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Validation of the boronate sensor ContPY1 as a specific probe for fluorescent detection of hydrogen peroxide in plants

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Studying the implication of hydrogen peroxide in biological processes in plants remains a challenge due to the current shortcomings of H_2O_2-responsive probes. The use of ContPY1, a new fluorescent probe, which is highly selective and sensitive for H_2O_2, was investigated. To validate the use of ContPY1 on plants, we have generated protocols employing cells suspensions and leaves, and measured specifically H_2O_2 production by plants using spectrofluorometry and microscopy.

In biological processes, an important reactive oxygen species (ROS) is hydrogen peroxide (H_2O_2) due to its high stability and its ability to diffuse across cell membranes through water channels.[1] It has been suggested that H_2O_2 could act as a direct cell-to-cell messenger.[2] Furthermore, H_2O_2 is well known for its involvement in wounding response and in plant/pathogen interactions. It is also directly implicated in the hypersensitive response (HR).[3,4] Following pathogen recognition, the earliest detectable reactions are the opening of specific ion channels and the formation of reactive oxygen intermediates, such as superoxide anion, H_2O_2 and hydroxyl radical.[5] The major challenge for practical sensing of H_2O_2 in biological environments is the availability of water-soluble probes that respond to H_2O_2 selectively over other competing cellular ROS, such as superoxide, nitric oxide, and lipid peroxides.[6] To date, several types of dyes are used: the Amplex Red/peroxidase system,[7] phosphine-containing fluorophores,[8] luminescent lanthanide complexes,[9] the genetically encoded biosensor HyPer,[10] and the 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) which is currently the most commonly employed dye to measure H_2O_2 in plant.[11-13] The key limitations of H_2O_2-responsive probes are the interfering background fluorescence from competing ROS, some potential side reactions with thiols that are present in high concentration in the cells, the need for external activating enzymes, the lack of membrane permeability, and/or the lack of water solubility requiring the use of organic co-solvents. DCFDA can diffuse passively through the cellular membrane and react with intracellular esterases generating 2',7'-dichlorodihydrofluorescein (DCFH), a non-fluorescent compound. Fluorescence is only observed when DCFH is oxidized to 2',7'-dichlorofluorescein (DCF). The specific drawbacks of DCFDA are its susceptibility to autoxidation, an increased background fluorescence upon continued exposure to light,[6] a lack of specificity toward hydrogen peroxide, and the necessity of the simultaneous presence of cellular peroxidases and H_2O_2 to oxidize DCFH into DCF.[14,15]

This paper describes the validation of ContPY1, a new fluorescent probe, as a specific sensor of H_2O_2 in plants. This compound, first reported by Chang (2011), is highly selective for, and sensitive to, H_2O_2. It also possesses properties amenable to biological imaging applications, among which its passive loading into living cells. This type of fluorescent probes presents several advantages: high sensitivity, high stability, low background emission, long lifetime, low environmental sensitivity, high specificity, and independence from an activating enzyme.[6] The Chang group used these fluorescent probes to study the production of H_2O_2 in living cells of different organisms, but not in plants, which are more complex due to the nature of their cell wall and the presence of chloroplast subcellular specific compartments. For the present study, protocols were developed to follow hydrogen peroxide production with ContPY1 on protoplasts and cell cultures of *Arabidopsis thaliana*, using a spectrofluorimeter, and on *Arabidopsis thaliana* (L.) Heynh. leaves by fluorescence microscopy.

ContPY1 was synthetized as described by Dickinson and Chang[16] excepted for the final purification step in which the crude reaction product was solubilized in ether and petroleum ether was added slowly until a light pink solid precipitated. The

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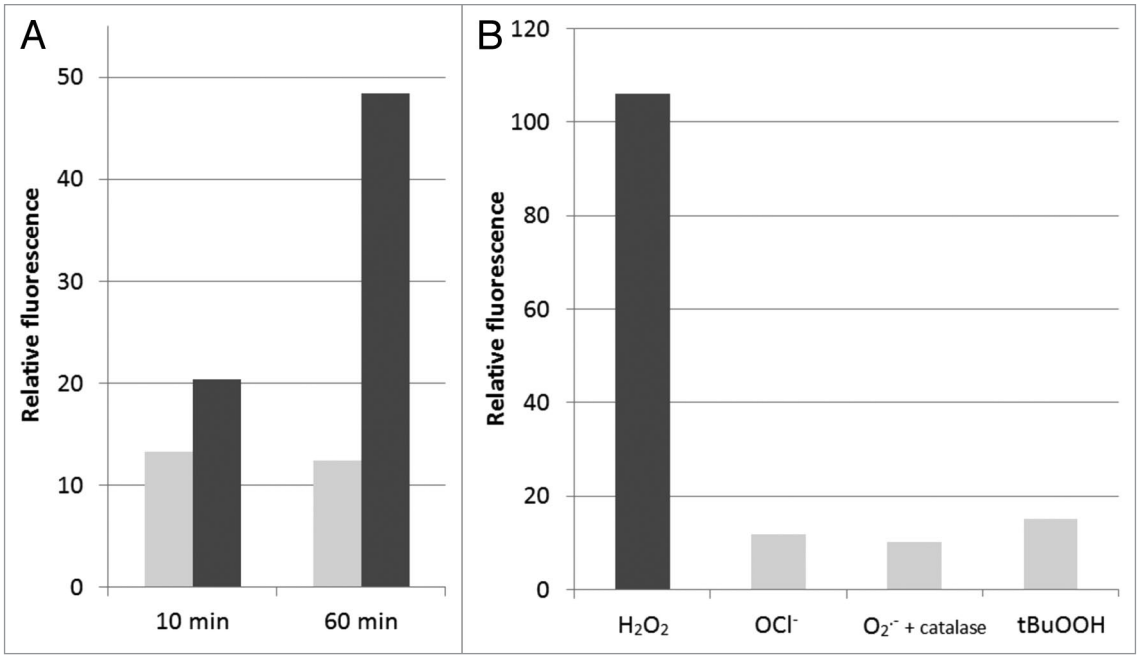

Figure 1. (**A**) Relative fluorescence of the ContPY1 probe (5 µM in HEPES pH 7), the control in light gray and in presence of H_2O_2 (250 µM) in dark gray. (**B**) Relative fluorescence of the ContPY1 probe (5 µM in HEPES pH7) in presence of H_2O_2 (1.5 mM) (dark gray) and with other reactive oxygen species (1.5 mM) (light gray).

presence of the desired ContPY1 was confirmed by spectroscopic analysis, including the observation of a molecular ion pic at 553 for [M^+] in the mass spectrum. The final product was solubilized at 500 µM in DMSO as a stock solution and stored at –20°C.

ContPY1 fluorescence in the presence of hydrogen peroxide was assessed spectrofluorimetrically (Fluoroskan Ascent FL with excitation band of 485 nm and emission band of 538 nm) 10 and 60 min after addition of 5 µl of a 10 mM H_2O_2 solution to 195 µl of a 5 µM ContPY1 solution in HEPES (pH 7) and comparing these results with a control solution lacking H_2O_2 (**Fig. 1A**). The relative emission of the control was stable over time and appeared to correspond to the basal level of fluorescence for a 5 µM solution of ContPY1 in HEPES at pH7. Addition of H_2O_2 resulted in a strong fluorescence after 60 min. This result confirmed the ability of the ContPY1 probe to fluoresce in the presence of H_2O_2 over a time scale of at least one hour. To confirm the specificity described by Chang,[6] 3 other reactive oxygen species were tested. A concentration of 1.5 mM was chosen for H_2O_2, OCl^-, $O_2^{\cdot-}$ (with catalase to suppress H_2O_2 production by spontaneous dismutation of $O_2^{\cdot-}$) and tBuOOH (**Fig. 1B**). Potassium superoxide and sodium hypochlorite were used to generated $O_2^{\cdot-}$ and OCl^- respectively. Without catalase, the H_2O_2 was generated by spontaneous dismutation of $O_2^{\cdot-}$ and reacted with ContPY1 to show a relative fluorescence response (data unpublished). In the presence of 5 µM of ContPY1, only H_2O_2 was able to increase the relative fluorescence after 60 min of incubation.

As ContPY1 had never been used on plants, the response of ContPY1 and DCFDA was compared during cell elicitation, a process that generates large amounts of H_2O_2. The literature describes successful ROS detection with DCFDA.[12,17] The response of *A. thaliana* cells to COS-OGA (a complex of chitosan oligomers (COS) and oligogalacturonides (OGA) with the capacity to activate pathogen defense-signaling pathways and oxidative burst), probably linked to oxidative burst, has been amply demonstrated.[18] A cell culture of *A. thaliana* ecotype Landsberg erecta (L-MM1) was maintained as described by Meunier et al.[19] Protoplasts were prepared by overnight digestion at 25°C[20] of a cell suspension. For the experiments on cell culture, 6 ml of a 3-d-old cell culture were obtained by sedimentation and re-suspended in 9 ml of Gamborg medium (3.2 g/l Gamborg/B5, 15 g/l sucrose, and phosphate buffer adjusted to pH 5.7 with KOH). After washing, the protoplasts were re-suspended in MMG solution (4 mM MES, 73 g/l mannitol, and 3 g/l $MgCl_2.6H_2O$) to obtain a concentration of about 10^5 protoplasts/ml. DCFDA and ContPY1 were added at a final concentration of 5 µM. Two hundred µl of cells or protoplasts suspensions were dispensed in 96 well black polystyrene cell culture microplates with 8 replicates by treatment. Then, 6 µl of COS-OGA (360 ppm) for elicited microwells or 6 µl of culture medium (Gamborg medium for cell or MMG solution for protoplast) for control microwells were added at the beginning of the experiment. The relative fluorescence of the control and elicited microwells was measured by spectrofluorimetry 60 min after elicitation and the background response was subtracted (measurement of relative fluorescence just after COS-OGA addition) (**Fig. 2**). Addition of COS-OGA to cell suspensions induced a significant increase of the relative fluorescence of DCFDA and ContPY1 (**Fig. 2A** and **B**). The dissimilarity of scale values between ContPY1 and DCFDA could be explained by their difference of brightness and the difference between

the amount of H_2O_2, solely detected by ContPY1, and the total quantity of ROS measured indiscriminately by DCFDA. The respective optical and functional properties of both fluorochromes and their compatibility with optical parameters of the spectrofluorimeter could also partially explain these differences. Regarding the measurement of elicited protoplasts and cells suspension with ContPY1, our results revealed a significant increase of the relative fluorescence, when compared with the response of non-elicited protoplasts and cells suspension (**Fig. 2A** and **C**). In contrast to what was observed when DCFDA-based detection was applied to cells suspension (**Fig. 2B**), no significant difference between elicited protoplasts and the control could be measured using DCFDA fluorescence (**Fig. 2D**). In addition, the extent of the fluorescence was particularly weak, both for the elicited protoplasts and the control. These weak values of the relative fluorescence measured with DCFDA in protoplasts could be explained by the loss of cell wall known to be as an important sources of ROS (**Fig. 2B** and **D**).[21]

In plant leaves, the release of hydrogen peroxide can be induced by wounding. Its appearance can be monitored with DAB or DCFDA probes.[4,22] To validate the use of ContPY1 on leaves, we incubated 2-week-old *A. thaliana* (Col-0) plants (grown under continuous light in a hydroponic culture)[23] in 30 µM of ContPY1 in W5 solution (2mM MES, 9 g/l NaCl, 18.4 g/l CaCl2.2H2O, and 0.37 g/l KCl) during 1 h. The ContPY1 concentration was increased compared with cells and protoplasts suspensions to decrease the illumination intensity during imaging. The plants were washed twice in W5. The leaves were placed on a microscope slide and wounded with a needle. For microscopy observations, an Axio Imager A1 min microscope (Carl Zeiss), equipped with a led (LED illumination Colibri.2 (Carl Zeiss) for fluorescence was employed. Excitation was performed at λ =

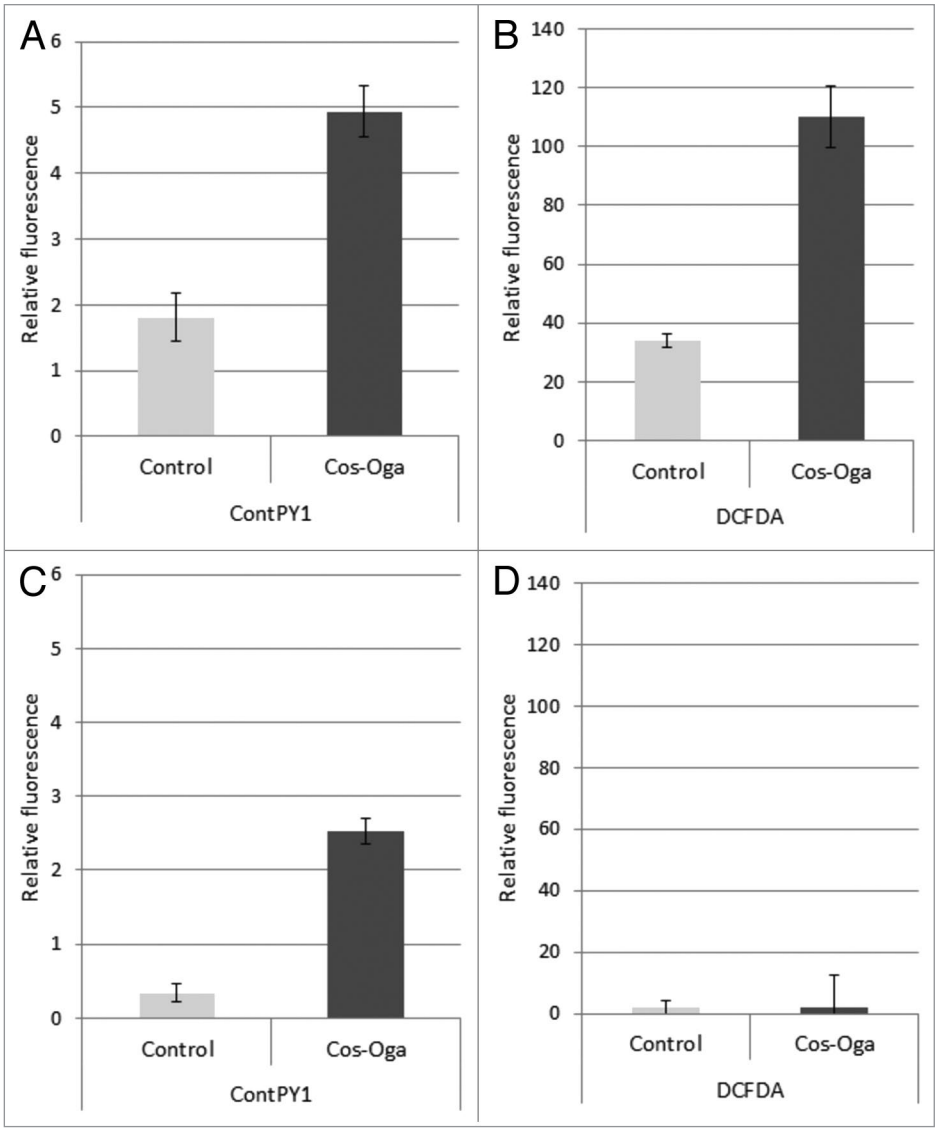

Figure 2. (**A**) and (**B**): Relative fluorescence of ContPY1 (**A**) and DCFDA (**B**) added to elicited suspension-cultured cells (COS-OGA) and non-elicited cells (Control). (**C**) and (**D**): measure of relative fluorescence of ContPY1 and DCFDA on protoplast of cell culture for elicited (COS-OGA) and non-elicited protoplast (Control). Notes: Results and standard deviation were based on 8 biological replicates.

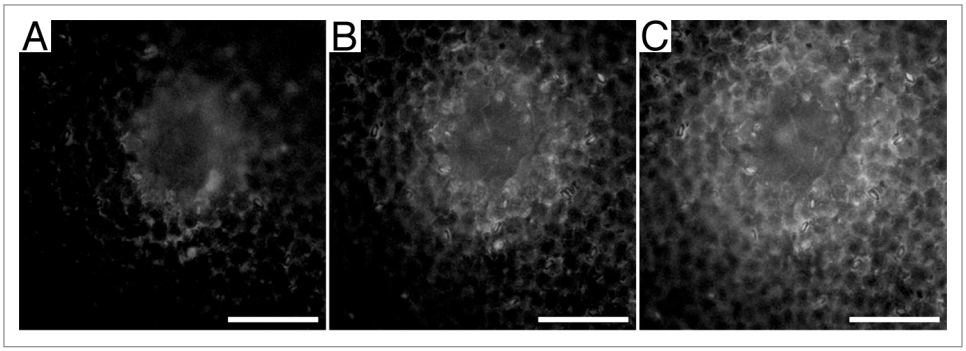

Figure 3. Time lapse acquisition of a wounded leaf of *Arabidopsis thaliana*. The leaves were incubated during 1 h in 30 µM ContPY1. (**A**), (**B**), and (**C**) represent wounding after 1, 20, and 40 min, respectively. Scale bars = 100 µm.

505 nm and a filter set 46HE (Carl Zeiss) (EX BP 500/25, BS FT 515, EM BP 535/30) appropriate for ContPY1 was used for the detection of fluorescence. Pictures were automatically captured after 1, 20, and 40 min with the AxioVision 4.8 software (Carl Zeiss). Images were taken with a Plan-Neofluar 40X/0.75 objective and an Axiocam MRc camera (Carl Zeiss). The same acquisition conditions were maintained for the 3 images. **Figure 3** shows the increase of fluorescence in close proximity to the wounds after respectively 1, 20, and 40 min.

In summary, these results demonstrate the ability of ContPY1 to specifically detect hydrogen peroxide in cells, protoplast suspensions, and plants leaves. ContPY1 possesses several advantages over other probes, namely: specificity for hydrogen peroxide, stability, low environmental sensitivity, and independence from any activating enzyme.[6] ContPY1 can also be used to detect both intra and extra cellular hydrogen peroxide production. This property stands in stark contrast with DCFDA which requires an enzymatic hydroxylation by intracellular esterases to be transformed into DCFH in order to react with hydrogen peroxide only in the intracellular medium.[24,25] Moreover, ContPY1 does not require genetic construction in plants, like the encoded biosensor HyPer.[10] ContPY1 thus represents a powerful tool for the study of hydrogen peroxide production in a large variety of plants and cell suspensions.

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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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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