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Article Addendum

Chitosan as a MAMP, searching for a PRR

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Chitosan, a deacetylated chitin derivative, behaves like a general elicitor, inducing a non-host resistance and priming a systemic acquired immunity. The defence responses elicited by chitosan include rising of cytosolic H^+ and Ca^{2+}, activation of MAP-kinases, callose apposition, oxidative burst, hypersensitive response (HR), synthesis of abscissic acid (ABA), jasmonate, phytoalexins and pathogenesis related (PR) proteins. Putative receptors for chitosan are a chitosan-binding protein, recently isolated, and possibly the chitin elicitor-binding protein (CEBiP). Nevertheless, it must be pointed out that biological activity of chitosan, besides the plant model, strictly depends on its physicochemical properties (deacetylation degree, molecular weight and viscosity), and that there is a threshold for chitosan concentration able to switch the induction of a cell death programme into necrotic cell death (cytotoxicity).

Recognition of microbe-associated molecular patterns (MAMPs), by pattern recognition receptors (PRRs), represents the major trait of innate immunity common to plants and animals. In plant immunity, MAMPs, more commonly known as general elicitors, include lipopolysaccharides (LPS), peptidoglycans, flagellin and fungal cell wall fragments (chitin/chitosan oligomers), phospholipids, oxylipins, fatty acids, sterols, proteins, double stranded RNA and methylated DNA, able to elicit a host defence response by binding to specific PRRs. In this view, chitosan, a deacetylated chitin derivative, behaves like a general elicitor, inducing a non-host resistance, by a PRR-mediated recognition, and priming a systemic acquired immunity (or systemic acquired resistance, SAR).[1] The defence responses elicited by chitosan include: raising of cytosolic Ca^{2+}, activation of MAP-kinases, callose apposition, oxidative burst, hypersensitive response (HR), synthesis of abscissic acid (ABA), jasmonate, phytoalexins and pathogenesis related proteins (PR) (Fig. 1 and Table 1).[2-20]

Recently, in their work entitled 'Early events induced by chitosan on plant cells', Amborabé and colleagues[21] provided a novel and original insight on the early processes elicited by chitosan in plant. They showed that the effect of chitosan on the plasma membrane H^+-ATPase activity occurred at least 30 min after treatment, i.e., earlier than other events triggered by chitosan and mentioned above (callose, oxidative burst, HR, phytoalexins, PR proteins). However, the references provided by the authors are somewhat incomplete and, according to our opinion, they did not consider some important topics related to chitosan-induced resistance in plant.

In their discussion, they hypothesized on the presence of a putative receptor for chitosan, without taking into account the work of Chen and Xu[22] on the isolation of a chitosan-binding protein, possibly a receptor. They also did not consider that chitosan induces the expression of a receptor-like kinase (RLKs) gene[15] and the activation of MAP-kinase pathway in different plant species.[12-15] Moreover, a plasma membrane receptor for chitin has been identified in rice cells, both at gene and protein level.[23] The mature chitin elicitor-binding protein (CEBiP) structurally differs from the two major classes of PRRs, the receptor-like proteins (RLPs) and the RLKs, both groups containing extracellular leucine-reach reapeats (LRRs). CEBiP has a transmembrane domain at the C terminus, but lacks of LRRs and intracellular kinase domains normally present in RLKs, like the receptor FLAGELLIN SENSITIVE 2 (FLS2). Two lysine motifs (LysM) are present in the extracellular portion of CEBiP, involved in chitin perception. It is supposed that receptors with extracellular LysM motifs are responsible for chitin sensing, such as Nod-factor receptor kinases (NFR1 and NFR2), involved in the symbiotic

Table 1 **Defence responses elicited by chitosan**

Plant responses	Ref.
Calcium transient	3,11
Plasma membrane H^+-ATPase inhibition	21
MAP-kinase activation	12,15
Callose apposition	4,16,18
Reactive oxygen species	12,13,14,20
Hypersensitive response/ Programmed cell death	11,14,20
Abscisic acid	19
Jasmonate	6
Phytoalexins	5,10,17
Pathogenesis related proteins	2,8,9,13,15
Systemic acquired resistance	14,18

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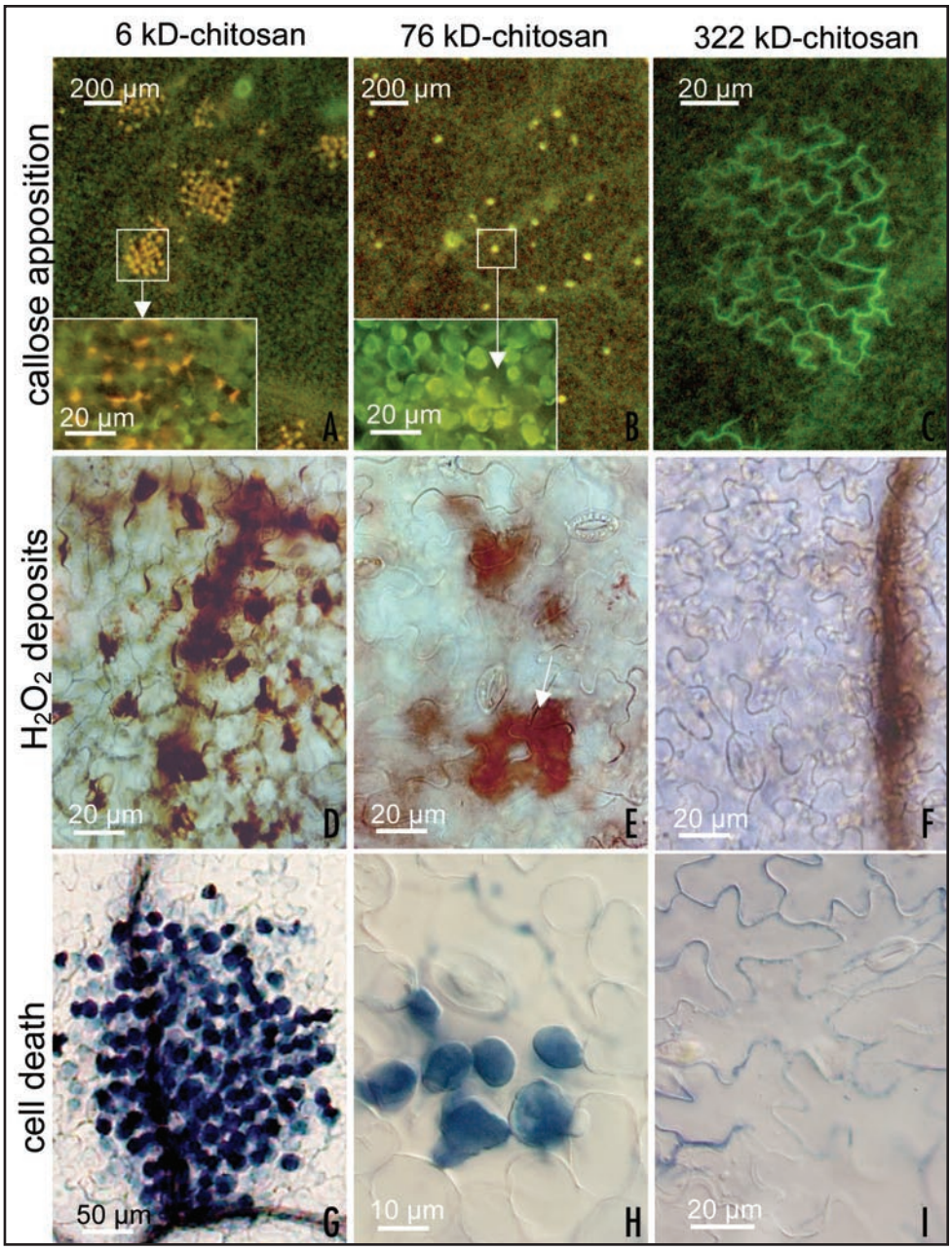

Figure 1. Different responses induced on *Phaseolus vulgaris* leaves by treatment with solutions of chitosan with 85% deacetylation degree and different molecular weights; all solutions have been prepared at 0.15% w/v in 0.05 M acetic acid and adjusted at pH 5.6. Callose detection with aniline blue (A–C) 12 h after treatment shows that 76 kD-chitosan elicits the formation of a network of small bright yellow fluorescent spots (B) due to callose apposition between the plasmalemma and the cell wall of some mesophyll cells (see the enlargement in the inset), while 6 kD-chitosan induces lesions involving numerous cells fluorescing in yellow-orange, possibly as consequence of the overlap of phenolics autofluorescence and callose fluorescence (see the enlargement in the inset). In 322 kD chitosan-treated leaves numerous green-fluorescent patches (C), due to chitosan deposits, are present on the leaf epidermis along cell walls, but rarely callose apposition is present. Detection of H_2O_2 deposits (as brownish precipitates in D–F) with 3-3′-diaminobenzydine (DAB), 24 h after 6 kD-chitosan treatment, indicates that the lesions in (A) are constituted by necrotizing cells as a consequence of extensive H_2O_2 deposition (D). At the same time, leaves treated with 76 kD-chitosan show moderate H_2O_2 deposits limited to the same mesophyll cells involved in callose apposition, and often localized around the substomatal cavity into which chitosan can permeate (E, arrow). No H_2O_2 deposition is present in 322 kD-chitosan treated plants (F). Evans blue staining to detect dead cells (stained in blue) 24 h after treatment (G–I) shows that the lesions in (A) have already evolved in extensive necrotic cell death, while only some of the cells with callose deposition visible in (B) had turned to programmed cell death (H) (as previously shown with other techniques). No dead cells are present in leaves treated with 322 kD-chitosan (I).

signaling between leguminous plants and arbuscular mycorrhiza fungi or rhizobial bacteria in root nodule formation.[23,24] Therefore, it is possible that chitosan recognition also occurs by a putative chitosan-binding protein with extracellular LysM domains, the latter playing a key role in chitin recognition. Interestingly, knockdown of CEBiP gene by RNA interference resulted in the suppression of the chitin-induced defence response, whereas treatment with LPS did not affect ROS generation in CEBiP-RNAi cell lines.[23]

Amborabé and colleagues,[21] while discussing the chitosan-induced cytotoxicity, did not consider that this elicitor, depending both on its concentration and its physiochemical properties (deacetylation degree, molecular weight and viscosity),[5,16,25] can activate a HR, i.e., a programmed cell death (PCD) phenomenon at the onset of the SAR. In other words, it exists a threshold concentration, for each chitosan type, able to switch PCD into necrotic cell death (cytotoxicity), that should be evaluated for each considered plant model.[11,14,20] In this view, it is of fundamental importance, when using chitosan as elicitor, to assess and report its physiochemical properties, as well as to consider that the type of acid solvent may be determinant for the biological activity.[14]

Finally, as well as for other elicitors, the concentration and physicochemical properties of chitosan employed in field experiments on plant induced resistance are decisive in determining the induction of priming (the capacity for augmented defence expression in plant after pathogen challenge) or the activation of plant direct defences, the latter a less effective defence strategy and more costly in term of plant fitness.[26,27]

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