RESEARCH PAPER

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Specific localization and measurement of hydrogen peroxide in *Arabidopsis thaliana* cell suspensions and protoplasts elicited by COS-OGA

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H_2O_2 acts as an important signaling molecule during plant/pathogen interactions but its study remains a challenge due to the current shortcomings in H_2O_2-responsive probes. In this work, ContPY1, a new molecular probe developed to specifically detect H_2O_2 was used to study the elicitation of *Arabidopsis thaliana* cells by a complex of chitosan oligomers (COS) and oligogalacturonides (OGA). The comparison of cell suspensions, protoplasts of cell suspensions and leaf protoplasts treated with different inhibitors gave indications on the potential sources of hydrogen peroxide in plant cells. The relative contribution of the cell wall, of membrane dehydrogenases and of peroxidases depended on cell type and treatment and proved to be variable. Our present protocol can be used to study hydrogen peroxide production in a large variety of plant species by simple protocol adaptation.

While plants are constantly in contact with pathogens, disease remains the exception. In plants, each cell has the possibility to detect the presence of pathogens, to activate resistance mechanisms and to emit signals which orchestrate both spatial and temporal development of this resistance. Programmed cell death (PCD) is one of these resistance processes and the signaling pathways include reactive oxygen species (ROS).

The first observations of ROS generation during plant-pathogen interactions were made on potato tubers infected by *Phytophthora infestans*.[1] The most important ROS is H_2O_2 because it is rather stable and can diffuse across cell membranes or through water channels.[2] H_2O_2 acts as an important signal molecule during the plant/pathogen interaction and is directly involved in the hypersensitive response (HR) process.[3] Following pathogen recognition, the earliest detectable reactions are the opening of specific ion channels and the formation of reactive oxygen intermediates, such as $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$.[4] Cell wall peroxidases, NADPH oxidases of the plasmalemma and chloroplasts may be mainly involved in these reactions as well as peroxisome and mitochondria.[5,6,7,8]

Among several challenges, the discrimination between various interaction pathways between plants and pathogens has still to be clarified. One way to reduce the complexity of this problem is to use elicitors. Plants have the capacity to detect potentially pathogenic microorganisms by recognizing conserved molecular motifs and to respond by producing ROS.[9] Transcriptional analysis of elicited cell suspensions have shown that the transcript and protein profiles of these cell suspensions are similar to those found in plants in response to pathogen attack.[10] Despite the abundance of studies on the different sources and mechanisms of this oxidative burst, many aspects of this response and of the specific contribution of hydrogen peroxide still remain obscure.

In this work, ContPY1, a new molecular probe developed to specifically detect H_2O_2 in plants[11] was used to study the elicitation by COS-OGA, an elicitor present during host-pathogen interaction. COS-OGA is a complex of chitosan oligomers (COS) and oligogalacturonides (OGA) with the capacity to activate plant defense-signaling pathways and initiate oxidative bursts.[12] To spatially resolve H_2O_2 production and to specifically distinguish the contribution of the intracellular *vs* extracellular cell compartments, three different model cell types were used: 1) heterotrophic cell suspensions of *Arabidopsis thaliana* with cell walls, plasmalemma and cytoplasm, but without fully mature chloroplasts (*A. thaliana* cell suspension ecotype Landsberg erecta (L-MM1) was maintained in a liquid growth medium as described by Meunier et al.[13]); 2) protoplasts obtained from heterotrophic cell suspensions of *A. thaliana* with a functional plasmalemma but no cell wall, nor fully mature chloroplasts (protoplasts were prepared by overnight digestion at 25 °C as described by Messiaen et al.[14]). Finally, 3) we prepared leaf protoplasts with functional plasmalemma and chloroplasts but without cell wall (4-wk-o *A. thaliana* (Col-0) plants grown under continuous light in hydroponic culture[15] were used to prepare protoplasts as described by Yoo et al.[16]). A distinction was made between heterotrophic and autotrophic cell types because autotrophic cells use photosynthesis to grow and need light and CO_2 supply, in contrast

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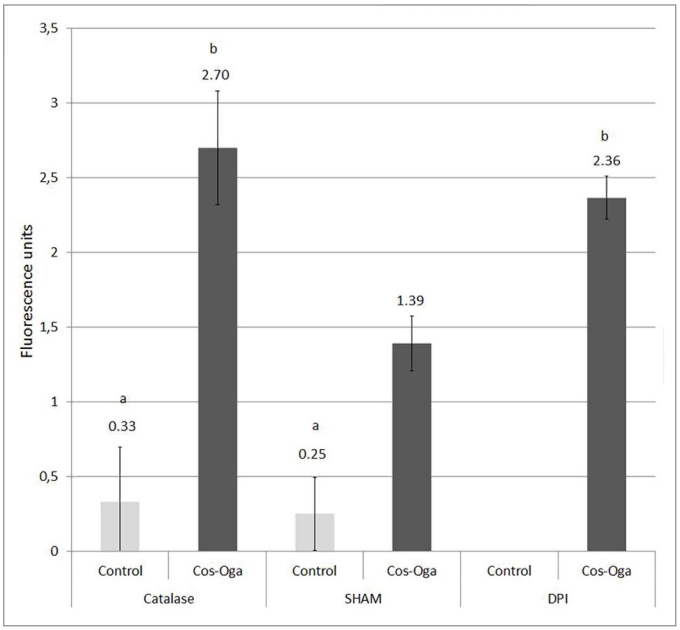

Figure 1. Means and standard deviations of ContPY1 fluorescence caused by the presence of H_2O_2 60 min after COS-OGA elicitation. The fluorescence of 200 µl of *Arabidopsis* suspension cells (approximately 600,000/ml) in presence of catalase, SHAM or DPI is shown. The FU values for control (1.81) and COS-OGA (4.94) treated suspension cells were obtained from a previous study.[11] A non-significant difference between FU was tagged with a similar letter (Statistical analysis: Tukey test $P < 0.05$)

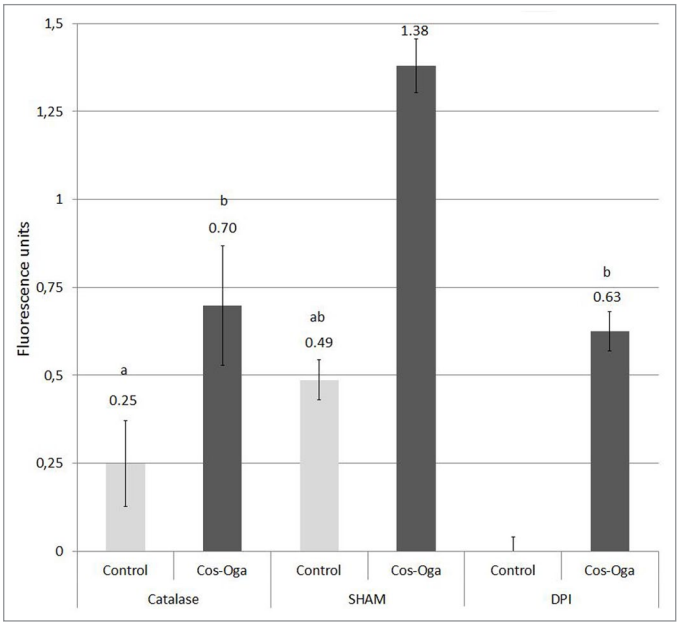

Figure 2. Means and standard deviations of ContPY1 fluorescence detected in presence of H_2O_2 60 min after COS-OGA elicitation. The fluorescence of 200 µl protoplasts from A. thaliana suspension cells (approximately 100,000/ml) in presence of catalase, SHAM or DPI is shown. The FU values for control (0.34) and COS-OGA (2.53) treated suspension protoplasts were obtained from a previous study.[11] A non-significant difference between FU was tagged with a similar letter (Statistical analysis: Tukey test $P < 0.05$)

to classical cell suspensions based on heterotrophic cells. By using different treatments (catalase, salicylhydroxamic acid (SHAM) and diphenyleneiodonium (DPI)) on these three cell types, we have been able to better understand the potential sources of hydrogen peroxide after elicitation.

For experiments on cell suspension, cells from 6 ml of a three days-old culture were obtained by sedimentation and re-suspended in 9 ml Gamborg medium (3.2 g/l Gamborg/B5, 15 g/l sucrose and phosphate buffer adjusted at pH 5.7 with KOH).[17] After washing, protoplasts from cell suspensions and leaves were re-suspended in MMG solution (4 mM MES, 73 g/l mannitol and 3 g/l MgCl2.6H2O) to obtain a final concentration of about 100,000 protoplasts/ml and 25,000 protoplasts/ml, respectively. Different protoplasts concentrations were tested to optimize measurement of ContPY1 fluorescence. For suppressing extracellular hydrogen peroxide, a catalase treatment (0.035 mg/ml, Sigma C-9322) was performed before re-suspension of cells or protoplasts. Twenty µM diphenyleneiodonium (DPI, Cayman 81050), an inhibitor of NADPH oxidases and of other flavin-containing oxidases that bind flavoproteins close to their NAD(P)H-binding site[18,19] or 2 mM salicylhydroxamic acid (SHAM, Sigma #5607), an inhibitor of peroxidases,[20] were added to Gamborg medium or MMG solution just before re-suspension. ContPY1 was synthetized as described by Dickinson and Chang.[21] The final product was solubilized at 500 µM in dimethyl sulfoxide and stored at -20 °C. ContPY1 was used at a final concentration of 5 µM. For measurement of relative fluorescence, a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific, Vantaa, Finland) was used. The experiments were performed in the dark. We choose excitation filter at 485 nm and emission filter at 538 nm. Two hundred µl of cell or protoplast suspensions were dispensed in 96-well black polystyrene cell culture microplates with eight replicates by treatment. Then, 6 µl of COS-OGA (360 ppm) for elicited microwells or 6 µl of culture medium (Gamborg medium for cells or MMG solution for protoplasts) for control microwells were added at the beginning of the experiment. To offset the initial background, the relative fluorescence measured immediately after elicitation was subtracted from the relative fluorescence measured 60 min after elicitation.

To evaluate the origin and the potential sources of H_2O_2 production detected by ContPY1, cell suspension (**Fig. 1**), protoplasts from cell suspension (**Fig. 2**) and protoplasts from leaves (**Fig. 3**) were treated with catalase, SHAM or DPI.

On the **Figure 1**, in the presence of catalase or SHAM, the control cells lost nearly all fluorescence, indicating that most H_2O_2 was extracellular and probably originated from peroxidases since catalase does not cross the plasmalemma and SHAM does not inhibit membrane dehydrogenases. DPI completely prevented H_2O_2 production, indicating that the source of electrons probably came from NAD(P)H. In the presence of COS-OGA, H_2O_2 generation by cell suspensions was always significantly higher than the respective controls. The H_2O_2 detected in the presence of catalase (FU 2.70) must have been localized inside the cells. In the presence of the elicitor, both SHAM (FU 1.39) and DPI (FU 2.36) penetrated inside the cells and partly reduced H_2O_2 production. The DPI treatment indicated that H_2O_2 production in the presence of COS-OGA

did not depend on NAD(P)H. Comparison of the SHAM and the catalase treatments suggests that approximately 51% of the hydrogen peroxide produced in response to SHAM+elicitor originated from a mechanism located inside the cells.

Protoplasts obtained from *A. thaliana* cell suspensions (**Fig. 2**) had a very low relative fluorescence level as compared with whole cells (**Fig. 1**), which indicated the importance of the cell walls in hydrogen peroxide production. The control fluorescence was intracellular, as deduced from the catalase (FU 0.25) treatment, and was not due to peroxidases since SHAM had no real effect on the control (FU 0.49). Once again, electrons used for basal H_2O_2 production by these non-elicited heterotrophic protoplasts came from NAD(P)H as shown by the absence of fluorescence when DPI was added. COS-OGA triggered also a H_2O_2 production by protoplasts. The intra cellular production of hydrogen peroxide was measured by ContPY1 in presence of catalase (FU 0.7). The presence of SHAM (FU 1.38) had less effect than catalase and DPI (FU 0.63) treatment.

Control leaf protoplasts from *A. thaliana* displayed higher background fluorescence (FU 5.93) of the ContPY1 probe, as compared with cells and protoplasts from suspensions (**Fig. 3**). When leaf protoplasts were treated with catalase (FU 3.28), they lost almost 45% of their fluorescence, which suggests that about 55% of H_2O_2 was present in the extracellular medium. However, in the presence of SHAM (FU 5.91), the basal level of H_2O_2 was not altered compared with the untreated control. The use of DPI on control leaf protoplasts (FU 4.4) indicated that roughly 25% of H_2O_2 could have originated from NAD(P)H. When leaf protoplasts were elicited with COS-OGA, the basal level of probe fluorescence was moderately increased (FU 7.64). The addition of catalase (FU 5.42) revealed that H_2O_2 induced by COS-OGA was essentially intracellular. The SHAM treatment (FU 7.53) had no effect and DPI treatment (FU 5.95) reduced moderately the H_2O_2 production.

For microscopic observations (**Fig. 4**), an Axio Imager A1 min microscope (Carl Zeiss, Göttingen, Germany) equipped with LED illumination (Colibri.2, Carl Zeiss, Göttingen, Germany) was used for fluorescence observations. Excitation was performed at λ = 505 nm with a filter set 46HE (Carl Zeiss, Göttingen, Germany) (EX BP 500/25, BS FT 515, EM BP 535/30) appropriate for ContPY1 detection. Co-localization was realized by multichannel acquisition using AxioVision4.8 software (Carl Zeiss, Göttingen, Germany). Images were taken with a Plan-Neofluar 40X/0.75 objective and an Axiocam MRc camera (Carl Zeiss, Göttingen, Germany). On **Figure 4**, suspension cells treated with COS-OGA displayed intra- and extra-cellular fluorescence. The most intense spots of fluorescence appeared to originate from many small intracellular and spherical structures ranging between 1 and 3 µm in diameter. Cell suspension protoplasts showed an important intra- and extra-cellular fluorescence (**Fig. 4B**) but no cellular structure could be distinguished. The leaf protoplast (**Fig. 4C**) also displayed intra and extra cellular fluorescence. The chloroplasts (dark spherical structure showed as green with color camera (data not shown)) seemed to co-localize with the areas of intense fluorescence.

Cell walls, plasmalemma and chloroplasts have all been described to contribute importantly to hydrogen peroxide production in

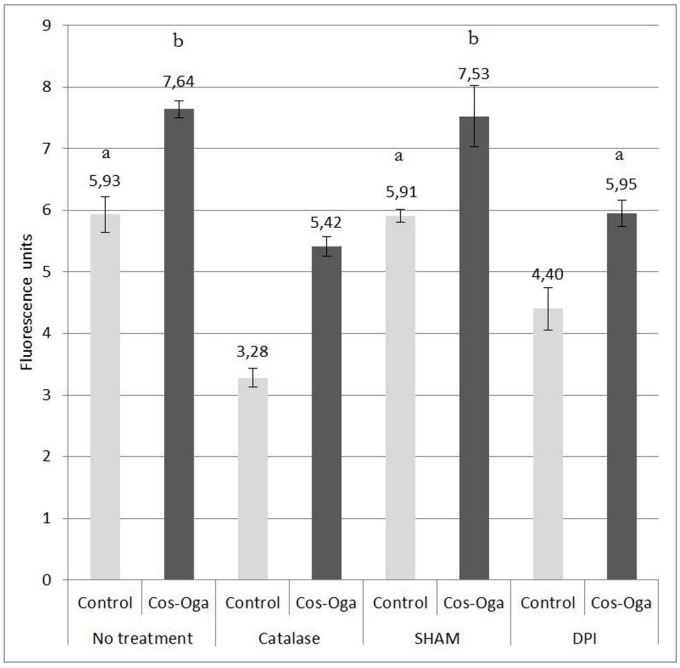

Figure 3. Means and standard deviation of ContPY1 fluorescence detected in presence of H_2O_2 60 min after COS-OGA elicitation. The fluorescence of 200 µl leaf protoplasts obtained from *A. thaliana* Col-0 (approximately 25,000/ml) in presence of catalase, SHAM or DPI was compared with control cells. A non-significant difference between readings was tagged with a similar letter (Statistical analysis: Tukey test $P < 0.05$)

plant cells.[5,22,23] In summary the present study shows the contribution of these compartments to H_2O_2 production during plant/pathogen interaction by using the elicitor COS-OGA which is a complex of oligochitosan and oligogalacturonides, to simulate the presence of a pathogen. Heterotrophic and autotrophic cells differed by the presence or absence of green chlorophyll pigmentation under brightfield microscopy and by measure of their spectrum of absorbance at 440 nm and 660 nm (data not shown).

The results obtained here were normalized with the control data of a previous study[11] that had been realized on the same heterotrophic suspension cells and protoplasts with the same protocol and without treatment by catalase, SHAM and DPI. The FU values for control (1.81) and COS-OGA (4.94) treated suspension cells, and the FU values for control (0.34) and COS-OGA (2.53) treated suspension protoplasts. Here, our results reveal large impact of catalase, SHAM and DPI treatments on suspension cells and protoplasts.

The production of hydrogen peroxide in controls of both heterotrophic suspension cells and protoplasts seemed to require NAD(P)H as an electron source, as indicated by the effect of DPI[24]. These authors showed that the O2-reducing activity of peroxidases producing $O_2^{\cdot-}$ and H_2O_2 in the presence of NADH is strongly inhibited by DPI. The impact of catalase revealed a difference between these two cell types: H_2O_2 production sites seemed to be mainly extracellular for cell suspensions and intracellular for protoplasts prepared from cell suspensions. The extracellular production of H_2O_2 by cell suspension was due to peroxidases as deduced from the effect of SHAM. Protoplasts from cell suspensions showed an

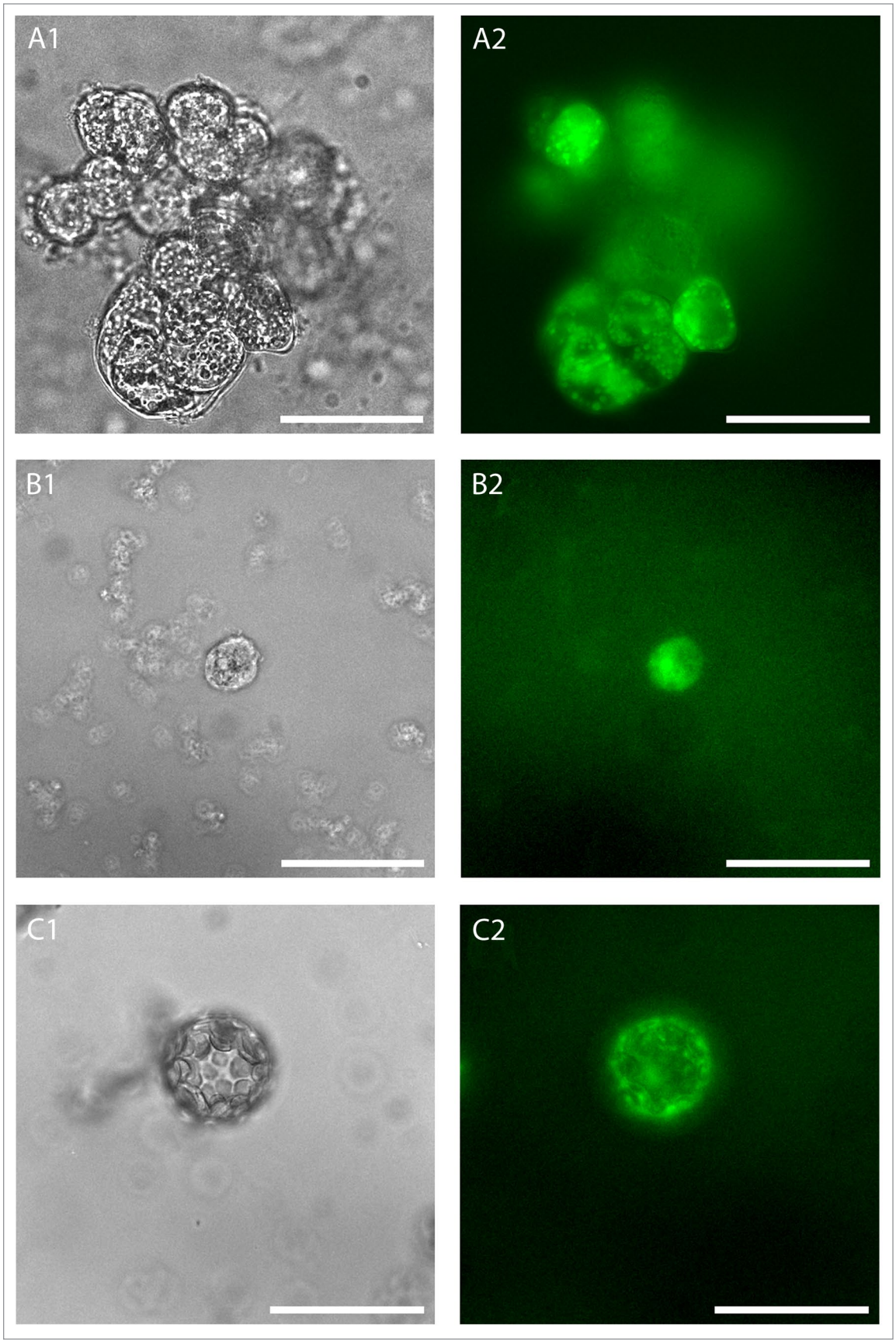

Figure 4. Fluorescence imaging of hydrogen peroxide one hour after COS-OGA elicitation on *A. thaliana*. (**A**) cells suspension; (**B**) protoplasts from cells suspension; (**C**) Leaf protoplasts. ContPY1 was used to detect hydrogen peroxide. Scale bar = 50 µm.

intracellular production of H_2O_2 independent of peroxidase activity. Cell wall peroxidases are known to contribute to the apoplastic ROS oxidative burst.[22,25] However, results obtained from protoplasts suggest an undefined intracellular source of H_2O_2 that is independent of light (experiments were performed in the dark) and cell wall peroxidases but dependent upon NAD(P)H as an electron source. The photosynthetic leaf protoplasts showed a high basal level of hydrogen peroxide generation. This production seemed mainly intracellular, independent of peroxidase activity and, contrary to both other cell types, independent of NAD(P)H. These results also suggest a difference in hydrogen peroxide homeostasis between heterotrophic and autotrophic cell types.

After COS-OGA elicitation, cell suspension and protoplasts strongly increased hydrogen peroxide production, in contrast to leaf protoplasts which responded relatively weakly. In contrast to the control, H_2O_2 production by cell suspensions was mainly intracellular and NAD(P)H- and peroxidase-independent. The photosystems were not active since experiments were all performed in the dark. Fluorescence microscopy also showed an intracellular fluorescence associated to spherical structures with diameters ranging between 1 and 3 µm, maybe not fully mature chloroplasts.[26] The source of H_2O_2 production in protoplasts from cell suspensions was also strongly modified after elicitation. Upon COS-OGA addition, protoplasts from cell suspensions showed an important extracellular production of H_2O_2 that remained highly dependent on NAD(P)H as an electron source and became partially peroxidase-dependent. Thus, both peroxidases and RboH seem to be potential candidates for this extracellular hydrogen peroxide production. Nevertheless, for about 25% of the hydrogen peroxide production, the response was intracellular and of an unknown origin. The leaf protoplasts revealed mainly an intracellular hydrogen peroxide production but did not show any drastic modification after elicitation by COS-OGA. The fluorescence microscopy of leaf protoplasts revealed co-localization of highly fluorescent patches and chloroplasts despite the fact that the experiment was realized in the dark.

From these experiments, the following conclusions can be drawn. First, our results corroborate those of O'Brien et al. (2012) who estimates that peroxidases account for approximately 50% of the hydrogen peroxide production during the oxidative burst in cell suspension.[10] According to Zurbriggen et al. (2009), chloroplast-derived ROS are essential for the progression of leaf cell death during biotic stress, but do not play any significant role in the induction of defense-associated genes.[27] The presence of fully mature chloroplasts in leaf protoplasts could explain the high level of basal hydrogen peroxide production caused by the protoplasting process, followed by a relatively weaker increase after elicitation. The impact of DPI on cell suspensions and their protoplasts confirms a flavin-containing oxidase system as a potential intracellular source of ROS as described by Allan and Fluhr.[28] However, our results indicate that part of this intracellular H_2O_2 was produced from DPI-insensitive mechanisms, suggesting that other important intracellular mechanisms are probably at work. Second, the mechanisms that carry out normal H_2O_2 production during unstressed biological processes are not necessarily the same as the ones involved after elicitation. Thus, plant defense signaling would modulate the contribution of different mechanisms of H_2O_2 production. Third, in terms of response to COS-OGA elicitation, cell wall peroxidases and NAD(P)H oxidases do not seem to be the only and most important actors in H_2O_2 production.

Finally, the observations of drastic differences between these three A. thaliana cell types suggest that plant cells are able to orchestrate differently their sources of H_2O_2 production as a function of their needs and capabilities. A good picture of the hydrogen peroxide homeostasis of plants will therefore be only possible by studying intact plants. This paper provides a basis for a better understanding of hydrogen peroxide production and allows its study in intact A. thaliana plant. ContPY1 could now be used for the specific detection of hydrogen peroxide produced in response to different types of plant and microorganisms interactions. Our protocol enables the study of a large range of plant species by minor protocol adaptations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Fluoroskan Ascent FL, AxioVision4.8 software, Axio Imager A1 min microscope, LED illumination Colibri.2, Plan-Neofluar 40X/0.75 objective and an Axiocam MRc camera.

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