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Reactive oxygen species as transducers of sphinganine-mediated cell death pathway

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Long chain bases or sphingoid bases are building blocks of complex sphingolipids that display a signaling role in programmed cell death in plants. So far, the type of programmed cell death in which these signaling lipids have been demonstrated to participate is the cell death that occurs in plant immunity, known as the hypersensitive response. The few links that have been described in this pathway are: MPK6 activation, increased calcium concentrations and reactive oxygen species (ROS) generation. The latter constitute one of the more elusive loops because of the chemical nature of ROS, the multiple possible cell sites where they can be formed and the ways in which they influence cell structure and function.

Key words: hydrogen peroxide, long chain bases, programmed cell death, reactive oxygen species, sphinganine, sphingoid bases, superoxide

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A new transduction pathway that leads to programmed cell death (PCD) in plants has started to be unveiled.[1,2] Sphingoid bases or long chain bases (LCBs) are the distinctive elements in this PCD route that naturally operates in the entrance site of a pathogen as a way to contend its spread in the plant tissues.[2,3] This defense strategy has been known as the hypersensitive response (HR).[4,5]

As a lately discovered PCD signaling circuit, three connected transducers have been clearly identified in Arabidopsis: the LCB sphinganine (also named dihydrosphingosine or d18:0); MPK6, a mitogen activated kinase and superoxide and hydrogen peroxide as reactive oxygen species (ROS).[1,2] In addition, calcium transients have been recently allocated downstream of exogenously added sphinganine in tobacco cells.[6]

Contrary to the signaling lipids derived from complex glycerolipid degradation, sphinganine, a metabolic precursor of complex sphingolipids, is raised by de novo synthesis in the endoplasmic reticulum to mediate PCD.[1,2] Our recent work demonstrated that only MPK6 and not MPK3 (commonly functionally redundant kinases) acts in this pathway and is positioned downstream of sphinganine elevation.[2] Although ROS have been identified downstream of LCBs in the route towards PCD,[1] the molecular system responsible for this ROS generation, their cellular site of formation and their precise role in the pathway have not been unequivocally identified. ROS are produced in practically all cell compartments as a result of energy transfer reactions, leaks from the electron transport chains, and oxidase and peroxidase catalysis.[7]

Similar to what is observed in pathogen defense,[3] increases in endogenous LCBs may be elicited by addition of fumonisin B1 (FB1) as well; FB1 is a mycotoxin that inhibits ceramide synthase. This inhibition results in an accumulation of its substrate, sphinganine and its modified forms, leading to the activation of PCD.[1,2,8] The application of FB1 is a commonly used approach for the study of PCD elicitation in Arabidopsis.[1,2,9-11]

An early production of ROS has been linked to an increase of LCBs. For example, an H_2O_2 burst is found in tobacco cells

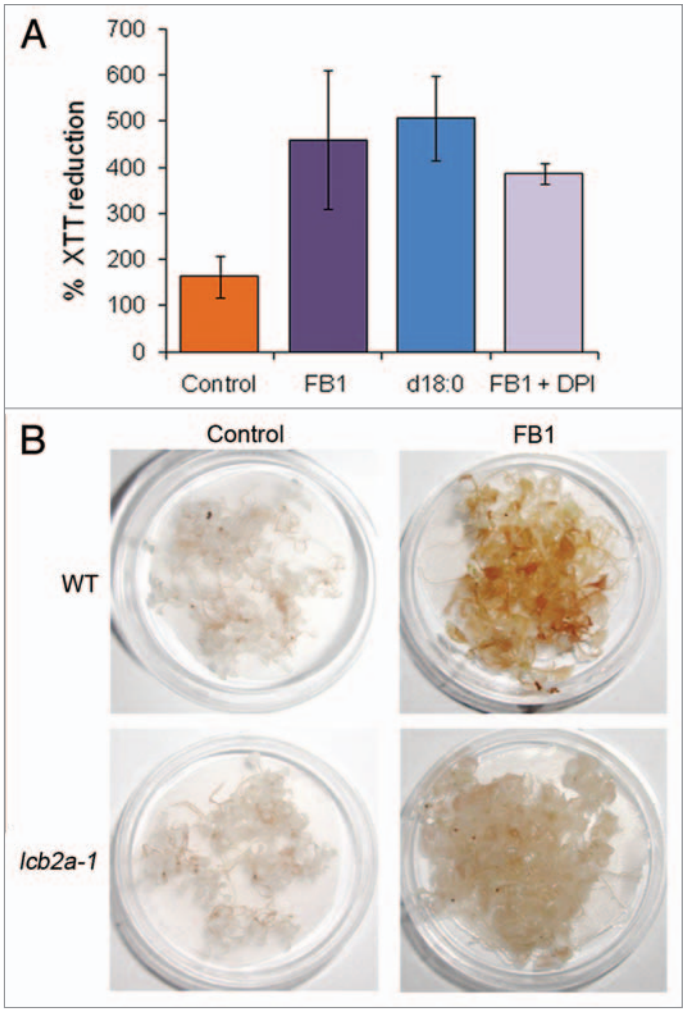

Figure 1. ROS are produced at early and long times in the FB1-induced PCD in *Arabidopsis thaliana* (Col-0). (A) Superoxide formation by Arabidopsis protoplasts is NADPH oxidase-independent and occurs 60 min after FB1 or sphinganine (d18:0) exposure. Protoplasts were obtained from a cell culture treated with cell wall lytic enzymes. Protoplasts were incubated with 10 μM FB1 or 10 μM sphinganine for 1 h. Then, cells were vacuum-filtered and the filtrate was used to determine XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt] reduction as described in references 28 and 29. DPI was used at 50 μM. (B) H_2O_2 formation in Arabidopsis wt and *lcb2a-1* mutant in the presence and absence of FB1. Arabidopsis seedlings were exposed to 10 μM FB1 and after 48 h seedlings were treated with DAB (3,3-diaminobencidine) to detect H_2O_2 according to Thordal-Christensen et al.[30]

after 2–20 min of sphinganine supplementation,[12] and superoxide radical augmented in the medium 60 min after FB1 or sphinganine addition to Arabidopsis protoplasts (**Fig. 1A**). In consonance with this timing, both superoxide and H_2O_2 were detected in Arabidopsis leaves after 3–6 h exposure to FB1 or LCBs.[1] However, the source of ROS generation associated with sphinganine elevation seems to not be the same in both species: in tobacco cells, ROS formation is apparently dependent on a NADPH oxidase activity, a ROS source consistently implicated in the HR,[13,14] while in Arabidopsis, superoxide formation was unaffected by diphenyliodonium (DPI), a NADPH oxidase inhibitor (**Fig. 1A**). It is possible that the latter oxidative burst is due to an apoplastic peroxidase,[15] or to intracellular ROS that diffuse outwards.[16,17] These results also suggest that both tobacco and Arabidopsis cells could produce ROS from different sources.

It has been suggested that the H_2O_2 burst associated with the sphinganine signaling pathway leads to the expression of defense-related genes but not to the PCD itself in tobacco cells.[12] It is possible that ROS are involved in the same way in Arabidopsis, since defense gene expression is also induced by FB1 in Arabidopsis.[9] In this case, it will be important to define how the early ROS that are DPI-insensitive could contribute to the PCD manifestation mediated by sphinganine.

The generation of ROS (4–60 min) found in Arabidopsis was associated to three conditions: the addition of sphinganine (**Fig. 1A**), FB1 (**Fig. 1A**) or pathogen elicitors.[15] This is consistent with the MPK6 activation time, which is downstream of sphinganine elevation and occurs as early as 15 min of FB1 or sphinganine exposure.[2] All of them are events that appear as initial steps in the relay pathway that produces PCD.

In order to explore a possible participation of ROS at more advanced times of PCD progression, we detected in situ H_2O_2 formation in Arabidopsis seedlings previously exposed to FB1 for 48 h. As shown in **Figure 1B**, formation of the brown-reddish precipitate corresponding to the reaction of H_2O_2 with 3,3'-diaminobenzidine (DAB) was only visible in the FB1-exposed wild type plants, as compared to the non-treated plants. However, when *lcb2a-1* mutant seedlings were used, FB1 exposure had a subtle effect in ROS formation. This mutant has a T-DNA insertion in the gene encoding subunit LCB2a from serine palmitoyltransferase (SPT), which catalyzes the first step in sphingolipid synthesis[18] and the mutant has a FB1-resistant phenotype.[2] These results indicate that mutations in the *LCB1*[1] and *LCB2a*[2] genes (coding for the subunits of the heterodimeric SPT) that lead to a non-PCD phenotype upon the FB1 treatment, are unable to produce H_2O_2. In addition, they suggest that high levels of hydrogen peroxide are produced at advanced times in the PCD mediated by LCBs in Arabidopsis.

Exposure of Arabidopsis to an avirulent strain of *Pseudomonas syringae* produces an endogenous elevation of LCBs as a way to implement defense responses that include HR-PCD.[3] In this condition, we clearly detected H_2O_2 formation inside chloroplasts (**Fig. 2A**). When ultrastructure of the seedlings tissues exposed to FB1 for 72 h was analyzed, integrity of the chloroplast membrane system was severely

affected in Arabidopsis wild-type seedlings exposed to FB1.[2] Therefore, we suggest that ROS generation-LCB induced in the chloroplast could be responsible of the observed membrane alteration, as noted by Liu et al. who found impairment in chloroplast function as a result of H_2O_2 formation in this organelle from tobacco plants. Interestingly, these plants overexpressed a MAP kinase kinase that activated the kinase SIPK, which is the ortholog of the MPK6 from Arabidopsis, a transducer in the PCD instrumented by LCBs.[2]

In addition, we have detected alterations in mitochondria ultrastructure as a result of 72 h of FB1 exposure (**Fig. 2B**). These alterations mainly consist in the reduced number of cristae, the membrane site of residence of the electron transport complexes. In this sense, it has been shown that factors that induce PCD such as the victorin toxin, methyl jasmonate and H_2O_2 produce alterations in mitochondrial morphology.[20-22] In fact, some of these studies propose that ROS are formed in the mitochondria and then diffuse to the chloroplasts.[22-24]

It is reasonable to envisage that damage of the membrane integrity of these two organelles reflects the effects of vast amounts of ROS produced by the electron transport chains.[25,26] Recent evidence supports the destruction of the photosynthetic apparatus associated to the generation of ROS in the HR.[26] At this time of PCD progression, ROS could be contributing to shut down the energy machinery in the cell, which ultimately would become the point of no-return of PCD[27] as part of the execution program of the cell death mediated by LCBs.

In conclusion, we propose that ROS can display two different functional roles in the PCD process driven by LCBs. These roles depend on the time of ROS expression, the cellular site where they are generated, the enzymes that produce them, and the magnitude in which they are formed.

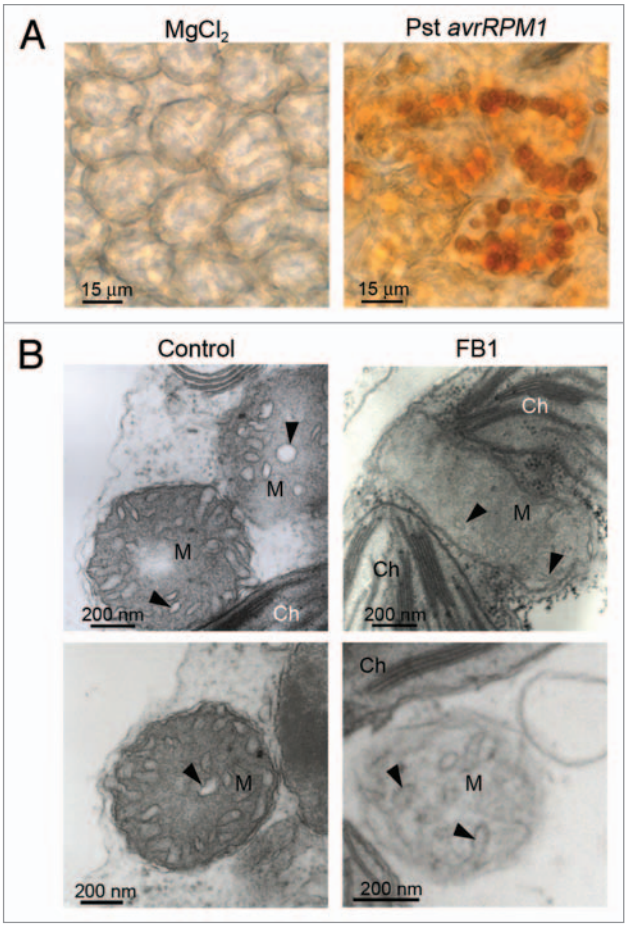

Figure 2. Conditions of LCBs elevation produce H_2O_2 formation in the chloroplast and perturbation in the membrane morphology of mitochondria. (A) Exposure of Arabidopsis leaves to the avirulent strain *Pseudomonas syringae* pv. *tomato* DC3000 (*avrRPM1*) (or Pst *avrRPM1*) induces H_2O_2 formation in the chloroplast. Arabidopsis leaves were infiltrated with 1 x 10^8 UFC/ml Pst *avrRPM1* and after 18 h, samples were treated to visualize H_2O_2 formation with the DAB reaction. Controls were infiltrated with 10 mM $MgCl_2$ and then processed for DAB staining. Then, samples were analyzed in an optical photomicroscope Olympus Provis Model AX70. (B) Effect of FB1 on mitochondria ultrastructure. Wild type Arabidopsis seedlings were treated with FB1 for 72 h and tissues were processed and analyzed according to Saucedo et al.[2] Ch, chloroplast; M, mitochondria; PM, plasma membrane. Arrows show mitochondrial cisternae. Bars show the correspondent magnification.

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