Insights into the plant defense mechanisms induced by *Bacillus* lipopeptides

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Cyclic lipopeptides as elicitors of systemic resistance

Members of the *Bacillus* genus are well known for the production of multiple bioactive molecules potentially inhibitory for phytopathogens. Among these antimicrobial compounds, cyclic lipopeptides (cLPs) of the surfactin, iturin and fengycin families have well-recognized biocontrol-related activities (Ongena and Jacques 2008). By facilitating biofilm formation and/or cell spreading, surfactins and iturins may first contribute to an efficient root colonization by the producing strain. Secondly, due to their antifungal activity, the involvement of iturins and fengycins has been demonstrated in the antibiosis-based biocontrol of *Bacillus* strains against various pathogens and in different plant species. A third way for beneficial *Bacillus* isolates to provide plant protective effect is through the stimulation of the plant immune system (Induced Systemic Rresistance). We recently demonstrated that surfactins and fengycins retain such eliciting activity. In bean, tomato and tobacco, pure cLPs provided a significant induced protective effect similar to the one induced by living cells of the producing strain. Experiments conducted on bean and tomato showed that overexpression of both surfactin and fengycin biosynthetic genes in the naturally poor producer *B. subtilis* strain 168 was associated with a significant increase in the potential of the derivatives to induce resistance (Ongena et al. 2007).

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Surfactins and, to a lower extend fengycins, can thus be specifically perceived by the host plant to mount some defensive response against subsequent pathogen ingress. We have shown that such macroscopic disease

reduction may be related to some metabolic changes associated with plant defense responses. Upon root treatment with lipopeptide-overproducers, major changes were first observed in the so-called oxylipin pathway which generates a wide array of biologically active secondary metabolites derived from unsaturated fatty acids (Blée 2002). The activity of the lipoxygenase enzyme (LOX) introducing molecular oxygen into linolenic and linoleic acids to yield either 9- or 13-hydroperoxides is significantly increased in infected leaves in the first 48 h after *Botrytis* inoculation. This is concomitant with an increase in the global activity of all hydroperoxide-degrading enzymes expressed as lipid hydroperoxidase activity. It is thus obvious that some of the end-products involved in the defense response of plants such as jasmonates or fungitoxic oxylipins may accumulate in the primed tissues to take part in the restriction of pathogen ingress. As revealed by TLC bioassay with *C. cucumerinum*, a clear accumulation of non-polar antifungal compounds also occurred in the infected leaves of plants bacterized with lipopeptide over-producers. Preliminary data strongly suggest that these compounds do not derive from the oxylipin metabolic route but rather are of phenolic nature. They may correspond to phenylpropanoids deriving from the PAL enzyme.

On another hand, surfactin-type lipopeptides are the main products from *B. subtilis* S499 that are recognized by tobacco cells in culture to mount early defense-related responses. Treatment with surfactin, but not iturin and fengycin, triggers a general modification of ion fluxes due to membrane depolarization. Extracellular medium alkalinisation, most probably mediated by plasma membrane H^+-ATPase, is coupled with K^+ and chloride effluxes. Surfactin treatment also induces a significant production of reactive oxygen species within minutes. The complete inhibition of this surfactin-induced oxidative burst by DPI shows that it mainly originates from the NADPH oxidase system. As revealed by the use of various chelators and/or channel blockers, both pH change and oxidative burst are regulated by Ca^{2+} influx, phospholipases A2/C and by dynamic changes in protein phosphorylation.

Some stimulation of defense enzymes is delayed but also occurs within 6-9 h post surfactin treatment. By analogy with whole plants, enhanced LOX activity indicates the possible induction of the oxylipin pathway. The perception of surfactin by tobacco cells also resulted in the stimulation of the PAL enzyme and in obvious changes in the phenolic content suggesting some reorientation of the phenylpropanoid pathway. Further metabolic profiling studies are being performed to confirm this hypothesis. All these results allowed us to dress a first picture of the early and later events elicited by surfactin (Fig. 1).

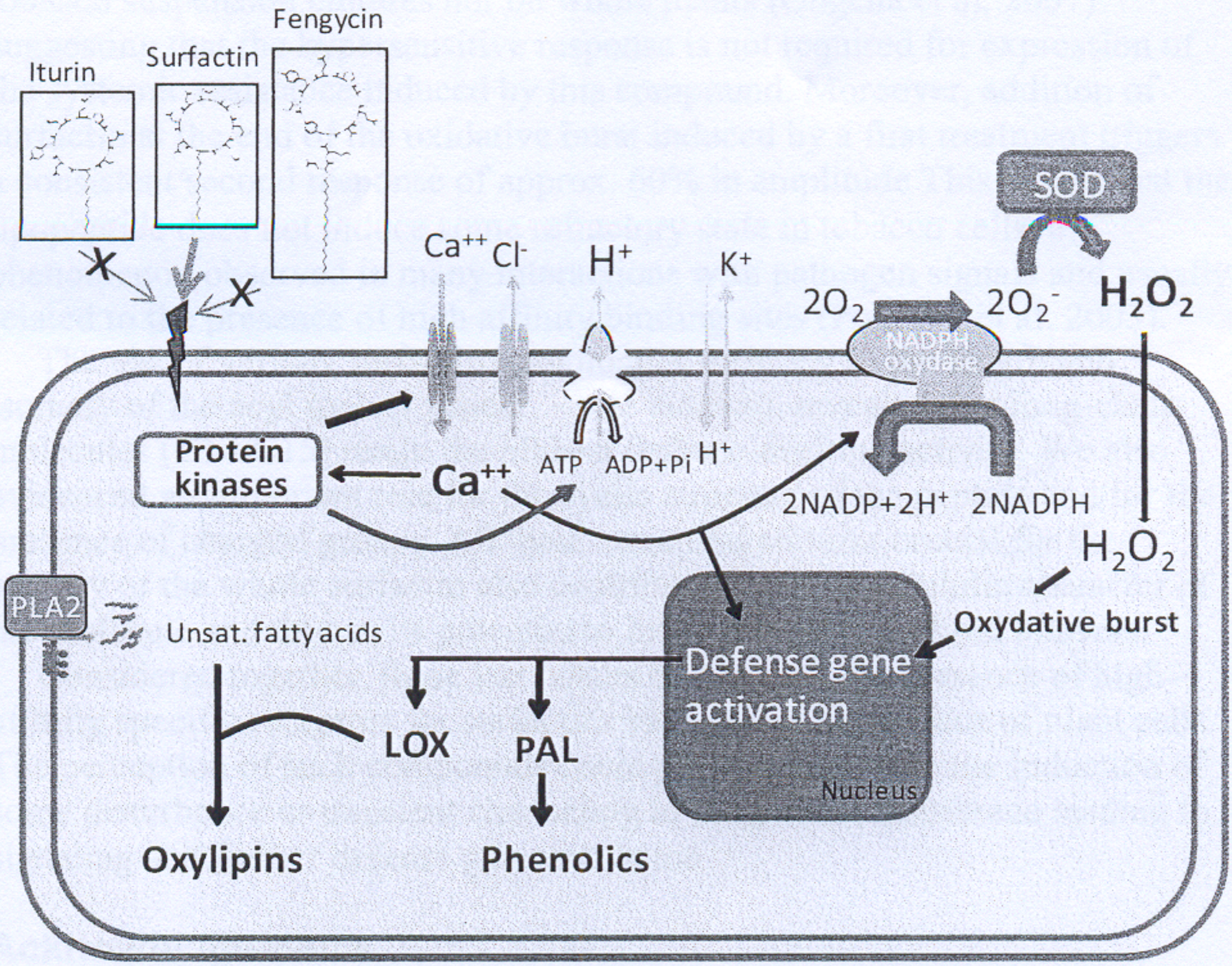

Fig. 1. Summary of the defense-related response of tobacco cells following elicitation by the surfactin lipopeptide.

Specific aspects in surfactin perception

Calcium and MAPkinase-dependent alkalinization and oxidative burst are also commonly found to be activated following perception of elicitors from phytopathogens (PAMPs). Similarly, activations of LOX and PAL enzymes are also common features of the plant defense response to pathogens or other stresses. However, there are several specific aspects underpinning the interaction between *Bacillus* cLPs and plant cells. First and by contrast with PAMPs that are perceived at nanomolar concentrations, surfactins are active in the micromolar range suggesting the absence of a high-affinity recognition system at the surface of tobacco cells. Secondly, the early oxidative burst observed upon treatment with the lipopeptide is usually considered as non-specific. It was not followed by a second one typically observed with other elicitors and which is concomitant with the developpement of HR and associated cell death (Garcia-Brugger et al. 2006). As a matter of fact, surfactin in the range 1-10 µM, do not cause any significant cell death in

tobacco suspension cultures nor on whole plants (Ongena et al. 2007) suggesting that the hypersensitive response is not required for expression of the systemic resistance induced by this compound. Moreover, addition of surfactin at the end of the oxidative burst induced by a first treatment triggers a consistent second response of approx. 60% in amplitude This means that the lipopeptide does not induce some refractory state in tobacco cells, a phenomenon observed in many interactions with pathogen signals and usually related to the presence of high affinity binding sites (Poinssot et al. 2003).

The test of various surfactin homologues differing in the length and isomery of the acyl moiety (linear, iso or anteiso), revealed that long-chain molecules (C14/C15) retain the highest defense-eliciting activity. We also evidenced an important role for the cyclic structure of the peptide and for the presence of charged groups. All these structural features crucial for the activity of the whole surfactin also contribute to the amphiphilic character of the molecule and thus to its potential to insert into phospholipid bilayers.

Considered together, these last results talk against the presence of high-affinity specific receptors for surfactins harbored at the surface of plant cells. The perception of such compounds could preferably rely on the induction of some disturbance or transient channeling in the plasma membrane leading to signaling and further defense gene activation.

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