MPMI Vol. 22, No. 4, 2009, pp. 456–468. doi:10.1094/MPMI-22-4-0456. © 2009 The American Phytopathological Society

Insights into the Defense-Related Events Occurring in Plant Cells Following Perception of Surfactin-Type Lipopeptide from *Bacillus subtilis*

E. Jourdan,[1] G. Henry,[1] F. Duby,[2] J. Dommes,[2] J. P. Barthélemy,[3] P. Thonart,[1] and M. Ongena[1]

[1]Centre Wallon de Biologie Industrielle, Faculté Universitaire des Sciences Agronomiques de Gembloux/Université de Liège, Belgium; [2]Département des Sciences de la Vie/Biologie Moléculaire et Biotechnologie Végétales, Université de Liège, Belgium; [3]Service de chimie analytique, Faculté Universitaire des Sciences Agronomiques de Gembloux/Université de Liège, Belgium

Submitted 27 October 2008. Accepted 14 December 2008.

Multiple strains of *Bacillus subtilis* were demonstrated to stimulate plant defense responses, and cyclic lipopeptides may be involved in the elicitation of this induced systemic resistance phenomenon. Here, we further investigated molecular events underlying the interaction between such lipopeptides and plant cells. Addition of surfactin but not fengycin or iturin in the micromolar range to tobacco cell suspensions induced defense-related early events such as extracellular medium alkalinization coupled with ion fluxes and reactive oxygen species production. Surfactin also stimulated the defense enzymes phenylalanine ammonia lyase and lipoxygenase and modified the pattern of phenolics produced by the elicited cells. The occurrence of these surfactin-elicited early events is closely related to Ca^{2+} influx and dynamic changes in protein phosphorylation but is not associated with any marked phytotoxicity or adverse effect on the integrity and growth potential of the treated tobacco cells. Reduced activity of some homologues also indicates that surfactin perception is dictated by structural clues in both the acyl moiety and cyclic peptide part. Our results suggest that these molecules could interact without irreversible pore formation but in a way sufficient to induce disturbance or transient channeling in the plasma membrane that can, in turn, activate a biochemical cascade of molecular events leading to defensive responses. The present study sheds new light not only on defense-related events induced following recognition of amphiphilic lipopeptides from *Bacillus* spp. but also more globally on the way elicitors from beneficial bacteria can be perceived by host plant cells.

To prevent pathogen invasion, plants have evolved several defense strategies. Preformed physical barriers such as cell walls or constitutively produced antimicrobial compounds can slow down or inhibit pathogen colonization. In a more active way, plants can recognize, via cell surface receptors, some pathogen-associated molecular patterns (PAMPs) harbored by nonspecific elicitors constitutively produced by the pathogen (Gómez-Gómez 2004; Jones and Dangl 2006; Montesano et al. 2003). Plants can also perceive avirulence (Avr) proteins that are specific pathogen effectors which interfere with defense-related signal transduction pathways triggered by general elicitors (Chisholm et al. 2006; Van Loon et al. 2006). Recognition of Avr proteins by the corresponding plant resistance (R) proteins activates a variety of defense response, including the hypersensitive response (HR) (Heath 2000) characterized by a rapid production of oxidative species, programmed cell death around the infection site and the synthesis of pathogenesis-related (PR) involved in defense against pathogen (Greenberg and Yao 2004). HR will restrict pathogen growth and confer a kind of local resistance (Kombrink and Schmelzer 2001) but plants can also develop a systemic form of resistance that extends to all organs following localized interaction. Systemic acquired resistance (SAR) is the best-characterized phenomenon activated after a first infection by an incompatible necrotizing pathogen and renders the host plant more resistant to a subsequent attack by a range of virulent pathogens on the same or another organ (Durrant and Dong 2004; Sticher et al. 1997). Such a systemic immunization can also be triggered by specific strains of plant-growth-promoting rhizobacteria (PGPR) via so-called PGPR-induced systemic resistance (ISR), effective against a broad range of diseases (Bakker et al. 2007; Kloepper et al. 2004; Ongena and Thonart 2006; Van Loon et al. 1998).

ISR can be globally viewed as a three-step process that includes bacterial elicitor perception, systemic signal transduction, and defense gene expression leading to enhanced responsive capacity of the host plant. ISR-associated signal transduction and defense mechanisms are being well documented, even if comparatively less well understood than in the case of SAR (Pieterse et al. 2001; Van Loon and Bakker 2005). By contrast, very little is known about the molecular events governing the early interaction between ISR-inducing bacteria and the plant cell. Some molecules responsible for the ISR-eliciting activity of these PGPR strains have been characterized and may be cell-surface components (Coventry and Dubery 2001; Duijff et al. 1997; Meziane et al. 2005; Reitz et al. 2002), iron-regulated metabolites (Audenaert et al. 2002; De Meyer et al. 1999; Leeman et al. 1996; Ongena et al. 2005c; Ran et al. 2005), volatiles (Ryu et al. 2004), quorum-sensing signals (Schuhegger et al. 2006), and antibiotics compounds (De Vleesschauwer et al. 2006; Iavicoli et al. 2003; Siddiqui and Shaukat 2003; Raaijmakers et al. 2006).

Among this last group, some cyclic lipopeptides (LPs) were recently identified as ISR elicitors. Massetolide A produced by *Pseudomonas fluorescens* SS101 is involved in ISR-eliciting

E. Jourdan and G. Henry contributed equally to this article.

Corresponding author: M. Ongena; Telephone: +32 81 62 23 05; Fax: +32 81 61 42 22; E-mail: ongena.m@fsagx.ac.be

activity in tomato against *Phytophthora infestans* (Tran et al. 2007). Moreover, we have demonstrated that surfactins and fengycins produced by *Bacillus subtilis* S499 can also act as elicitors of ISR (Ongena et al. 2007). These *Bacillus* LPs were mostly studied for their antagonistic activity against a wide range of potential phytopathogens, including viruses, bacteria, fungi, and oomycetes (Haas and Défago 2005; Leclère et al. 2005; Ongena et al. 2005a; Zahir et al. 2004). It is now clear that such compounds may act not only as antagonists but also by facilitating root colonization (Bais et al. 2004; Hofemeister et al. 2004) and by reinforcing the host resistance potential (Ongena et al. 2007). Fengycins are cyclic lipodecapeptides with a β-hydroxy fatty acid chain saturated or not with a length of 14 carbons (C14) to 18 carbons (C18) (Schneider et al. 1999; Vanittanakom et al. 1986). The surfactin family encompasses structural variants isolated from various *Bacillus* spp. but all members are heptapeptides interlinked with a β-hydroxy fatty acid to form a cyclic lactone ring structure (Peypoux et al. 1999) (Fig. 1). They are among the most powerful biosurfactants known, with exceptional emulsifying and foaming properties. Because of their amphiphilic nature, surfactins can also readily associate and tightly anchor into lipid layers (Deleu et al. 2003; Heerklotz and Seelig 2007; Sheppard et al. 1991).

By contrast to the numerous investigations conducted with some PAMPs used as models for the study of early defense-related events (Garcia-Brugger et al. 2006; Gómez-Gómez 2004; Zhao et al. 2005), very little information is available about perception mechanisms of ISR-specific elicitors by plant cells. Through this work, we wanted to provide a first picture of the metabolic changes that can be induced upon recognition of the different *Bacillus* LPs by tobacco suspension. In fact, their ISR activity on whole plants suggests the existence of some signal perception and response system in the host. The early events identified were also used as markers of the induced defensive state to test the activity of structural LP variants and then appreciate the relative importance of specific substructures for the elicitation of defense reactions in order to understand the molecular aspects of their perception by plant cells.

RESULTS

Involvement of LPs in the induction of extracellular alkalinization in tobacco cell cultures.

Medium alkalinization takes part in the plant defense-associated early responses to various biotic elicitors (Boller 1995; Felix et al. 1999) and we used this readily measurable phenomenon on suspension-cultured tobacco cells to test the eliciting activity of products secreted by *B. subtilis* S499. To this end, supernatant samples were collected at various time points during in vitro growth in a specific medium optimized for the production of cyclic LPs (Fig. 2A). Crude supernatant samples were prepurified on C-18 cartridge to yield the corresponding MeOH40 extract (fractions eluted with 40% methanol) containing molecules with intermediate hydrophobicity, and the MeOH100 extract (fractions eluted with pure methanol), retaining more apolar compounds and LPs of the surfactin, fengycin, and iturin families. The effect on tobacco cells of C-18 extracts prepared from samples collected every hour all over

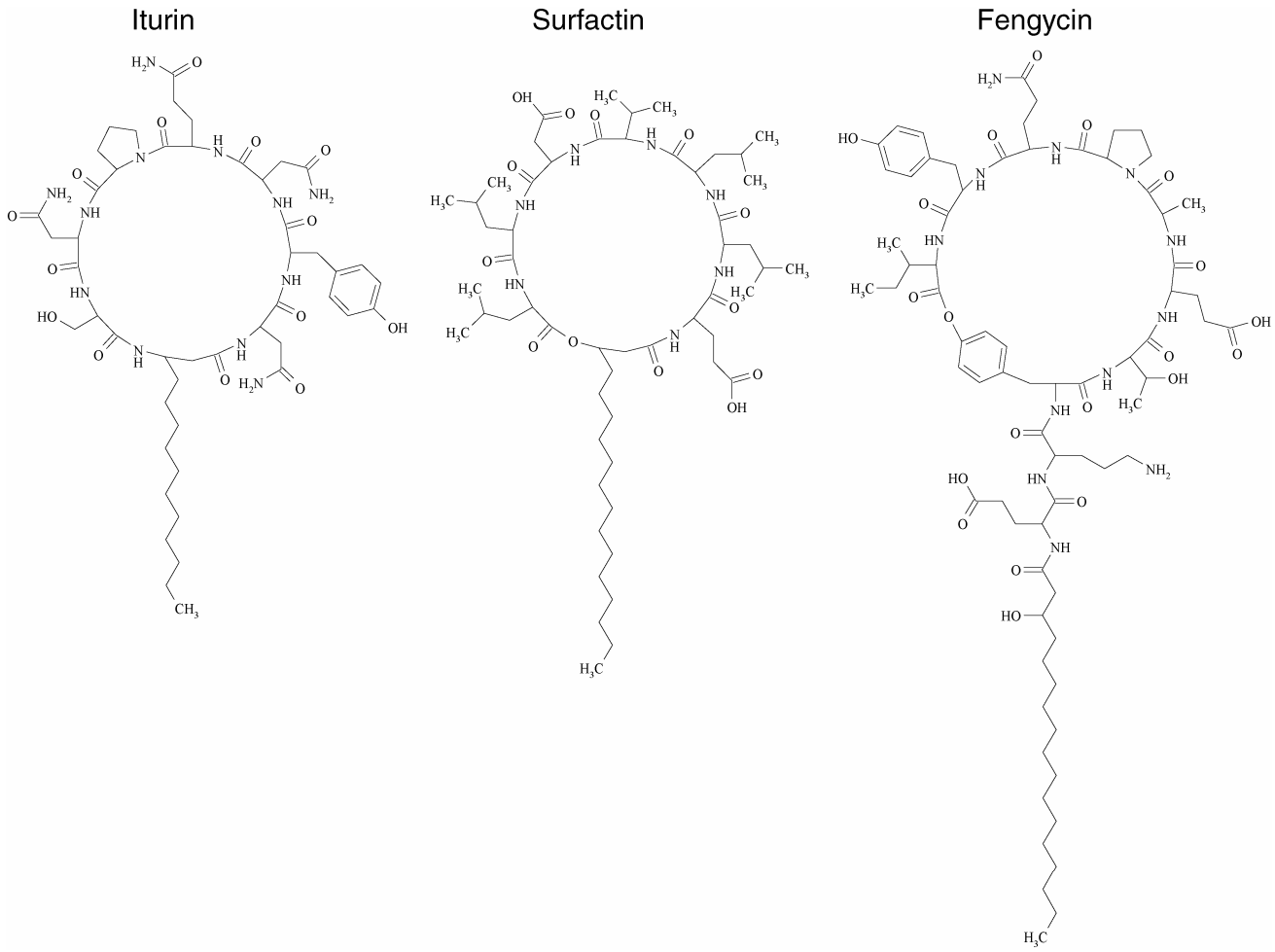

Fig. 1. Structures of representative members of the three lipopeptide families synthesized by *Bacillus* spp.

the culture time were first estimated with indicator paper (data not shown). MeOH40 extract did not induce any pH change, suggesting that no metabolite with intermediate hydrophobicity produced by strain S499 upon such culture conditions are active in triggering an alkalinization response by tobacco cells. The same applies for MEOH100 fractions obtained from samples collected before surfactin production, indicating that apolar metabolic products that could accumulate early in the exponential growth phase are not active. By contrast, indicator paper color changes could be clearly visualized by using MeOH100 extracts prepared from culture samples collected then after. pH increases induced by these samples were more precisely measured with pH probe. A weak but significant pH alkalinization was observed by treating tobacco cells with extract from 16-h-old culture, corresponding to the apparition of surfactin in the supernatant. Stronger pH alkalinizations were observed when MeOH100 extracts from samples containing higher surfactin concentrations were added to the cells (Fig. 2B). These first results suggest that these LPs are involved in the induction of alkalinization because it exactly correlates with their appearance and accumulation trend in the medium (Fig. 2A). However, a contribution of other metabolites with a similar production kinetic, or of fengycins, whose production is only 3 to 4 h delayed compared with surfactins (data not shown), could not be ruled out at this stage. By contrast, iturins that accumulate later in the culture are seemingly not involved because iturin-rich samples collected after 72 h of growth (data not shown) did not show enhanced activity compared with those collected at 54 h with reduced iturin content.

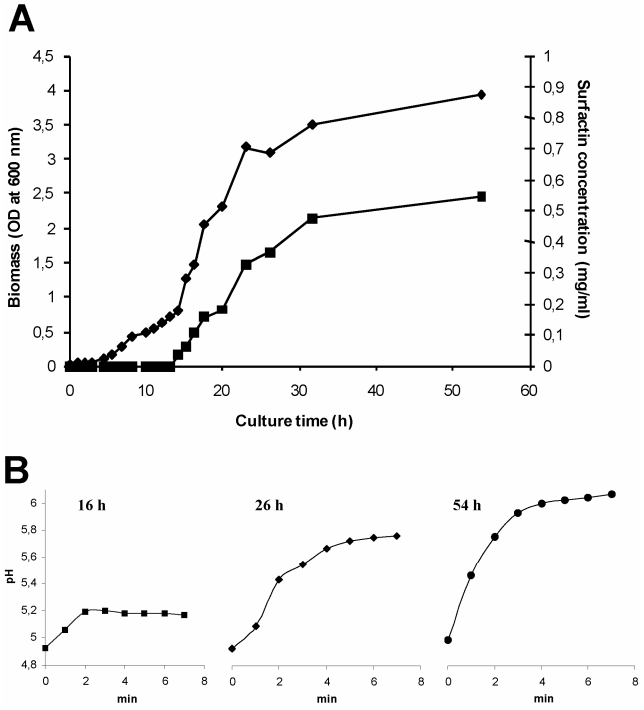

Fig. 2. Alkalinization response of tobacco cells induced by *Bacillus subtilis* S499 culture supernatant extracts. **A,** Bacterial growth (◆) and surfactin production (■) kinetics. Biomass was estimated by optical measurements at 600 nm. Surfactin concentration was determined by high-performance liquid chromatography. **B,** Extracellular pH modification of tobacco cell culture induced by treatment with S499 supernatant extracts resulting from C-18 solid phase extraction. Tobacco cells suspension were treated with 50 µl of the resulting methanolic solutions prepared from supernatant samples collected after 16, 26, and 54 h of culture. It corresponded to final surfactin concentrations in contact with plant cells of 0.7, 2.5, and 5.2 µM, respectively. Control consisted of cells treated with the same volume of 100% methanol.

Surfactin as main inducer of alkalinization response and oxidative burst.

In a first approach to get more information about the relative alkalinization-inducing activities of the three LP families, we tested MeOH100 extracts from various derivatives of the *B. amyloliquefaciens* FZB42 strain affected in the biosynthesis of different LPs. The AK3 mutant efficiently produces surfactin but fengycin and iturin biosyntheses are suppressed. The CH1 and CH2 derivatives retain fengycin and iturin synthesis, respectively, but are impaired in the production of the two other LP families. LP production by these strains was qualitatively checked and quantified by high-performance liquid chromatography (HPLC). MeOH extracts were diluted in order to obtain a final LP concentration of 2 µM after addition into the tobacco cell culture medium. A strong alkalinization response was observed following treatment of tobacco cells with the AK3 extract but neither CH1 nor CH2 extracts induced a significant pH increase compared with cells treated with methanol and used as control (Fig. 3), suggesting that surfactins are mainly involved in the alkalinization response.

Purified surfactins, fengycins, and iturins from S499 were also tested individually as a 99% pure mixture of homologues for their alkalinization effect. Surfactins but not iturins and fengycins induced a significant and transient pH increase upon addition at a concentration of 2 µM (Fig. 4A). Higher doses of the last two LPs were also not effective, as illustrated by the weak ΔpH_{max} of 0.2 to 0.25 obtained by treating plant cells with compounds at 20 µM. However, as illustrated in Figure 4B, a first treatment with fengycins or iturins did not preclude a full alkalinization response of the cells upon subsequent addition of surfactins. By contrast, a dose-dependent response was clearly observed with surfactins and a 50% effective con-

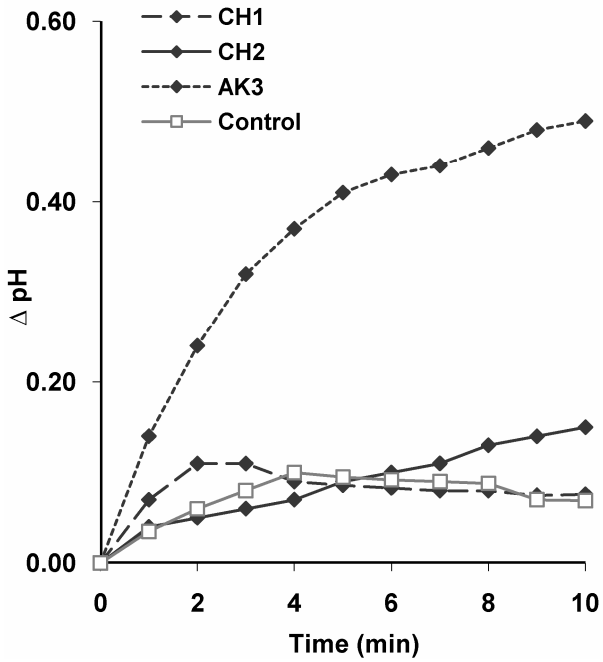

Fig. 3. Variation in extracellular pH of culture medium of tobacco cells in response to culture extracts from *Bacillus amyloliquefaciens* FZB42 mutants. The three mutants of strain FZB42 that are affected in surfactin (surf), fengycin (feng), or iturin (itu) production are CH2 (surf$^-$/feng$^-$/itu$^+$), CH1 (surf$^-$/feng$^+$/itu$^-$), and AK3 (surf$^+$, feng$^-$, itu$^-$). Lipopeptides were extracted from culture broths of *Bacillus amyloliquefaciens* FZB42 mutants by using C-18 solid phase extraction cartridges. Tobacco cells suspension cultures were treated with 21 µl of culture extracts dissolved in 100% methanol to obtain a final lipopeptide concentration of 2 µM. Control consisted of cells treated with same volume of 100% methanol. Data are from one representative assay.

centration (EC_{50}) corresponding to the half-maximal alkalinization inducing activity was calculated at 2.5 μM on the basis of the curve presented in Figure 4C. A concentration of 20 μM was not more effective than 10 μM, suggesting a saturation of the phenomenon as already observed in other studies (Bourque et al. 1998; Felix et al. 1999). ΔpH_{max} varied with age, cell density, and the initial pH of different batches of the cell culture, but the response to a given dose of surfactin was highly reproducible within a given batch of cells. On the other hand, tobacco cells were also treated with Triton X-100 used as positive control, miming the effect of an anionic detergent (such as surfactin) that could provoke pore formation leading to medium alkalinization due to passive efflux or influx of ions through the plasma membrane. By contrast with the response induced by similar concentrations of surfactins, Triton X-100 only induced a constant, slow, and limited response over the time of measurement (Fig. 4A).

The extracellular alkalinization response following elicitation usually occurs as a consequence of altered ion fluxes across the plasma membrane. In addition to the entry of calcium into the cells (see below), ion chromatography analyses from three independent assays revealed a significant efflux of potassium, nitrate, and chloride concomitant to pH increase. Indeed, the concentrations of K^+, $NO3^-$, and Cl^- in the extracellular medium increased by 0.68 ± 0.06 mM, 0.29 ± 0.21 mM, and 24 ± 2 μM, respectively, over the 15 min following surfactin addition (5 μM), whereas they remained fairly unchanged in the methanol-treated control cell cultures.

The rapid generation of reactive oxygen species or oxidative burst has also been demonstrated to be typically involved in early events associated with the plant defense response following pathogen perception (Apel and Hirt 2004). Hydrogen peroxide release by LP-treated tobacco cells was investigated using the luminol-based chemiluminescence assay. A rapid and transient oxidative burst was clearly stimulated within minutes by pure surfactins but not fengycins and iturins added at the same concentration (Fig. 5A). As in the case of alkalinization, no marked oxidative response could be observed by treating with higher amounts of these last two LPs. The relative surfactin-induced H_2O_2 accumulation peaked after 8 to 10 min when the LP was present at concentrations of 10 μM or higher (Fig. 5A). This corresponded to H_2O_2 concentrations in the range of 4 μM upon treatment with 10 or 20 μM. This burst was transient and returned to near basal level within 25 min and no further H_2O_2 release occurred within the next 4-h period (data not shown). At lower doses (2 to 5 μM), reduced but significant H_2O_2 accumulations were also measured, allowing the calculation of an EC_{50} value of 2.3 μM (Fig. 5B).

Regulatory aspects of surfactin-induced alkalinization and oxidative burst.

In plant–pathogen interactions, calcium is known to have an important role in the regulation of early events after elicitor perception (Lecourieux et al. 2006). In order to determine whether Ca^{2+} is also involved in surfactin-induced alkalinization and oxidative burst, the effects of both EGTA as calcium-chelator and $LaCl_3$ as calcium channel inhibitor were tested in surfactin-treated tobacco cell suspensions. $LaCl_3$ addition at a final concentration of 500 μM (inhibitory for channels in the plasma membrane but not those within the cells) (Pineros and Kochian 2003) before treatment with surfactin totally inhibited

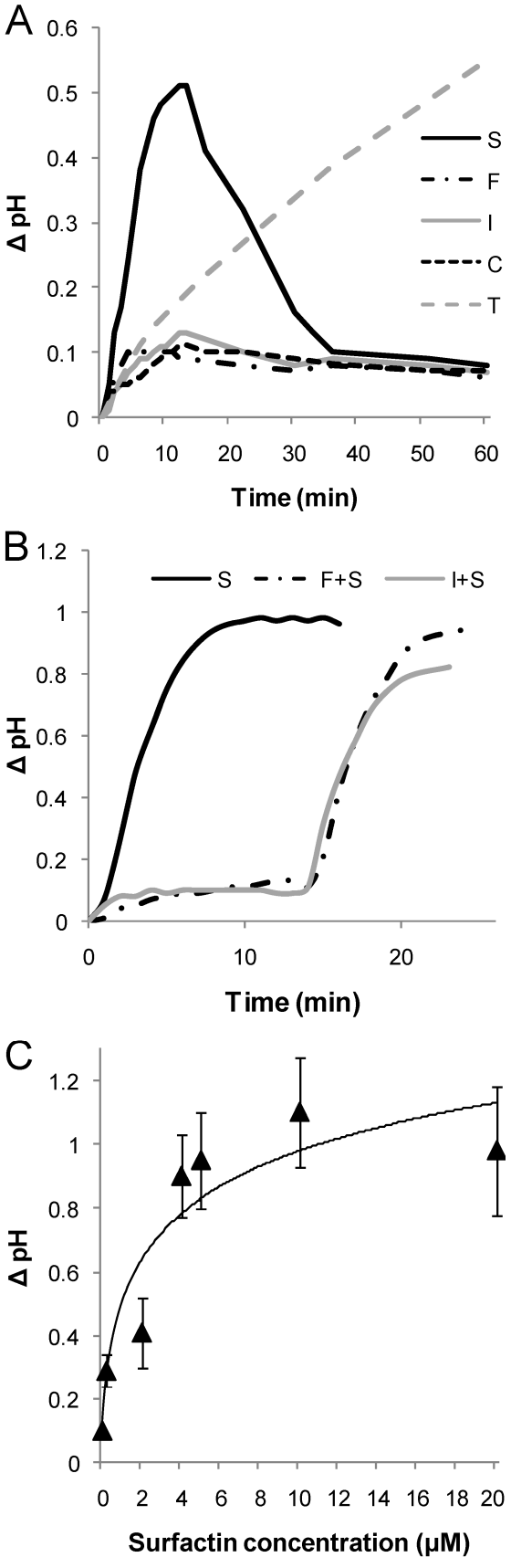

Fig. 4. Extracellular alkalinisation induced by *Bacillus* lipopeptides upon treatment of tobacco cell cultures. **A,** Effect of purified iturins (I), fengycins (F), or surfactins (S) added at a final concentration of 2 μM to exponentially growing cells, compared with methanol-treated control (C) or to triton-treated cultures (T). **B,** Alkalinization response observed upon addition of surfactins (5 μM) alone (S) or 12 min after pre-treatment with fengycins (F+S) or iturins (I+S) at the same concentration. **C,** Dose-response curve observed for surfactin-induced alkalinisation. Representative data from three replicates are presented.

the oxidative burst and pH change induced by the LP (Fig. 6). A drastic effect on surfactin activity was also observed by preincubating tobacco cells in the presence of 1 mM EGTA, confirming the crucial role of Ca^{2+} influx in the occurrence of both phenomena. Incubation of plant cells in the presence of the serine/threonine protein kinase inhibitor K252a (2 μM) prior to surfactin addition also resulted in a marked decrease in the amplitude of both alkalinization (reduced by 54%) and H_2O_2 accumulation, which was almost completely inhibited (Fig. 6). In addition, diphenyleneiodonium (DPI) at 2 μM, which is an inhibitor of NADPH oxidase involved in the oxidative burst (Pugin et al. 1997), completely inhibited the active oxygen species (AOS) production stimulated by 10 μM surfactin but only partially affected (maximal inhibition 22%) pH change (Fig. 6). Phospholipase A2 is thought to be involved in expression of the plant defense response to various stimuli (Chandra et al. 1996; Lee et al. 1997; Navarez-Vasquez et al. 1999; Roos et al. 1999). The effect of the inhibitor arachidonic acid trifluoromethyl ketone ($AACOF_3$) was also tested to assess the involvement of the enzyme in signal transduction upon surfactin elicitation. $AACOF_3$ caused a 53% decrease in pH changes when added (33 μM) 30 min prior to the elicitation with 2 μM LP.

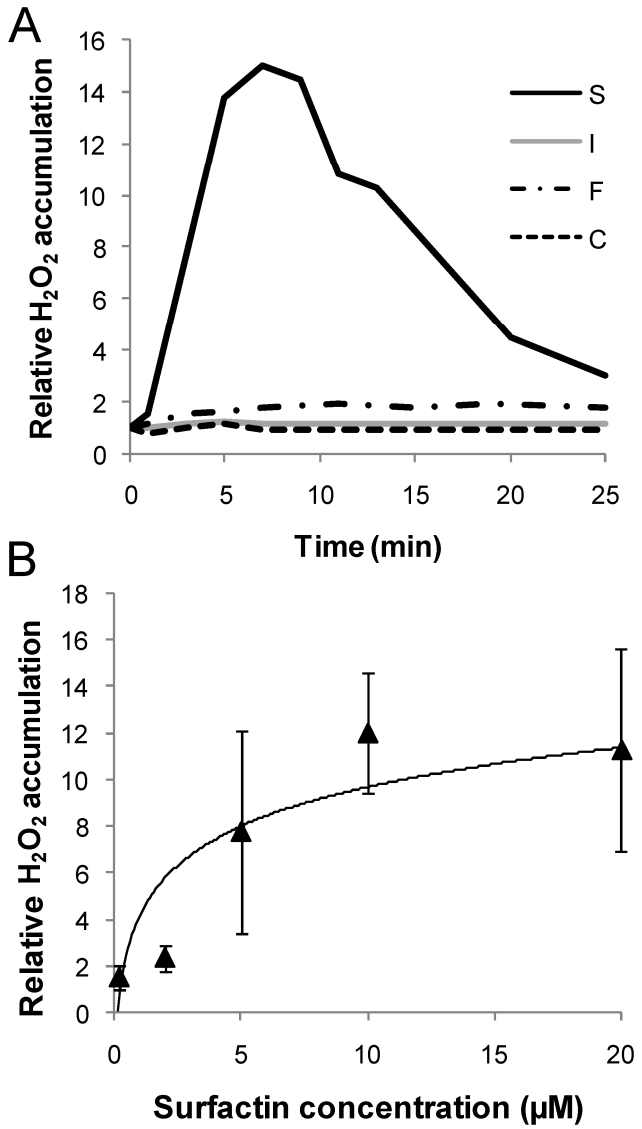

Fig. 5. Lipopeptide-induced oxidative burst in tobacco cell suspensions. **A,** Hydrogen peroxide accumulation in the medium observed upon addition of iturins (I), fengycins (F), and surfactins (S) at 10 μM compared with the methanol control. **B,** Dose-response curve observed for surfactin-induced oxidative burst. Curves in A are from an individual but representative experiment and data in B were calculated from three independent assays.

Limited cell death triggered by surfactin.

Viability of tobacco cells was determined in the presence of increasing concentrations of surfactin by using Evans blue coloration (Baker and Mock 1994). Both microscopic observation and spectrophotometric quantification did not reveal any significant cell mortality in the first hours after surfactin addition at the concentrations used compared with the methanol-treated control (data not shown). Data obtained for mortality assessment 24 h after LP addition showed a limited adverse effect of surfactins on tobacco cell viability (Fig. 7) compared with benzylamine, reported as a powerful cell-death-inducer agent (Kawano et al. 2000) (data not shown), or Triton X-100, used for calibration. No significant cell death was observed following treatment with 2 μM surfactin, and mortality rates of approximately 25% were induced by surfactin added at a concentration of 10 to 20 μM (Fig. 7B). In addition, the integrity of cells was evaluated by measuring intracellular protein release and only minor leaks, if any (4 to 7% increase in extracellular proteins), were induced during the first 5 h after treatment with 2 to 20 μM surfactin (data not shown).

Stimulation of defense-related metabolic pathways.

Phenylalanine ammonia lyase (PAL) catalyzing the deamination of phenylalanine to yield cinnamic acid is a key enzyme involved in plant defense because it represents the entry of the phenylpropanoid pathway (Dixon et al. 2002; Zhao et al. 2005). PAL activity in tobacco cells was first tested spectrophotometrically on extracts prepared from cells collected at various time points after surfactin addition. Results are compared in Figure 8A with those obtained for untreated cells and for cells treated with methyl jasmonate as a positive control overexpressing PAL (Sharan et al. 1998). A very slow increase in PAL activity was observed in methanol-treated control cells but a strong induction of PAL activity was obtained within 6 h after surfactin addition to reach a maximal value after 9 h. Compared with the control, PAL activity was 4.1 and 8.6 times higher in the presence of 10 and 20 μM surfactin, respectively. In addition, hybridization on Northern blots also revealed a strong accumulation of PAL transcripts in tobacco cells treated with surfactin (Fig. 8B), suggesting that de novo enzyme synthesis is induced by this LP. In a third approach, stimulation of the phenylpropanoid pathway was also investigated by analyzing the differential accumulation of PAL-derived phenolics secreted by tobacco cells. Different samples from tobacco cell culture supernatants were purified by liquid–liquid extraction and analyzed by HPLC. Reversed-phase diode array detector (DAD)-HPLC analyses of prepurified supernatant extracts collected 10 h after treatment of tobacco cells with 10 μM surfactin revealed significant accumulation or decrease of several compounds (Fig. 8C). Cinnamic, salicylic, hydroxy-benzoic, and benzoic acids could be identified among accumulating compounds on the basis of their retention time and ion fragmentation profile in liquid chromatography-ESI/mass spectrometry (LC-ESI/MS). Apparition of salicylic acid and a threefold increase in cinnamic acid were detected 5 h after treatment and were maintained until 24 h after surfactin addition (data not shown). On the other hand, ferulic and coumaric acids were poorly resolved under the HPLC conditions used; however, significant 1.5- and 2.4-fold decreases in their concentrations after surfactin addition were observed by using quantitative LC-MS. Other surfactin-induced phenolics were detected on the basis of their UV-visible spectral properties but could not be reliably identified so far.

Plant lipoxygenase (LOX) is involved in the defense response to various pathogen-induced or wound-induced stresses (Shah 2005). This enzyme catalyzes the incorporation of molecular oxygen in polyunsaturated fatty acids to yield the corresponding fatty acid hydroperoxides. These highly reactive compounds are, in turn, converted by other enzymes into a wide array of bioactive oxylipins (Blée 2002). Thus, possible increase of LOX activity in surfactin-treated tobacco cells was also tested to provide an additional example of defense-related pathway induction by this LP. Evolution of LOX activity was monitored spectrophotometrically by measuring hydroperoxide production from the C18:3 linolenic acid. A significant stimulation of

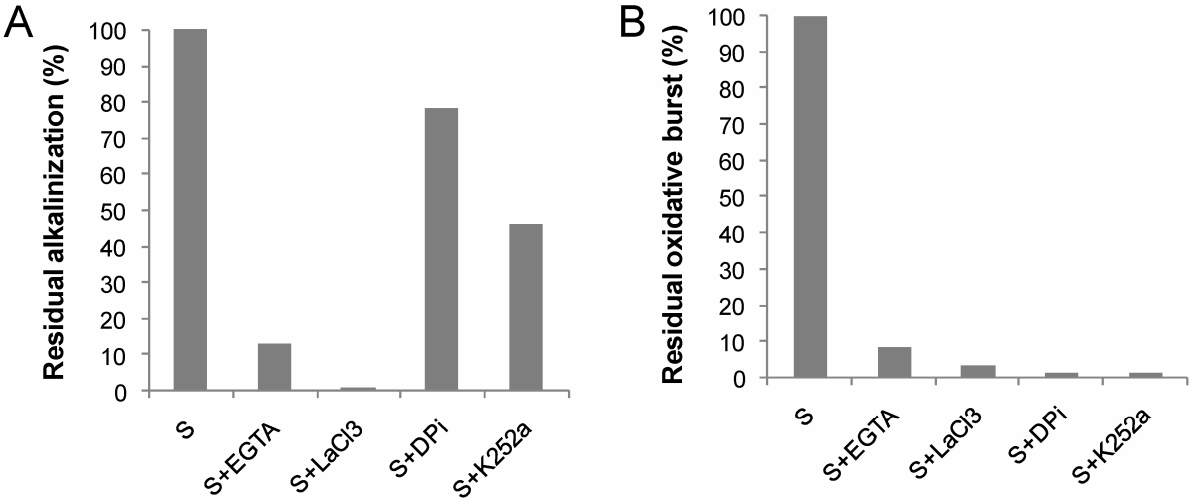

Fig. 6. Suppression or inhibition of the surfactin-induced alkalinization (measured 10 min after treatment) and oxidative burst (measured 8 min after treatment) reactions by the calcium chelator EGTA, the calcium channel blocker $LaCl_3$, the NADPH oxidase inhibitor, diphenyleneiodonium (DPI), and the Ser/Thr protein kinase inhibitor K252a added to *Nicotiana tabacum* cell cultures prior to elicitation with the lipopeptide (2 or 10 μM for alkalinization or oxidative burst assays, respectively). Mean values from two experiments are represented.

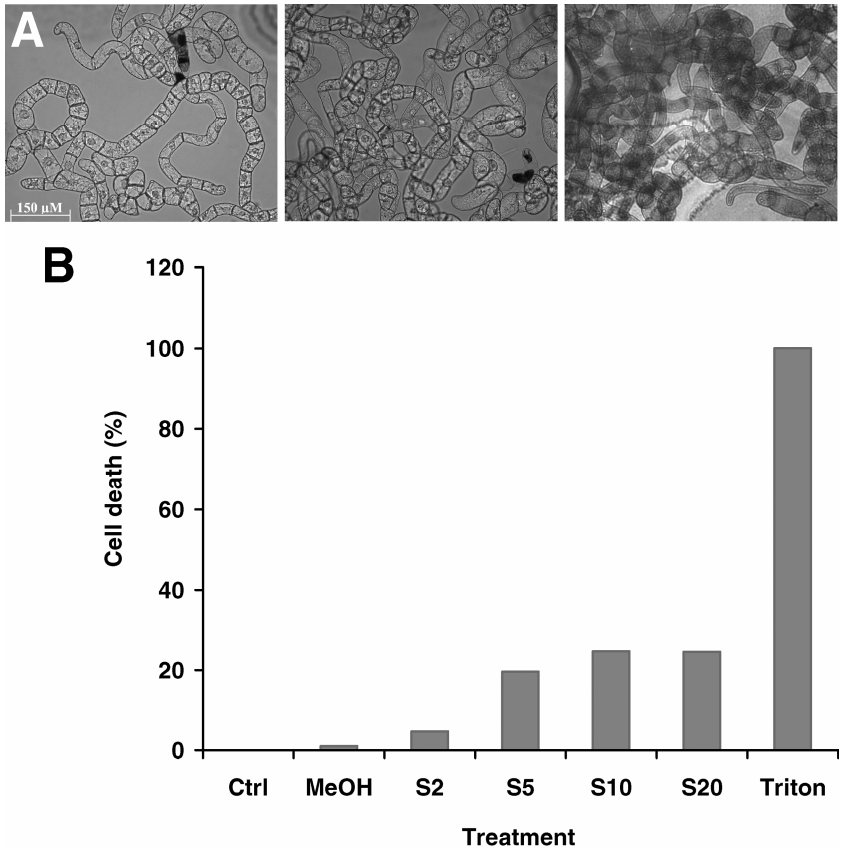

Fig. 7. Evaluation of tobacco cell mortality upon treatment with surfactin. **A,** From left to right, tobacco cells treated with 100% methanol, 2 μM surfactin, and triton X-100. Pictures were taken 24 h after treatment of cells. Dead cells are blue dyed. **B,** Cell death caused by increasing surfactin concentration on tobacco cell suspension after 24 h of incubation. Cultures were treated with purified surfactins from *Bacillus subtilis* S499 to obtain final concentrations in the medium of 2 μM (S2), 5 μM (S5), 10 μM (S10), or 20 μM (S20). Purified surfactins were dissolved in 100% methanol. Control (Ctrl) treatment represents untreated cells and MeOH are cells treated with the same volume of 100% methanol. Tobacco cell suspensions were treated with Triton X-100 (0.1 mM) as a positive control inducing strong cell mortality. Data were obtained using optical density of sonicated washed cells colored with Evans blue.

LOX activity was specifically observed in surfactin-treated cells during the first hours to reach a fivefold higher level after 9 h compared with control cells (Fig. 9). Higher LOX activity was maintained in elicited cells during at least 21 h. As in the case of PAL, the addition of methyl jasmonate also induced a strong LOX response proving adequate tobacco cell reactivity.

Structural requirements for surfactin elicitor activity.

As revealed after separation by HPLC and identification by matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry, the surfactin mixture from strain S499 is mainly composed of six homologues with C12 to C15 linear saturated acyl chains and two for iso-C14 and iso-C15 forms. These homologues were purified by semipreparative HPLC and were tested individually on tobacco cells, except for the two C13 and the two C15 forms that could not be completely resolved and were tested as single samples. Means calculated from three independent experiments revealed that homologues with the shortest lipid chains (C12 and C13) failed to induce any significant pH shift (Fig. 10). By contrast, C14 and C15 surfactins triggered a significant alkalinization response that probably accounts for most of the activity of the mixture sample. A loss of eliciting capacity also was observed for the C12 homologue whereas C14 surfactins retained almost the same potential to trigger H_2O_2 accumulation compared with the mixture (data not shown). Interestingly, chain ramification may also be important for elicitor activity because a limited but significant difference was observed between cells treated with linear (C14n) or ramified (C14i) surfactin homologues (Fig. 10B). Moreover, the synthesis of C14 surfactin with linear and methylated forms of the peptide part was performed to appreciate the importance of these traits. Both modifications, considered individually, led to a significant decrease of the eliciting activity on tobacco cells (Fig. 10B) and the lin-

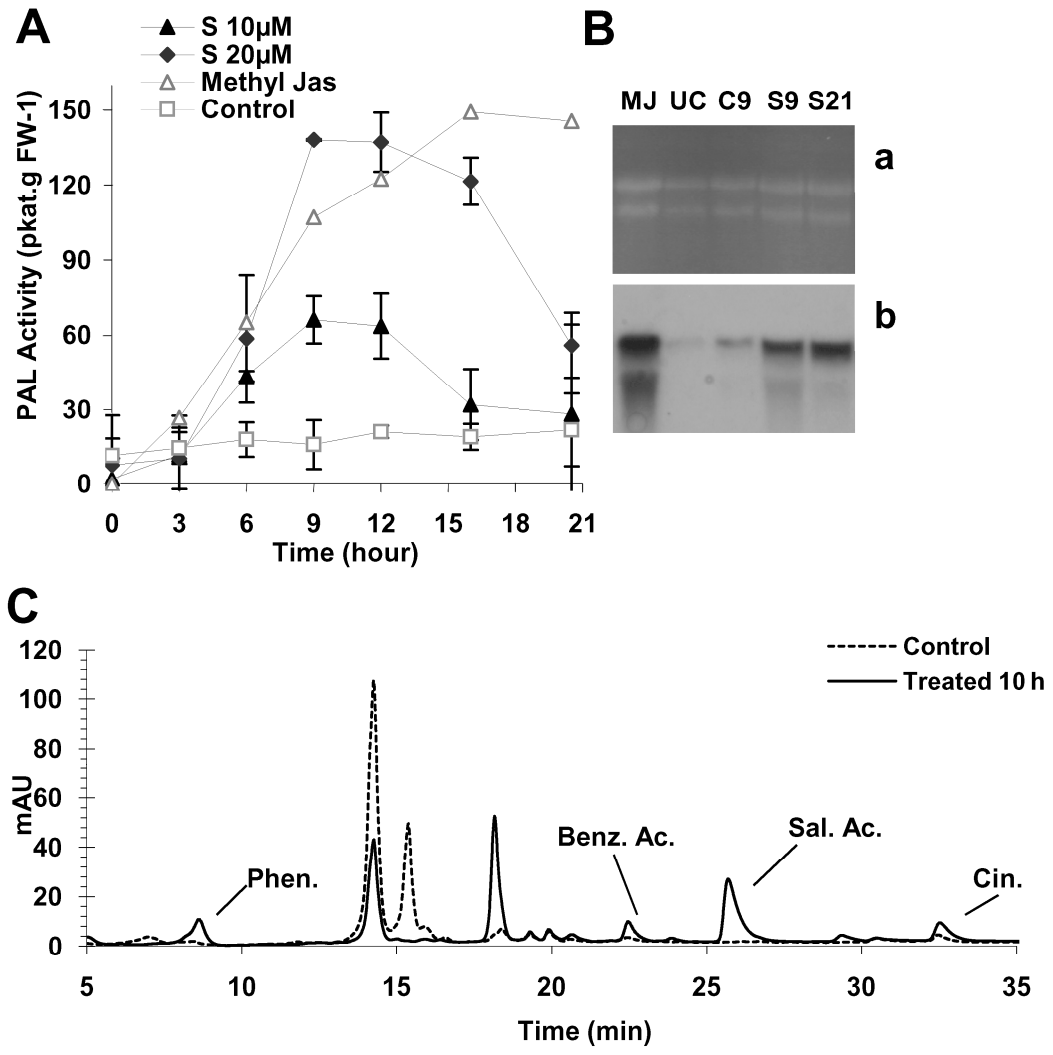

Fig. 8. Stimulation of phenylalanine ammonia-lyase (PAL) and changes in the phenolic pattern in tobacco cells treated with surfactin. **A,** Time course of PAL activity following treatment with two different surfactin concentrations and with methyl jasmonate. Control consisted of cells treated with the same volume of 100% methanol. Data are means and standard deviations from two independent experiments, except for treatment with methyl jasmonate, which are from of one experiment. **B,** Expression pattern of PAL genes in tobacco cells. Total mRNA were extracted from untreated (same volume of methanol) tobacco cells (UC), cells that have been treated with methyl jasmonate as positive control (MJ), or 9 and 21 h after treatment with 20 µM *Bacillus* surfactin (S9 and S21, respectively). A negative control (C9), corresponding to RNA extraction of tobacco cells 9 h after addition of methanol, is also shown. Gel loading represented in (a) and the upper band in (b) corresponding to PAL transcripts was revealed using the probes as described in Materials and Methods. This assay for PAL gene expression was repeated with similar results in an independent experiment. **C,** High-performance liquid chromatography profile obtained for the analysis of phenolics in tobacco cell culture supernatants. Phenolic compounds were extracted with ethyl acetate from supernatant of cells treated with methanol (Control) or of cells 10 h after treatment with surfactin (Treated 10 h). Compounds eluting in some peaks were identified by comparison of their retention time with those of authentic standards (Phen, phenylalanine; Benz. Ac., benzoic acid; Sal. Ac., salicylic acid; Cin. Ac., cinamic acid) and on the basis of the molecular weight and fragmentation in liquid chromatography mass spectrometry.

earized and methylated form retained only approximately 25% of the activity.

DISCUSSION

In light of works reported in the past decade, a global picture of the ISR phenomenon induced by beneficial bacteria is being addressed, depicting the cascade of signaling events from the activation of transcription factors to the phenotypic expression of the phenomenon (Van Wees et al. 2008). However, molecular mechanisms underlying early steps in the perception of elicitors from these rhizobacteria by the host plant are still poorly understood (Van Loon 2007). Based on our recent work showing the ability of LPs produced by *B. subtilis* S499 to trigger ISR in tomato and bean (Ongena et al. 2007), in this work we have further investigated some aspects of the responses induced by these LPs in cultured plant cells. By two different approaches, the present work demonstrates that surfactin-type LPs are the main products from *B. subtilis S499* that are recognized by tobacco cells to mount early defense-related responses.

Treatment with surfactin in micromolar concentrations triggers extracellular medium alkalinization. The extracellular alkalinization response upon treatment with PAMPs is usually related to a general modification of ion fluxes due to plasma membrane depolarization mainly induced by calcium influx and chloride efflux (Garcia-Brugger et al. 2006). In surfactin-elicited cells, both phenomena occur and are coupled with K^+ efflux whereas the alkalinization was only slightly affected by pretreatment with DPI, showing that increase in extracellular pH is not based on proton consumption for H_2O_2 production via the NADPH oxidase or superoxide dismutase system as observed for other elicitors (Pugin et al. 1997). Therefore, medium alkalinization following LP addition mostly results from other mechanisms and a Ca^{2+} activation of the plasma membrane H^+-ATPase could be involved because blocking calcium entry resulted in complete inhibition of pH increase upon surfactin treatment. In addition, the decrease in proton concentration corresponding to a ΔpH of 0.95 units is similar to the concomitant increase in the amounts of potassium ions we measured in the external medium over the same 15-min laps of time. This also suggests that alkalinization provoked by surfactin mainly originates from K^+/H^+ exchange across the plasma membrane through the ATPase. Upon surfactin treatment, we also observed significant production of reactive oxygen species within minutes. Data obtained from the complete inhibition of surfactin-induced oxidative burst by DPI show that this phenomenon mainly originates from the NADPH oxidase system, which forms superoxide anion radicals that are further converted into hydrogen peroxide via superoxide dismutase. However, this first burst, considered to be nonspecific, was not followed by a second one typically observed with other elicitors and which is concomitant with the development of the HR and associated cell death (Desender et al. 2006). Thus, it is worth noting here that, in contrast to protein elicitors such as elicitin, harpin, or cryptogein (Dong et al. 1999; Dorey et al. 1999; Koehl et al. 2003; Strobel et al. 1996), surfactin did not cause cell death in tobacco suspension cultures or on whole plants (Ongena et al.

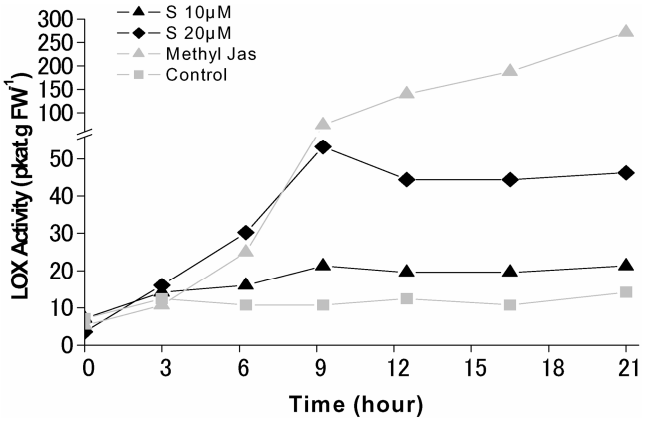

Fig. 9. Time course of tobacco cells lipoxygenase (LOX) activity induced by treatment with surfactin. Data are mean values from two independent experiments.

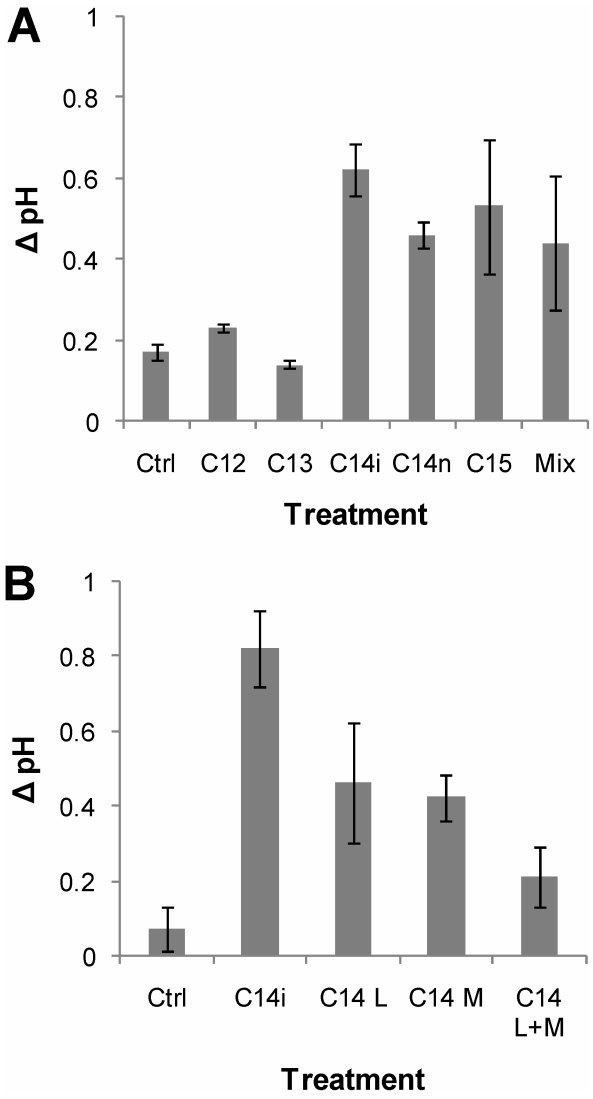

Fig. 10. Structure–activity relationship of surfactin in eliciting pH alkalinization of tobacco cells medium. **A,** Medium alkalinization response (measured 15 min after addition of surfactin homologues) to treatment of tobacco cells with surfactin homologues with variable lipid chain from 12 carbons (C12) to 15 carbons (C15). For surfactin with 14 carbons in the lipid chain, a linear chain (C14n) and a ramified chain (C14i) were tested. Cells were treated with purified surfactin dissolved in 100% methanol. Treatments were added to obtain a final surfactin concentration in cell suspension of 2 µM. The mixture of the different surfactin homologues was used as a positive control (Mix). Data are means and standard variations calculated from three independent experiments. **B,** Influence of structural modifications in the peptide core of C14i surfactin on tobacco cell alkalinization response. This isoform of the C14 homologue was either methylated on the carboxy groups of the two aspartate residues (C14 M), synthesised in a linearized form (C14 L), or both (C14 L+M). The natural C14i homologue and the modified surfactins were added to obtain a final concentration of 4 µM in the cell suspension. Control consisted of cells treated with same volume of 100% methanol (Ctrl). Data are means and standard variations calculated from three independent experiments.

2007), suggesting that that the HR is not required for expression of the systemic resistance induced by this compound.

Intracellular pH modification can lead to the activation of defense-specific pathways and activate the synthesis of intracellular and systemic signaling compounds (Desender et al. 2007). H_2O_2 generation also plays multiple roles in plant defense. In addition to a direct protective effect, reactive oxygen species may favor cell-wall cross-linking, induce cell death, or activate an array of defense genes and the biosynthesis of antimicrobial proteins and phytoalexins (Apel and Hirt 2004; Fedoroff 2006). Reactive oxygen species also play a potential role in systemic signaling, leading to the establishment of SAR (Fobert and Despres 2005). As revealed by the use of chelator and channel blocker, both pH change and oxidative burst are regulated by Ca^{2+} influx into surfactin-elicited tobacco cells. Therefore, in the case of surfactin, as for PAMPs, elicitor-induced Ca^{2+} spiking is one of the earliest events that may act as a master messenger for almost all downstream response reactions. On the other hand, oxidative burst and extracellular alkalinization were totally suppressed and significantly reduced, respectively, by the protein kinase inhibitor K252a. Thus, the occurrence of these surfactin-elicited early events is closely related to dynamic changes in protein phosphorylation. In addition, our results also show that the use of the specific inhibitor of phospholipase A2 significantly reduced extracellular alkalinization, suggesting a key role of the enzyme that has been suggested to play a regulatory role in the expression of plant defense mechanisms associated with pathogenesis, wound-induced signal transduction, or elicitation by systemin, oligosaccharides, and ergosterol (Chandra et al. 1996; Lee et al. 1997; Navarez-Vasquez et al. 1999; Roos et al. 1999).

Phospholipase A2 catalyses the hydrolysis of glycerophospholipids, resulting in the liberation of free fatty acids, among which are LOX substrates linoleate and linolenate (Zhao et al. 2005). Thus, phospholipase involvement may also be viewed as a start point for the expression of the defense-related LOX pathway. Activation of the LOX enzyme is a common feature of the plant defense response to pathogens, wounding, and stress (Baysal and Demirdoven 2007; Feussner and Wasternack 2002; La Camera et al. 2004; Shah 2005) and also occurs later upon surfactin treatment (Fig. 9). It indicates a possible induction of the so-called oxylipin pathway leading to a wide range of bioactive metabolites, among which are signaling jasmonates or various compounds with fungitoxic activity (Matsui 2006; Prost et al. 2005). We have already demonstrated that key enzymes of the LOX pathway were activated in resistant tomato plants following induction by surfactin overproducers (Ongena et al. 2007). Time-course monitoring showed that LOX and hydroperoxide-degrading enzyme activities were significantly enhanced and it correlated with disease symptom reduction. Stimulation of the entire metabolic route leading to oxylipins was also evidenced in bean (Ongena et al. 2004) and tomato (A. Adam, *unpublished data*) treated with the rhizobacterium *Pseudomonas putida* BTP1.

Perception of surfactin by tobacco cells also resulted in the stimulation of PAL, a key enzyme in phenylpropanoid metabolism leading to a large array of phenolics, including precursors for cell wall reinforcement, antifungal compounds, or salicylic acid, which play an important role in disease resistance (Dixon et al. 2002). As a result of PAL stimulation, surfactin treatment leads to major changes in the phenolic pattern produced by tobacco cells. All the accumulating or appearing compounds could not be identified; however, clear increases in cinnamic acid, hydroxyl-benzoic acid, and salicylic acid apparition were observed concomitantly with decreases in ferulic and coumaric acid amounts. On this basis, we hypothesize a reorientation of the phenylpropanoid pathway, starting from cinnamate to salicylic acid via the stimulation of enzymes such as benzoic acid 2-hydroxylase, to the detriment of the other branch initiated by the cinnamic acid 4-hydroxylase to other phenolic compounds. Nevertheless, a use of coumaric, ferulic, and other acids as precursor for synthesis of other defense molecules such as scopoletin (tobacco phytoalexin) or lignin precursors for cell-wall reinforcement cannot be rejected. Further metabolic profiling studies are being performed to confirm this hypothesis. Interestingly, treatment of potato tuber cells with purified *Bacillus* LPs also resulted in the accumulation of some plant phenolics involved in or derived from the phenylpropanoid metabolism (Ongena et al. 2005a). Increased concentrations of cinnamic acid were also observed together with higher amounts of chlorogenic acid that may act directly as an antimicrobial defense compound and that derive from the benzoate branch of the pathway (Maher et al. 1994). Again, p-coumaric, ferulic, sinapic, and caffeic acids that constitute building blocks for the synthesis of lignin did not accumulate. Otherwise, Audenaert and collaborators (2002) have observed some local PAL stimulations after treatment of tomato roots with *P. aeruginosa* 7NSK2, another ISR-inducing rhizobacteria.

Results from this study confirm that surfactin may thus constitute a novel class of microbial-associated molecular patterns that can be specifically perceived by plant cells, but also show that iturins and fengycins are poorly active in triggering defense response in tobacco cells. In bean and tomato plants, a role for surfactins but also for fengycins in resistance induction was demonstrated by the similar protective activity of purified compounds compared with the producing strain (Ongena et al. 2007). Treatment of potato tuber cells with purified fengycins but not surfactins and iturins resulted, as observed here in tobacco, in the accumulation of plant phenolics derived from the phenylpropanoid metabolism (Ongena et al. 2005a). By contrast, strain S499 can stimulate a systemic defense response in cucumber (Ongena et al. 2005b) but semipurified LP extracts failed to confer any protection, suggesting that they are not involved in the resistance triggering process in that plant. Thus, it is clear that the three LP families retain differential abilities to stimulate defense reactions, suggesting that specific functions in the peptide part of these molecules are important for their perception. However, by contrast with elicitors or avirulence factors in plant–pathogen interactions (Chisholm et al. 2006), there is no indication for the presence of receptors specific for bacterial LPs in plant cells. Further clues will certainly be gained by searching for labeled surfactin-binding protein factors associated with plasma membranes, or for a specific, reversible, and saturable high-affinity binding activity. That said, surfactin is active in micromolar concentrations and defense events are more intensively expressed at the higher surfactin dose than at the lower dose assayed. It means that, if there is some specific recognition system at the cell surface, it should be of quite low affinity compared with elicitors from pathogens. Moreover, addition of surfactin at the end of the oxidative burst induced by a first treatment with this molecule triggered a second oxidative response that conserved approximately 60% in amplitude (data not shown). This contrasts with the refractory state phenomenon usually observed in interactions with pathogen signals, indicating the presence of high-affinity binding sites for these PAMPs in the plasma membrane of plant cells.

Our results highlight the importance of the acyl chain and of the amphiphilic nature of surfactin, strongly suggesting that these LPs can mainly interact via a less-specific mechanism based on some destabilization of the lipid bilayer structure. Indeed, based on the amplitude of the alkalinization effect and oxidative burst, optimal responses were observed by treating tobacco cells with surfactins homologues containing the longer acyl chains with 14 and 15 carbons. Such an important role for

the length of the acyl chain has already been documented for other biological activities of LPs and is explained by the fact that it readily inserts into phospholipid bilayers (Bonmatin et al. 2003; Carrillo et al. 2003; Dufour et al. 2005; Eeman et al. 2006; Heerklotz and Seelig 2007; Youssef et al. 2005). In addition, our results also show that a cyclized and charged peptide part is also necessary for maximal activity. It agrees with the global model of a lipid bilayer destabilization process facilitated by the tridimensional form of the surfactin molecule featuring charged side chains protruding into the aqueous phase and apolar moieties inserting into the hydrophobic core of the membrane. However, as stated above, the presence of LPs was not associated with any marked phytotoxicity or adverse effect on the integrity and growth potential of the treated tobacco cells. This suggests that these molecules could interact without irreversible pore formation but in a way sufficient to induce disturbance or transient channeling in the plasma membrane that can, in turn, activate a biochemical cascade of molecular events leading to defensive responses. Interestingly, a similar hypothesis has been recently raised to explain the antiproliferative effect of surfactin on colon cancer LoVo cells that did not appear to be leaky or lysed but underwent significant changes in the expression of protein factors regulating cell survival (Kim et al. 2007). In the case of plant cells, this perturbation could remain limited due to the specific composition in sterols which is known to influence the LP interaction with biological membranes.

On the other hand, little information is available about the quantities and pattern of surfactin homologues secreted by strains growing in the rhizosphere. However, recent results from our laboratory indicate that rhizosphere factors somewhat influence the production of surfactins by *B. subtilis* strains, not only quantitatively but also qualitatively (V. Nihorimbere, *unpublished data*). Thus, different patterns of surfactin variants with specific activities may be naturally synthesized by a given *Bacillus* strain depending on the nutritional context imposed by the host plant and physicochemical parameters inherent to the soil. This could have an impact on the global biocontrol activity of the strain because some homologues within the particular family of surfactins may be more active than others at stimulating the plant immunity potential. In the same way, the variety of the LPs naturally synthesized may explain why some *Bacillus* strains are more efficient than others in reducing plant diseases via ISR or direct antagonism toward the pathogen.

Progress in the knowledge of PAMP perception during recent years has been coupled with a better understanding of the mechanisms inherent in pathogen-induced immunity. It clearly appears that plant responses to different pathogen signals converge at an early step after signal perception (He et al. 2007). Most of the early cellular defense events observed here following surfactin recognition are similar, again suggesting a convergence of the early cellular reactions with those initiated by PAMPs (Zhao et al. 2005), but it also appears to display some specificity in the response. Further experiments are necessary to elucidate the impact of the defense events identified here on the global protective reaction mounted in plant tissues following surfactin perception but this study contributes substantially to understanding the biochemical basis of the mechanism of action of this LP as an elicitor for beneficial rhizobacteria involved in the triggering of plant defense responses.

MATERIALS AND METHODS

Bacterial and plant cell growth.

The wild-type *B. subtilis* strain S499 was isolated from soil by L. Delcambe (CNPEM, Liège, Belgium) and studied in our laboratory for several years. *B. amyloliquefaciens* CH1, CH2, and AK3 strains were given by R. Borriss (Center of Bacterial Genetic, Biology Institute, Humboldt University of Berlin, Germany). *B. amyloliquefaciens* strains and *B. subtilis* S499 were cultured in aerobic condition in flasks containing optimized medium with the following composition per liter: 7 g of yeast extract, 30 g of casein peptone, 20 g of saccharose, 1.9 g of KH_2PO_4, 0.45 g of $MgSO_4$, 10 mg of citric acid, 3.6 mg of $MnSO_4 \cdot H_2O$, 0.014 mg of $ZnSO_4 \cdot 7H_2O$, 0.01 mg of H_3BO_3, 0.005 mg of $FeCl_3 \cdot 6H_2O$, 0.004 mg of $NaMoO_4$, 0.002 mg of KI, and 0.001 mg of $CuSO_4$, adjusted to pH 7 with KOH. All cultures were inoculated with a 16-h-old preculture and incubated at 30°C on a rotary shaker (120 rpm, Multi-Shaker PSU 20). Cellular concentration was measured by optical density at 600 nm.

Tobacco cells (*Nicotiana tabacum* L. cv. Bright Yellow-2) were cultivated in Murashige and Skoog medium (MP Biomedicals, Irvine, CA, U.S.A.) (4.4 g/liter) at pH 5.8, completed with saccharose at 30 g/liter, KH_2PO_4 at 0.2 g/liter, myo-inositol at 50 mg/ liter, thiamine at 2.5 mg/liter, and 2,4-dichlorophenoxyacetic acid at 0.2 mg/liter. Tobacco cells were grown at a constant temperature (28°C) in the dark on a rotary shaker (110 rpm, Multi-Shaker PSU 20) and subcultured weekly by the addition of 4 ml of cells to 100 ml of fresh liquid medium. Experiments were realized using cells in exponential growth phase after 5 to 6 days.

Semipreparative purification of surfactin homologues.

Milligrams of surfactin were obtained from AIBI (Agricultural University of Gembloux, Belgium) as a mixture of homologues with a purity of 95%. The different homologues were identified and relative proportions established by the combined use of MALDI-TOF mass spectrometry and reversed-phase HPLC as described in the next section (surfactins: A C13, 22%; B C13, 17%; A C14, 28%; A C15, 33%). A mean value of 1,050 amu for the different homologues present in the surfactin mixture was used as molecular weight to prepare the desired concentrations. The various homologues were purified from this mixture (solubilized in pure methanol) by semipreparative reverse-phase HPLC (HP 1100 series system; Hewlett-Packard) on a Chromspher 5 C-18 (250 by 10 mm, 5-µm packing; Chrompack) column by repeated injections of 100-µl aliquots. Surfactins were eluted isocratically with 78% acetonitrile in 0.1% trifluoroacetic (TFA) acid in milliQ water and at a flow rate of 4 ml/min. Samples were monitored spectrophotometrically at 280, 214, 254, 320, and 375 nm by means of a DAD. The collected fractions corresponding to each peak were pooled and evaporated to dryness (Speed-Vac Plus SC110A; Savant Instruments, Inc., Holbrook, NY, U.S.A.). The residues were weighted and resuspended in methanol to a concentration of 1 mg/ml, and adequate volumes were used to treat tobacco cells.

Quantification of surfactins.

Surfactin production by the strains was determined from 10-ml samples of culture supernatant collected after growth for 72 h. Supernatant samples were loaded on an Alltech Maxi-Clean SPE 900-mg C-18 column after activation and conditioning of the cartridge by flushing with ACN and water. After loading, the packing bed was washed successively with 10 ml of water and 5 ml of 40% ACN. The remaining material containing the elicitor then was desorbed with 1 ml of 100% methanol and evaporated to dryness. The residue was resolubilized in 80 µl of methanol and analyzed by HPLC on a Purosphere RP-18 analytical column (250 by 4.6 mm, 5-µm packing; Merck, Darmstadt, Germany) in the isocratic mode (78% ACN + 0.05% TFA in milliQ water). Amounts were calculated on the basis of the corresponding peak area at 280 nm.

The methylation of surfactins was carried out by dissolving 1 mg of surfactin in 1 ml of anhydrous methanol with 10 µl of concentrated HCl. The sample was kept at 22°C overnight. After adding an equal volume of distilled water, methylated surfactin was purified by HPLC (using the same method describe above). Linear derivatives of the C14 surfactin homologue were given by S. Dufour (Laboratoire de Biologie Industrielle, University of Gembloux, Belgium) and were synthesized using the method described by Dufour and associates (2005).

Determination of extracellular pH changes and hydrogen peroxide accumulation.

Extracellular pH variation was monitored with a glass pH electrode (Microprocessor pH Meter 211; Hanna Instruments) in 10 ml of agitated cell culture. The production of H_2O_2 was monitored by chemiluminescence from the ferricyanide-catalyzed oxidation of luminol using a luminometer (TD-20/20 Luminometer, Turner Designs, Fresno, CA, U.S.A.). Briefly, tobacco cells in exponential phase were filtrated on a Whatman filter and resuspended in HEPES solution (mannitol at 31.8 g/liter, HEPES at 480 mg/liter, K_2SO_4 at 90 mg/liter, and $CaCl_2$ at 73 mg/liter, adjusted to pH 7) to obtain a final concentration of cells of approximately 0.15 g/ml. After treatment with surfactin, a 100-µl aliquot of the cell suspension was added to 100 µl of phosphate buffer (50 mM, pH 7.9) and 100 µl of 1.1 mM luminol in phosphate buffer. The reaction was started by addition of 100 µl of $K_3[Fe(CN)_6]$, 14 mM freshly prepared, and the signal was integrated over the first 30 s after reaction start. AOS production was compared with calibration values established with known concentrations of H_2O_2. Lanthanum chloride and EGTA were added in the specified concentrations to cell suspensions 30 s before addition of surfactin while DPI and $AACOCF_3$ were added 10 and 30 min, respectively, prior to surfactin addition.

Quantification of ions fluxes.

Five-day-old tobacco suspension cells were equilibrated for 5 h in 10 mM MES, 5 mM sucrose, 0.5 mM $CaSO_4$, and 3 mM KOH, pH 5.7, for cells at approximately 150 mg/ml. Samples were taken every 5 min and then filtrated twice on 0.45- and 0.2-µM cellulose acetate membrane filters. Concentrations of nitrate and chlorure ions were measured by conductimetry after separation by ion chromatography (Dionex-120) using a 4-by-250-mm column (Ionpack AS9-HC) and a 9-mM sodium carbonate flow of 1 ml/min. Potassium ions were detected by flame emission spectroscopy (Eppendorf, Hamburg, Germany) with an air-propane flame. The final composition of the spectral buffer was 0.5% CsCl, 2.5% $Al(NO_3)_3$, and 0.1 N HCl.

Cell mortality determination.

A volume of 1.5 ml of treated tobacco suspension cells was filtered (Miracloth pore size 22 to 25 µm; Calbiochem) and resuspended in 1 ml of HEPES medium at pH 5.7. A 0.5% (wt/vol, distilled water) Evans Blue solution (100 µl) was added and the mixture incubated for 10 min at 27°C on a rotary shaker (110 rpm). Then, 800 µl was filtered and washed with 10 ml of water to remove nonfixed dye. The fixed dye was finally solubilized by suspending washed cells in 1 ml of a 50% MeOH/1% sodium dodecyl sulfate (SDS) solution at 55°C for 30 min. The quantification of dead cells was carried out by measuring the absorbance of the colored supernatant at 620 nm (Beckman Coulter AD 340, Indianapolis, IN, U.S.A.). A calibration curve was realized by treating tobacco cells with increasing concentrations of Triton X-100 0.9%. Pictures of cells were realized with an Axioskop2-type microscope (Axiocam camera, Axiovision 3.0 software; Carl Zeiss Jena GmbH, Jena, Germany).

PAL and LOX activities.

Cell suspension (5 ml) was filtrated, washed with 10 ml of cold distilled water, and resuspended in extraction buffer (50 mM borate buffer, 5% glycerol, and 5 mM β-mercaptoethanol, pH 8) to obtain a final concentration of 0.5 g/ml. Cells were then sonicated (Sonoplus, Bandelin Electronic, Berlin) during 1 min at 70% maximal power. After 10 min of incubation, cell suspensions were centrifuged for 10 min and supernatants were directly analyzed. The reaction mixture consisted of 200 µl of extract added to 2.8 ml of dosage buffer (35.7 mM sodium tetraborate, pH 8) containing 5.35 mM L-phenylalanine. PAL activity was measured by apparition of cinnamic acid at 290 nm. The reaction was performed at 40°C and absorbance was followed for 20 min.

For LOX measurements, cells were resuspended in 100 mM phosphate buffer, pH 7, containing $Na_2S_20_5$ at 0.4 g/liter and Tween 20 at 2.5 g/liter. After sonication and incubation for 1 h on ice, cells suspensions were centrifuged and supernatants were directly analyzed. A 50-µl aliquot was added to 2.940 ml of oxygened dosage buffer (phosphate buffer, pH 7) and reaction was initiated by adding 10 µl of substrate (18 mM linolenic acid in phosphate buffer and 0.05 M NaOH). LOX activity was measured by apparition of hydroperoxyde acid at 234 nm. Reaction was performed at 30°C and absorbance was followed for 20 min. The rates of increase were calculated from the initial linear portion of the curve and activities are expressed in pKatal.

Northern blot analyses.

cDNA probes (565 pb) were synthesized by polymerase chain reaction (PCR) amplification of genomic DNA using forward primer (5′-TCTTGAATGCTGGAGTTTTTGG-3′) for PAL1 and reverse primer (5′-TGTTCCATAATAGCAGCAG CCTC-3′) for PAL2. Primers were chosen by comparison on five conserved sequences of PAL genes (X78269, D17467, AB008200, AB 008199, and M84466; GenBank, Pubmed). Probes (TabPal1 and TabPab2) were cloned with pGEM-T easy vector (Promega Corp., Madison, WI, U.S.A.). For expression pattern, total RNA was extracted from tobacco cell powder (obtained by grinding fresh cells in liquid nitrogen and stocked at –80°C) by the phenol/SDS method. Total RNA (15 µg) was separated on formaldehyde gels, blotted onto Hybond N^+ membranes (Amersham, Little Chalfont, U.K.), and hybridized with a mix of DNA probes TabPal1 and TabPal2 labeled by random priming in the presence of [α-32P]dATP, according to the procedure recommended by the manufacturer (Random Primers DNA Labeling System; Invitrogen, Carlsbad, CA, U.S.A.). After hybridization, the blots were washed and then exposed to X-ray film (Fujifilm, Tokyo) for at least 24 h.

Phenolic analyses.

Analyses of phenolic compounds were realized on tobacco cell culture supernatants extracted with ethyl acetate (1:1, vol/vol). Extracts were concentrated (Speed-Vac Plus SC110A; Savant) and 10 µl was injected in HPLC on a lichrospher 100 RP C-18 column (250 by 4.6 mm, 5-µm packing; Merck), using acetonitrile/water with 0.1% TFA. Compounds were eluted with a gradient of acetonitrile as follows: (time in min/percent acetonitrile/percent 0.1% TFA/flow) 0:5:95:0.5, 2:5:95:0.5, 2.5:5:95:1, 5:5:95:1, 15:20:80:1, 25:25:75:1, 35:32:68:1, 36:95:5:1, 40:95:5:1, 41:5:95:1, and 46:5:95:1. Identification of phenolic compounds was performed with LC-MS. The HPLC system was a Waters Alliance 2690. Elution program and column used were the same as described above. The MS instrument was a Waters Micromass Quattro Ultima Platinum triple-quadrupole mass spectrometer running in positive-ion mode. The MS was tuned to optimize the signal of each stan-

dard of the various phenolics by direct injection of diluted solutions. The capillary voltage used was 2.6 kV, and the source and desolvatation temperatures were set at 115 and 250°C, respectively. The cone and desolvatation gas flows were 50 and 630 liters/h, respectively. The collision cell pressure was set at 2 μbars. Both instruments were controlled with the MassLynx software.

ACKNOWLEDGMENTS

This work received financial support from the Program F.R.F.C. no. 2.4624.06 (National Funds for Scientific Research, FRS-F.N.R.S, Belgium). E. Jourdan and M. Ongena are postdoctoral researcher and research associate, respectively, at the FRS-F.N.R.S. G. Henry is recipient of a grant from the Formation à la Recherche dans l'Industrie et l'Agriculture.

LITERATURE CITED

Apel, K., and Hirt, H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55:373-399.

Audenaert, K., Pattery, T., Cornelis, P., and Höfte, M. 2002. Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2:Role of salicylic acid, pyochelin, and pyocyanin. Mol. Plant-Microbe Interact. 15:1147-1156.

Bais, H. P., Fall, R., and Vivanco, J. M. 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol. 134:307-319.

Baker, C. J., and Mock, N. M. 1994. An improved method for monitoring cell-death in cell-suspension and leaf disc assays using Evans blue. Plant Cell Tissue Organ. Cult. 39:7-12.

Bakker, P., Pieterse, C. M. J., and Van Loon, L. C. 2007. Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology 97:239-243.

Baysal, T., and Demirdoven, A. 2007. Lipoxygenase in fruits and vegetables: A review. Enzyme Microb. Technol. 40:491-496.

Blée, E. 2002. Impact of phyto-oxylipins in plant defense. Trends Plant Sci. 7:315-321.

Boller, T. 1995. Chemoperception of microbial signals in plant cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46:189-214.

Bonmatin, J. M., Laprevote, O., and Peypoux, F. 2003. Diversity among microbial cyclic lipopeptides: Iturins and surfactins. Activity-structure relationships to design new bioactive agents. Comb. Chem. High Throughput Screen 6:541-556.

Bourque, S., Ponchet, M., Binet, M. N., Ricci, P., Pugin, A., and Lebrun-Garcia, A. 1998. Comparison of binding properties and early biological effects of elicitins in tobacco cells. Plant Physiol. 118:1317-1326.

Carrillo, C., Teruel, J. A., Aranda, F. J., and Ortiz, A. 2003. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochim. Biophys. Acta 1611:91-97.

Chandra, S., Heinstein, P. F., and Low, P. S. 1996. Activation of phospholipase A by plant defence elicitors. Plant Physiol. 110:979-986.

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. 2006. Host–microbe interactions: Shaping the evolution of the plant immune response. Cell 124:803-814.

Coventry, H. S., and Dubery, I. A. 2001. Lipopolysaccharides from *Burkholderia cepacia* contribute to an enhanced defensive capacity and the induction of pathogenesis-related proteins in *Nicotianae tabacum*. Physiol. Mol. Plant Pathol. 58:149-158.

Deleu, M., Bouffioux, O., Razafindralambo, H., Paquot, M., Hbid, C., Thonart, P., Jacques, P., and Brasseur, R. 2003. Interaction of surfactin with membranes: A computational approach. Langmuir 19:3377-3385.

De Meyer, G., Audenaert, K., and Höfte, M. 1999. *Pseudomonas aeruginosa* 7NSK2-induced systemic resistance in tobacco depends on in planta salicylic acid accumulation but is not associated with PR1a expression. Eur. J. Plant. Pathol. 105:513-517.

Desender, S., Klarzynski, O., Potin, P., Barzic, M. R., Andrivon, D., and Val, F. 2006. Lipopolysaccharides of *Pectobacterium atrosepticum* and *Pseudomonas corrugata* induce different defense response patterns in tobacco, tomato, and potato. Plant Biol. 8:636-645.

Desender, S., Andrivon, D., and Val, F. 2007. Activation of defense reactions in Solanaceae: Where is the specificity? Cell. Microbiol. 9:21-30.

De Vleesschauwer, D., Cornelis, P., and Höfte, M. 2006. Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances *Rhizoctonia solani* susceptibility in rice. Mol. Plant-Microbe Interact. 19:1406-1419.

Dixon, R. A., Achnine, L., Kota, P., Liu, C. J., Reddy, M. S. S., and Wang, L. J. 2002. The phenylpropanoid pathway and plant defense—a genomics perspective. Mol. Plant Pathol. 3:371-390.

Dong, H., Delaney, T. P., Bauer, D. W., and Beer, S. V. 1999. Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. Plant J. 20:207-215.

Dorey, S., Kopp, M., Geoffroy, P., Fritig, B., and Kauffmann, S. 1999. Hydrogen peroxide from the oxidative burst is neither necessary nor sufficient for hypersensitive cell death induction, phenylalanine ammonia lyase stimulation, salicylic acid accumulation, or scopoletin consumption in cultured tobacco cells treated with elicitin. Plant Physiol. 121:163-171.

Dufour, S., Deleu, M., Nott, K., Wathelet, B., Thonart, P., and Paquot, M. 2005. Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. Biochim. Biophys. Acta 1726:87-95.

Duijff, B. J., Gianinazzi-Pearson, V., and Lemanceau, P. 1997. Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. New Phytol. 135:325-334.

Durrant, W. E., and Dong, X. 2004. Systemic acquired resistance. Annu. Rev. Phytopathol. 42:185-209.

Eeman, M., Berquand, A., Dufrene, Y. F., Paquot, M., Dufour, S., and Deleu, M. 2006. Penetration of surfactin into phospholipid monolayers: Nanoscale interfacial organization. Langmuir 22:11337-11345.

Fedoroff, N. 2006. Redox regulatory mechanisms in cellular stress responses. Ann. Bot. 98:289-300.

Felix, G., Duran, J. D., Volko, S., and Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. 18:265-276.

Feussner, I., and Wasternack, C. 2002. The lipoxygenase pathway. Annu. Rev. Plant Biol. 53:275-297.

Fobert, P. R., and Despres, C. 2005. Redox control of systemic acquired resistance. Curr. Opin. Plant. Biol. 8:378-382.

Garcia-Brugger, A., Lamotte, O., Vandelle, E., Bourque, S., Lecourieux, D., Poinssot, B., Wendehenne, D., and Pugin, A. 2006. Early signaling events induced by elicitors of plant defenses. Mol. Plant-Microbe Interact. 19:711-724.

Gómez-Gómez, L. 2004. Plant perception systems for pathogen recognition and defense. Mol. Immunol. 41:1055-1062.

Greenberg, J. T., and Yao, N. 2004. The role and regulation of programmed cell death in plant-pathogen interactions. Cell. Microbiol. 6:201-211.

Haas, D., and Défago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3:307-319.

He, P, Shan, L, and Sheen, J. 2007. Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. Cell Microbiol. 9:1385-1396.

Heath, M. C. 2000. Hypersensitive response-related death. Plant Mol. Biol. 44:321-334.

Heerklotz, H., and Seelig, J. 2007. Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. Eur. Biophys. J. Biophys. Lett. 36:305-314.

Hofemeister, J., Conrad, B., Adler, B., Hofemeister, B., Feesche, J., Kucheryava, N., Steinborn, G., Franke, P., Grammel, N., Zwintscher, A., Leenders, F., Hitzeroth, G., and Vater, J. 2004. Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. Mol. Genet. Genomics 272:363-378.

Iavicoli, A., Boutet, E., Buchala, A., and Métraux, J. P. 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol. Plant-Microbe Interact. 16:851-858.

Jones, J. D., and Dangl, J. L. 2006. The plant immune system. Nature 444:323-329.

Kawano, T., Pinontoan, R., Uozumi, N., Miyake, C., Asada, K., Kolattukudy, P. E., and Muto, S. 2000. Aromatic monoamine-induced immediate oxidative burst leading to an increase in cytosolic Ca^{2+} concentration in tobacco suspension culture. Plant Cell Physiol. 41:1251-1258.

Kim, S. Y., Kim, J. Y., Kim, S. H., Bae, H. J., Yi, H., Yoon, S. H., Koo, B. S., Kwon, M., Cho, J. Y., Lee, C. E., and Hong, S. 2007. Surfactin from *Bacillus subtilis* displays anti-proliferative effect via apoptosis induction, cell cycle arrest and survival signaling suppression. FEBS (Fed. Eur. Biochem Soc.) Lett. 581:865-871.

Kloepper, J. W., Ryu, C. M., and Zhang, S. A. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259-1266.

Koehl, J., Obwald, W., Kohn, H., Elstner E, F., and Heiser, I. 2003. Different responses of two tobacco cultivars and their cell suspense on cultures to quercinin, a novel elicitin from *Phytophthora quercina*. Plant Physiol. Biochem. 41:261–269.

Kombrink, E., and Schmelzer, E. 2001. The hypersensitive response and its role in local and systemic disease resistance. Eur. J. Plant. Pathol. 107:69-78.

La Camera, S., Gouzerh, G., Dhondt, S., Hoffmann, L., Fritig, B., Legrand, M., and Heitz, T. 2004. Metabolic reprogramming in plant innate immunity: The contributions of phenylpropanoid and oxylipin pathways. Immunol. Rev. 198:267-284.

Leclère, V., Béchet, M., Adam, A., Guez, J. S., Wathelet, B., Ongena, M., Thonart, P., Gancel, F., Chollet-Imbert, M., and Jacques, P. 2005. Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. Appl. Environ. Microbiol. 71:4577-4584.

Lecourieux, D., Raneva, R., and Pugin, A. 2006. Calcium in plant defense-signaling pathways. New Phytol. 171:249-269.

Lee, S., Suh, S., Kim, S., Crain, R. C., Kwak, J. M., Nam, H. G., and Lee, Y. 1997. Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. Plant J. 12:547-556.

Leeman, M., den Ouden, F. M., Van Pelt, J. A., Dirkx, F. P. M., Steijl, H., Bakker, P. H. A. M., and Schippers, B. 1996. Iron availability affects induction of systemic resistance to Fusarium wilt of radish by *Pseudomonas fluorescens*. Phytopathology 86:149-155.

Maher, E. A., Bate, N. J., Ni, W., Elkind, Y., Dixon, R. A., and Lamb, C. J. 1994. Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. Proc. Natl. Acad. Sci. U.S.A. 91:7802-7806.

Matsui, K. 2006. Green leaf volatiles: Hydroperoxide lyase pathway of oxylipin metabolism. Curr. Opin. Plant. Biol. 9:274-280.

Meziane, H., Van der Sluis, I., Van Loon, L. C., Höfte, M., and Bakker, P. A. H. M. 2005. Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. Mol. Plant Pathol. 6:177-185.

Montesano, M., Brader, G., and Palva, E. T. 2003. Pathogen derived elicitors: Searching for receptors in plants. Mol. Plant Pathol. 4:73-79.

Navarez-Vasquez, J., Florin-Christensen, J., and Ryan, C. A. 1999. Positional specificity of a phospholipase a activity induced by wounding, systemin and oligosaccharide elicitors in tomato leaves. Plant Cell 11:2249-2260.

Ongena, M., and Thonart, P. 2006. Resistance induced in plants by non-pathogenic microorganisms: Elicitation and defense responses. Pages 447-463 in: Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues, 1st ed. A. Jaime and T. Da Silva, eds. Global Science Books, London.

Ongena, M., Duby, F., Rossignol, F., Fauconnier, M. L., Dommes, J., and Thonart, P. 2004. Stimulation of the lipoxygenase pathway is associated with systemic resistance induced in bean by a nonpathogenic *Pseudomonas* strain. Mol. Plant-Microbe Interact. 17:1009-1018.

Ongena, M., Jacques, P., Touré, Y., Destain, J., Jabrane, A., and Thonart, P. 2005a. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. Appl. Microbiol. Biotechnol. 69:29-38.

Ongena, M., Duby, F., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J., and Thonart, P. 2005b. *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. Appl. Microbiol. Biotechnol. 67:692-698.

Ongena, M., Jourdan, E., Schafer, M., Kech, C., Budzikiewicz, H., Luxen, A., and Thonart, P. 2005c. Isolation of an n-alkylated benzylamine derivative from *Pseudomonas putida* BTP1 as elicitor of induced systemic resistance in bean. Mol. Plant-Microbe Interact. 18:562-569.

Ongena, M., Adam, A., Jourdan, E., Paquot, M., Brans, A., Joris, B., Arpigny, J. L., and Thonart, P. 2007. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ. Microbiol. 9:1084-1090.

Peypoux, F., Bonmatin, J. M., and Wallach, J. 1999. Recent trends in the biochemistry of surfactin. Appl. Microbiol. Biotechnol. 51:553-563.

Pieterse, C. M. J., Van Pelt, J. A., Van Wees, S. C. M., Ton, J., Leon-Kloosterziel, K. M., Keurentjes, J. J. B., Verhagen, B. W. M., Knoester, M., Van der Sluis, I., Bakker, P. A. H. M., and Van Loon, L. C. 2001. Rhizobacteria-mediated induced systemic resistance: Triggering, signaling and expression. Eur. J. Plant. Pathol. 107:51-61.

Pineros, M. A., and Kochian, L. V. 2003. Differences in whole-cell and single-channel ion currents across the plasma membrane of mesophyll cells from two closely related *Thlaspi* species. Plant Physiol. 131:583-594.

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M. J., Kift, N., Carbonne, F., Griffiths, G., Esquerre-Tugaye, M. T., Rosahl, S., Castresana, C., Hamberg, M., and Fournier, J. 2005. Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol. 139:1902-1913.

Pugin, A, Frachisse, J. M., Tavernier, E., Bligny, R., Gout, E., Douce, R., and Guern, J. 1997. Early events induced by the elicitor cryptogein in tobacco cells: Involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway. Plant Cell 9:2077-2091.

Raaijmakers, J. M., de Bruijn, I., and de Kock, M. J. D. 2006. Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: Diversity, activity, biosynthesis, and regulation. Mol. Plant-Microbe Interact. 19:699-710.

Ran, L. X., Li, Z. N., Wu, G. J., Van Loon, L. C., and Bakker, P. A. H. M. 2005. Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. Eur. J. Plant. Pathol. 113:59-70.

Reitz, M., Oger, P., Meyer, A., Niehaus, K., Farrand, S. K., Hallmann, J., and Sikora, R. A. 2002. Importance of the O-antigen, core-region and lipid A of rhizobial lipopolysaccharides for the induction of systemic resistance in potato to *Globodera pallida*. Nematology 4:73-79.

Roos, W., Dordschbal, B., Steighardt, J., Hieke, M., Weiss, D., and Saalbach, G. 1999. A redox-dependent, G-protein-coupled phospholipase A of the plasma membrane is involved in the elicitation of alkaloid biosynthesis in *Eschscholtzia californica*. Biochim. Biophys. Acta 1448:390-402.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., and Pare, P. W. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol. 134:1017-1026.

Schneider, J., Taraz, K., Budzikiewicz, H., Deleu, M., Thonart, P., and Jacques, P. 1999. The structure of two fengycins from *Bacillus subtilis* S499. Z. Naturforsch. C: Biosci. 54:859-866.

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., Hutzler, P., Schmid, M., Van Breusegem, F., Eberl, L., Hartmann, A., and Langebartels, C. 2006. Induction of systemic resistance in tomato by *N*-acyl-L-homoserine lactone-producing rhizosphere bacteria. Plant Cell Environ. 29:909-918.

Shah, J. 2005. Lipids, lipases, and lipid-modifying enzymes in plant disease resistance. Annu. Rev. Phytopathol. 43:229-260.

Sharan, M., Taguchi, G., Gonda, K., Jouke, T., Shimosaka, M., Hayashida, N., and Okazaki, M. 1998. Effects of methyl jasmonate and elicitor on the activation of phenylalanine ammonia-lyase and the accumulation of scopoletin and scopolin in tobacco cell cultures. Plant Sci. 132:13-19.

Sheppard, J. D., Jumarie, C., Cooper, D. G., and Laprade, R. 1991. Ionic channels induced by surfactin in planar lipid bilayer membranes. Biochim. Biophys. Acta 1064:13-23.

Siddiqui, I. A., and Shaukat, S. S. 2003. Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: Importance of bacterial secondary metabolite, 2,4-diacetylpholoroglucinol. Soil Biol. Biochem. 35:1615-1623.

Sticher, L., Mauch-Mani, B., and Métraux, J. P. 1997. Systemic acquired resistance. Annu. Rev. Phytopathol. 35:235-270.

Strobel, N. E., Ji, C., Gopalan, S., Kuc, J. A., and He, S. Y. 1996. Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 HrpZ(Pss) protein. Plant J. 9:431-439.

Tran, H., Ficke, A., Asiimwe, T., Höfte, M., and Raaijmakers, J. M. 2007. Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. New Phytol. 175:731-742.

Vanittanakom, N., Loeffler, W., Koch, U., and Jung, G. 1986. Fengycin—A novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. J. Antibiot. 39:888-901.

Van Loon, L. C. 2007. Plant responses to plant growth-promoting rhizobacteria. Eur. J. Plant. Pathol. 119:243-254.

Van Loon, L. C., and Bakker, P. A. H. M. 2005. Induced systemic resistance as a mechanism of disease suppression by rhizobacteria. In: PGPR: Biocontrol and Biofertilization. Z. A. Siddiqui, ed. Springer Science, Dordrecht, The Netherlands.

Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36:453-483.

Van Loon, L. C., Rep, M., and Pieterse, C. M. J. 2006. Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. 44:135-162.

Van Wees, S. C. M., Van der Ent, S., and Pieterse, C. M. J. 2008. Plant immune responses triggered by beneficial microbes. Curr. Opin. Plant Biol. 11:443-448.

Youssef, N. H., Duncan, K. E., and McInerney, M. J. 2005. Importance of 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity. Appl. Environ. Microbiol. 71:7690-7695.

Zahir, Z. A., Arshad, M., and Frankenberger, W. T. 2004. Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Adv. Agron. 81:97-168.

Zhao, J., Davis, L. C., and Verpoorte, R. 2005. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv. 23:283-333.