to several hours [23]. In another report, Kawano found that cytosolic [Ca^{2+}] in tobacco cell increased and reached to a peak after treated with *N*-acetylchitohexaose for 90s. The peak reached its top 10 min later, and could last for 40 min [24].

Our results supported the above views. In our research, mesophyll protoplasts of cotton could quickly release H_2O_2 under the induction of pathogen elicitor at calcium condition, but didn't at non-calcium condition; Low concentration of H_2O_2 could induce cell wall calcium aggregation or intracellular calcium increase. Both of the results supported the above views.

However, questions were still unsolved, whether oxidative burst triggers calcium influx or calcium transient trigger oxidative burst, whether oxidative burst is the product or intermediate signal in calcium signaling pathway. Some research indicate that calcium fluxes appear to function both upstream and downstream of ROS production [25].

Previous studies basically involved in the influx of extracellular calcium; the change of cellular calcium in calcium store during oxidative burst was not investigated. This may be the results from the difficulties in simultaneously detection of cellular calcium and H_2O_2. According to the above views, our results showed that the mesophyll protoplasts of (no cell wall) cotton generated calcium but not H_2O_2 after elicitor induction at non-calcium condition, and epidermis guard cells of cotton (with cell wall) generated both calcium and H_2O_2 after elicitor induction at non-calcium condition, and that epidermis guard cells of cotton (with cell wall) generated neither calcium nor H_2O_2 after pectinase and SA induction. So the questions may be answered in the following way. Incompatible elicitors could release calcium in both intracellular calcium store and cell wall calcium store, but the release events are independent. Meanwhile, the release of intracellular calcium doesn't rely on H_2O_2 (although H_2O_2 can trigger extracellular calcium influx) and doesn't induce H_2O_2. Early stage oxidative burst only depends on extracellular calcium influx. Because incompatible elicitor release enough cell wall calcium beforehand, early stage oxidative burst could be induced by elicitor at non-calcium condition. SA, oligogalacturonides and other plant-derived extracellular signals could only induce intracellular calcium influx, so they couldn't induce oxidative burst at non-calcium condition. At last the whole mechanism of pathogen elicitor induced oxidative burst can be concluded as below.

Elicitor induction → change of cell wall state→ cell wall calcium release and aggregation→calcium influx →NADPH oxidase activation → early stage oxidative burst → H_2O_2 diffusion →…→ late stage oxidative burst

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