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Cyclic lipopeptides from *Bacillus subtilis* activate distinct patterns of defence responses in grapevine

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SUMMARY

Non-self-recognition of microorganisms partly relies on the perception of microbe-associated molecular patterns (MAMPs) and leads to the activation of an innate immune response. *Bacillus subtilis* produces three main families of cyclic lipopeptides (LPs), namely surfactins, iturins and fengycins. Although LPs are involved in induced systemic resistance (ISR) activation, little is known about defence responses induced by these molecules and their involvement in local resistance to fungi. Here, we showed that purified surfactin, mycosubtilin (iturin family) and plipastatin (fengycin family) are perceived by grapevine plant cells. Although surfactin and mycosubtilin stimulated grapevine innate immune responses, they differentially activated early signalling pathways and defence gene expression. By contrast, plipastatin perception by grapevine cells only resulted in early signalling activation. Gene expression analysis suggested that mycosubtilin activated salicylic acid (SA) and jasmonic acid (JA) signalling pathways, whereas surfactin mainly induced an SA-regulated response. Although mycosubtilin and plipastatin displayed direct antifungal activity, only surfactin and mycosubtilin treatments resulted in a local long-lasting enhanced tolerance to the necrotrophic fungus *Botrytis cinerea* in grapevine leaves. Moreover, challenge with specific strains overproducing surfactin and mycosubtilin led to a slightly enhanced stimulation of the defence response compared with the LP-non-producing strain of *B. subtilis*. Altogether, our results provide the first comprehensive view of the involvement of LPs from *B. subtilis* in grapevine plant defence and local resistance against the necrotrophic pathogen *Bo. cinerea*. Moreover, this work is the first to highlight the ability of mycosubtilin to trigger an immune response in plants.

Keywords: *Bacillus subtilis*, *Botrytis cinerea*, grapevine, induced resistance, lipopeptides, MAMP.

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INTRODUCTION

Non-self-perception in plant innate immunity initially relies on the recognition of microbe-associated molecular patterns (MAMPs) (Boller and Felix, 2009; Dodds and Rathjen, 2010). MAMP-triggered immunity (MTI) involves the activation of a wide range of defence responses and provides the plant with a basal level of resistance to pathogenic microorganisms. MTI defence responses are characterized by early signalling events, including ion fluxes, mitogen-activated protein (MAP) kinase cascade activation and the production of reactive oxygen species (ROS) (Garcia-Brugger *et al*., 2006). Key hormone molecules, such as salicylic acid (SA) and jasmonic acid (JA), are involved in the regulation of downstream defence genes (Robert-Seilaniantz *et al*., 2011). Interactions between these signal molecules allow the plant to activate and/or modulate an appropriate spectrum of responses, including the strengthening of plant cell physical barriers and the production of antimicrobial compounds that participate in the restriction of the pathogen (Dodds and Rathjen, 2010).

MAMPs are typically essential components of whole classes of pathogens, such as flagellin, EF-Tu, peptidoglycans, lipopolysaccharides (LPSs) from bacteria or chitin, and elicitins from fungi and oomycetes (Boller and Felix, 2009). Recently, rhamnolipids (RLs), which are bacterial molecules with amphiphilic properties, have been characterized as a new class of MAMPs perceived by plant cells, and activate an MTI in several plant species (Sanchez *et al*., 2012; Varnier *et al*., 2009; Vatsa *et al*., 2010).

Lipopeptides (LPs), another class of amphiphilic molecules, have also emerged as key players in the induction of plant immunity driven by beneficial microorganisms (Falardeau *et al*., 2013; Ongena and Jacques, 2008; Raaijmakers *et al*., 2010). LPs are produced by a variety of bacterial genera, such as *Streptomyces*, *Pseudomonas* and *Bacillus* (Raaijmakers *et al*., 2010). LPs consist of a short peptide chain linked to a lipid tail. They are synthesized by a non-ribosomal enzyme complex called non-ribosomal peptide synthetase (NRPS), which confers considerable structural diversity to the molecules and results in the production of linear, branched or cyclic compounds (Strieker *et al*., 2010). This structural diversity

is associated with a large range of functions for the microorganisms. For instance, they are used to modify surface tension, leading to improved motility (Leclere *et al*., 2006; Raaijmakers *et al*., 2010), to produce biofilms, which are important for surface attachment and root colonization (Bais *et al*., 2004), and to chelate metal ions (Raaijmakers *et al*., 2010). In addition, LPs also show direct antagonist activity toward viruses, mycoplasmas, bacteria, yeast, fungi and oomycetes (Coutte *et al*., 2010; Fickers *et al*., 2009; Khong *et al*., 2012; Ongena and Jacques, 2008; Ongena *et al*., 2007; Raaijmakers and Mazzola, 2012; Toure *et al*., 2004; van de Mortel *et al*., 2009). LPs are able to activate an induced systemic resistance (ISR), which is effective against several diseases in a large range of plant systems (Falardeau *et al*., 2013; Ongena and Jacques, 2008). For instance, the treatment of tomato roots with massetolide A leads to an increased resistance to the oomycete *Phytophthora infestans* in leaves (Tran *et al*., 2007). Fengycin and surfactin are able to trigger an ISR effective against *Botrytis cinerea* infection in tomato and bean (Ongena *et al*., 2007). Although LPs are known to be involved in ISR activation, little is known about their perception by plant cells. To date, the only available data have been reported by Jourdan *et al*. (2009), indicating that surfactin stimulates extracellular medium alkalinization, ROS production and the activity of defence-related enzymes, such as phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX), in tobacco cell suspensions.

Bacillus subtilis is known to produce three main families of cyclic LPs, namely surfactins, iturins and fengycins (Falardeau *et al*., 2013). In this study, we investigated the perception of these three families of LPs by grapevine plant cells. We also investigated the efficacy of each LP to protect grapevine leaves against the necrotrophic pathogen *Bo. cinerea*. We showed, for the first time, that grapevine plant cells perceive the three families of LPs, and that these compounds differentially activate the plant innate immunity system and trigger different levels of local resistance to the fungus. Interestingly, mycosubtilin is the most efficient stimulator of the innate immune response in grapevine. We also found that LP overproduction by *B. subtilis* is not critical in the activation of grapevine innate immunity driven by the bacterium, suggesting that other MAMPs may be involved in the process. Our results provide a comprehensive view of the involvement of LPs in grapevine plant defence and local protection against *Bo. cinerea*.

RESULTS

LP perception triggers early signalling events with different signatures

Extracellular alkalinization is an essential component of ion fluxes involved in plant defence (van Loon *et al*., 2008), and has been used as an efficient method to monitor chemosensory perception in cultured plant cells (Felix *et al*., 1993). We first carried out dose–response experiments on grapevine cell suspension cultures using concentrations of LPs ranging from 10 to 50 μg/mL. All LPs induced an alkalinization of the grapevine cell suspension medium (Fig. 1). However, we observed important differences between the different molecules. Plipastatin only caused a limited alkalinization of the culture medium, whereas cell suspensions incubated with surfactin and mycosubtilin exhibited a clear dose-dependent response with a strong alkalinization at 50 μg/mL (Fig. 1). Interestingly, mycosubtilin treatment displayed a long-lasting alkalinization response over several hours that did not return to baseline (data not shown). The subsequent experiments were carried out using a concentration of 50 μg/mL, which displayed a sustained alkalinization response with the different LPs. The kinase inhibitor K-252a was used to verify whether the proton fluxes were physiologically associated with plant cell signalling activation. The addition of K-252a strongly inhibited all LP-induced alkalinizations (Fig. S1, see Supporting Information). Moreover, no refractory state of the proton influx response, indicative of homologous desensitization (Felix *et al*., 1993; Smith *et al*., 2014),

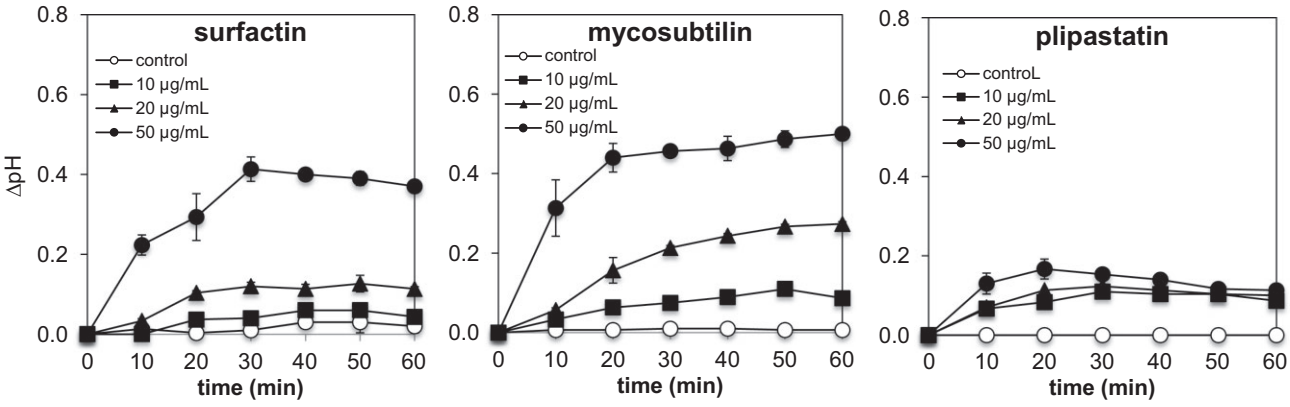

Fig. 1 Medium alkalinization of grapevine cell suspensions challenged by lipopeptides (LPs). Cells were treated with surfactin, mycosubtilin or plipastatin at 10, 20 and 50 μg/mL or dimethylsulphoxide (DMSO) 0.1% (control). Data presented are means of triplicate experiments ± standard deviation (SD).

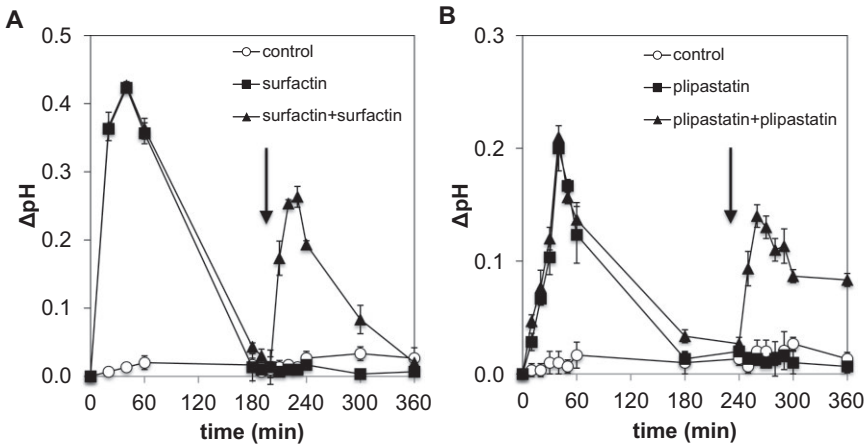

Fig. 2 Alkalinization response of grapevine cells exposed to consecutive additions of surfactin and plipastatin. Cells were sequentially treated with surfactin (A) or plipastatin (B) at 50 μg/mL. A second dose of LP was added (arrow) once the alkalinization response had returned to baseline. Data presented are means of triplicate experiments ± standard deviation (SD).

was observed with either surfactin or plipastatin (Fig. 2). As the alkalinization response was not transient with mycosubtilin, we were unable to test the refractory state with this molecule. Interestingly, only surfactin stimulated a significant and strong oxidative burst (Fig. S2, see Supporting Information). In addition, treatment with the three LPs did not activate calcium influx in grapevine cells (L. Trda and B. Poinssot, personal communication, Université de Bourgogne, UMR 1347 Agroécologie, Pôle Interactions Plantes Micro-organismes, 17 rue Sully, 21000 Dijon, France).

Perception of surfactin and mycosubtilin, but not plipastatin, stimulates defence gene expression

In order to compare grapevine gene expression profiles after LP challenge, we selected several reliable defence gene markers covering a large set of defence classes. We used the *chit4c* and *pin* genes (coding for an acidic chitinase and a protease inhibitor, respectively), which are strongly induced in the grapevine plant systems on elicitation (Bordiec *et al*., 2011). The *gluc* gene (coding for a basic glucanase) was used as a JA-regulated gene marker, whereas the *Vv17.3* gene was chosen as a marker of the SA-dependent signalling pathway in grapevine (Bordiec *et al*., 2011). The expression profile of these genes was monitored 9 and 24 h after inoculation of grapevine cells with the three LPs. Mycosubtilin induced strong expression of all defence markers (Fig. 3). *chit4c*, *pin* and *Vv17.3* were significantly up-regulated after challenge with surfactin, but at a lower level compared with mycosubtilin. Surprisingly, plipastatin did not significantly induce defence gene expression in these conditions. Using the Evans blue test, no cell death increase was detected in the cell suspensions treated with surfactin or plipastatin at 50 μg/mL (Fig. S3, see Supporting Information). However, when mycosubtilin was applied, a significant increase in cell death was observed (around 23% of the total cells compared with 11% in the control) at 24 h post-treatment.

As the gene induction profiles triggered by LPs and, especially, the ability of mycosubtilin to trigger an immune response were quite unexpected, we investigated gene expression in *Arabidopsis thaliana* challenged with LPs. Interestingly, treatment of *A. thaliana* leaves with purified LPs also led to a differential activation of defence genes and the overall response was comparable with that of grapevine (Fig. S4, see Supporting Information). Indeed, mycosubtilin was the most active in the induction of *PR1* expression, followed by surfactin. Plipastatin remained inactive. *PR4* expression was only slightly induced by mycosubtilin and surfactin, whereas *PDF1.2* expression remained unchanged.

Mycosubtilin and plipastatin, but not surfactin, display direct antifungal activity against *Bo. cinerea*

Although crude supernatant cultures containing LPs (iturins or fengycins) or *B. subtilis* strains producing these LPs are known to exhibit antagonistic activities towards a large range of fungi (Coutte *et al*., 2010; Falardeau *et al*., 2013; Leclere *et al*., 2005; Ongena and Jacques, 2008; Ongena *et al*., 2007; Romero *et al*., 2007; Toure *et al*., 2004), direct antifungal activity of the pure compounds against *Bo. cinerea* has not been described to date. Using an *in vitro* bioassay, we found that both mycosubtilin and plipastatin inhibited *Bo. cinerea* spore germination, whereas surfactin remained inactive (Fig. 4).

Surfactin and mycosubtilin treatments lead to long-lasting enhanced tolerance to *Bo. cinerea* in grapevine plants

We then investigated the efficiency of LPs to protect grapevine plants against the necrotrophic fungus *Bo. cinerea*. Vitro-plantlets were immersed into solutions of LPs, 48 h prior to infection with *Bo. cinerea*. A concentration of 10 μg/mL was used to investigate the elicitor properties of surfactin and mycosubtilin and to minimize a direct antifungal effect. However, because plipastatin did not display a strong elicitor activity, we increased the concentration of this molecule to 50 μg/mL. In addition, mycosubtilin was tested in these experiments at an antifungal concentration of

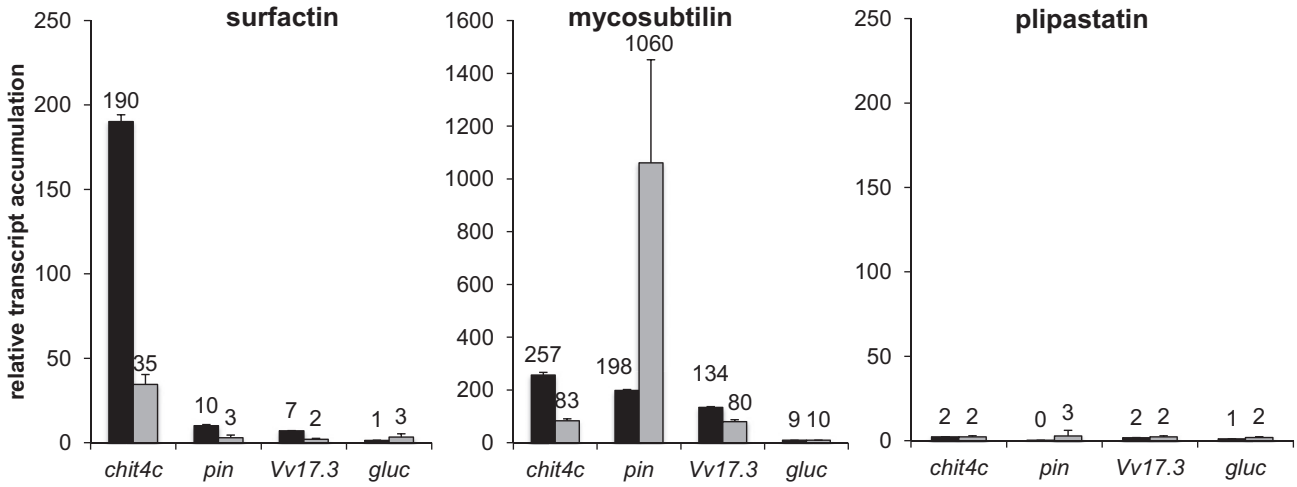

Fig. 3 Defence gene expression in response to lipopeptides (LPs). Transcript accumulation of genes encoding a chitinase (*chit4c*), a protease inhibitor (*pin*), a salicylic acid (SA)-regulated marker (*Vv17.3*) and a glucanase (*gluc*) was monitored at 9 h (black bars) and 24 h (grey bars) after challenge with surfactin, mycosubtilin and plipastatin. Analyses were performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The transcript level was calculated using the standard curve method from duplicate data, with the grapevine *EF-1α* gene as the internal control. Results are expressed as relative transcript accumulation (fold increase) over the dimethylsulphoxide (DMSO) 0.1% control. Data presented are means of triplicate experiments ± standard deviation (SD).

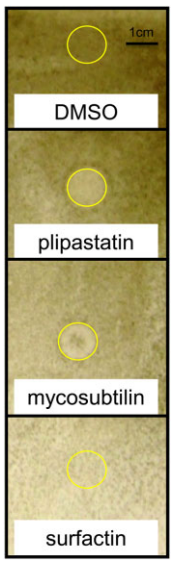

Fig. 4 Spore germination assay with lipopeptides (LPs). LPs were spotted onto a TLC (thin layer chromatography) membrane (10 μL for each LP at a concentration of 50 μg/mL; the surface area of the membrane covered by LP solution was delimited by a yellow circle). Conidia (1×10^5) from *Botrytis cinerea* were applied to the membrane and fungal growth was observed 96 h later. Data present one experiment of three with similar profiles.

50 μg/mL. Symptoms resulting from fungus colonization were monitored at 2 and 4 days post-inoculation (Fig. 5). Water control plants infected with *Bo. cinerea* without previous LP treatment displayed strong disease symptoms, with 41% and 97% of leaves presenting strong necrosis (lesions larger than 50% of the total leaf area) at 2 and 4 days, respectively. Furthermore, 56% of the leaves also presented small necrosis lesions (lesions smaller than 50% of the total leaf area) 2 days after infection. Similar results were observed after DMSO 0.1% treatments. However, 2 days after fungus inoculation of plants pretreated with surfactin (10 μg/mL), mycosubtilin (10 and 50 μg/mL) or plipastatin (50 μg/mL), leaves were significantly protected against *Bo. cinerea*. Indeed, depending on the LPs, 21%–29% of leaves were symptomless and strong necrosis was only observed in 12%, 10% and 5% of the leaves pretreated with mycosubtilin (10 μg/mL), surfactin (10 μg/mL) and mycosubtilin (50 μg/mL), respectively. Four days after infection, surfactin- and mycosubtilin-driven protection persisted, with more than 35% of the leaves without or with reduced symptoms, whereas plants pretreated with plipastatin completely lost their protection. Interestingly, we did not observe significant differences in the LP-driven protection by changing the time of elicitation (1–4 days prior to infection), except for mycosubtilin at the highest concentration, which was more efficient when applied 1 day prior to infection (data not shown). The diversity of immune responses, direct effects and local resistance triggered by LPs are summarized in Table 1.

Activation of defence responses by *B. subtilis* in grapevine is not exclusively dependent on LP production

An LP non-producer and three LP-overproducing strains of *B. subtilis* were used to investigate the implication of these molecules in *B. subtilis* perception. The strain BBG127 does not

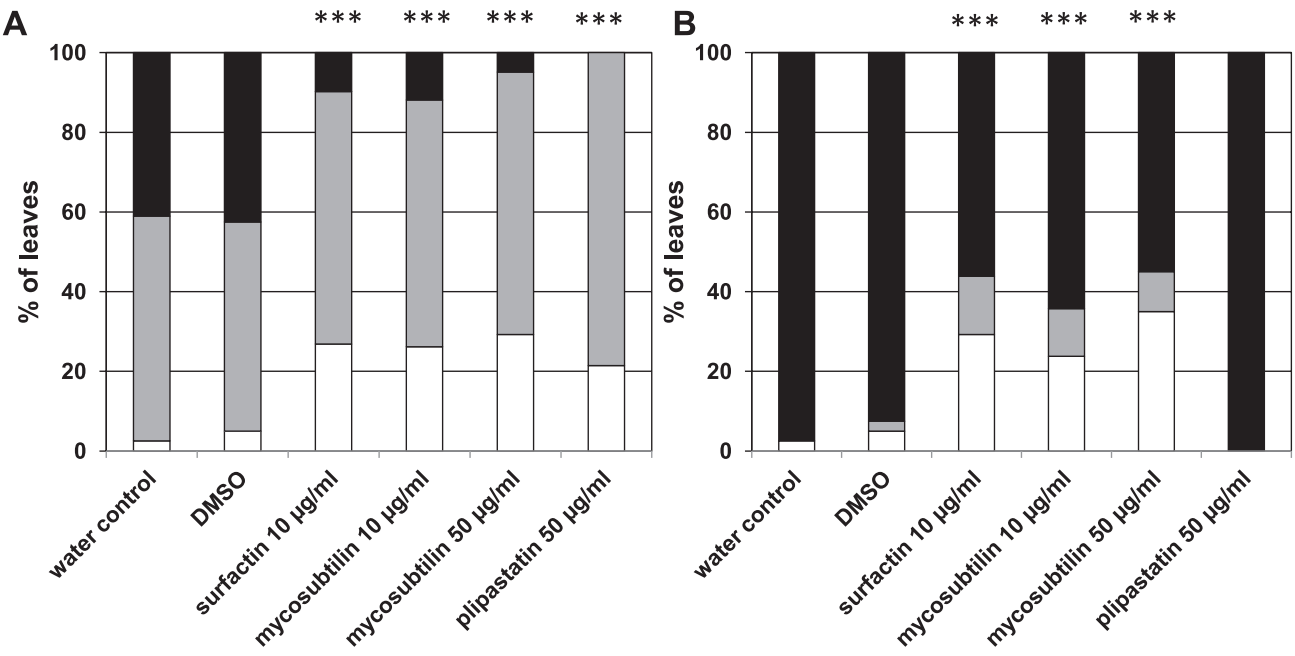

Fig. 5 Protection from *Botrytis cinerea* in grapevine plants challenged by lipopeptides (LPs). Leaves from vitro-plantlets were sprayed with solutions of LPs at the indicated concentrations or dimethylsulphoxide (DMSO) 0.1% or water. Two days later, leaves were placed on Petri dishes and inoculated with *Bo. cinerea* conidia (10^5 conidia/mL). Symptoms were scored 2 days (A) and 4 days (B) after inoculation by defining three lesion surface classes: no lesion (white bars), lesions affecting less than 50% of the leaf surface (grey bars) and lesions affecting more than 50% of the leaf surface (black bars). Results are means of three independent experiments ($n = 40$). Asterisks indicate statistically significant differences in the percentage of leaves in the different classes compared with the water control (Fisher's test, $***P = 0.001$).

Table 1 Diversity of immune responses, direct antifungal effects and local resistance triggered by lipopeptides (LPs).

	Mycosubtilin	Surfactin	Plipastatin
ΔpH	+++	++	+
Reactive oxygen species	+/–	+++	+/–
Ca^{2+}	–	–	–
Defence gene induction	+++	++	–
Local resistance to *Botrytis cinerea*	+++	+++	+
Spore germination inhibition	++	–	++

produce any LPs, whereas BBG131, BBG125 and Bs2504 overproduce solely surfactin, mycosubtilin and plipastatin, respectively. We first monitored the alkalinization response of grapevine cell cultures after inoculation with *B. subtilis* strains at concentrations of 10^5 and 10^7 colony-forming units (cfu)/mL (Fig. 6). We observed a clear alkalinization response after challenge of cells with the strain BBG127 at the highest concentration, whereas no significant ΔpH was observed with 10^5 cfu/mL. Perception of this strain stimulated a maximum increase in pH of about 0.7 units within 30 min. Similar ΔpH effects were detected with the strains BBG131 and Bs2504 overproducing surfactin and plipastatin, respectively. BBG125, which produces mycosubtilin, displayed a different profile from that of the other strains. The alkalinization response was significantly lower (ΔpH of about 0.3 units) at the concentration of 10^7 cfu/mL. The *chit4c* gene is the classical marker used to monitor defence induction following bacterial challenge in grapevine (Bordiec *et al*., 2011). We therefore monitored the expression of this defence marker gene after perception of the *B. subtilis* strains (Fig. 7). Challenge of the grapevine cells with the four strains significantly induced defence marker expression. The strain BBG131, overproducing surfactin, and the strain BBG125, overproducing mycosubtilin, were significantly more active than the non-producing strain BBG127, especially at the earliest time point. Intriguingly, the strain Bs2504, overproducing plipastatin, stimulated the defence marker gene to a lower level than did the BBG127 control strain. Using Evans blue assay, we did not monitor any cell death at 9 and 24 h after inoculation of cell suspensions with the different strains of *B. subtilis* (Fig. S3). Surprisingly, when vitro-plantlets were immersed into LP- or non-LP-producing bacterial solutions prior to infection with *Bo. cinerea*, we only observed a slight enhanced protection with the mycosubtilin overproducer strain BBG125 (Fig. S5, see Supporting Information). This result suggests that, although being perceived by grapevine plant cells, *B. subtilis* is not very effective in triggering local resistance to the fungus in this plant.

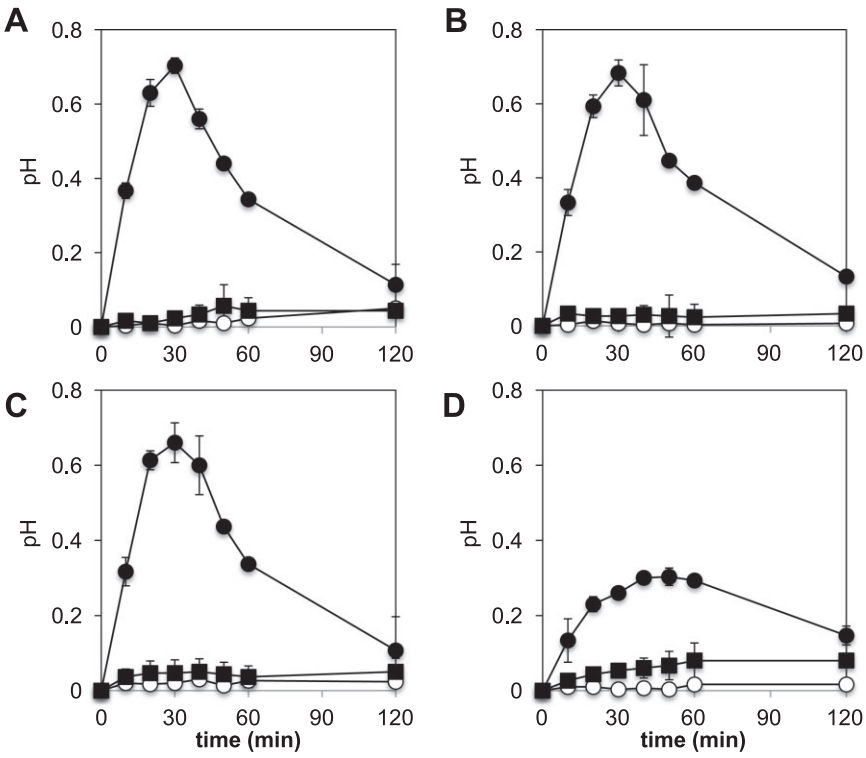

Fig. 6 Alkalinization response after perception of *Bacillus subtilis* not producing or overproducing single lipopeptides (LPs). Cells were treated with LP non-producer strain BBG127 (A), strain BBG131 overproducing surfactin (B), strain Bs2504 overproducing plipastatin (C) and strain BBG125 overproducing mycosubtilin (D) at 10^5 colony-forming units (cfu)/mL (filled squares), 10^7 cfu/mL (filled circles) or $MgCl_2$ (open circles). Data presented are means of triplicate experiments ± standard deviation (SD).

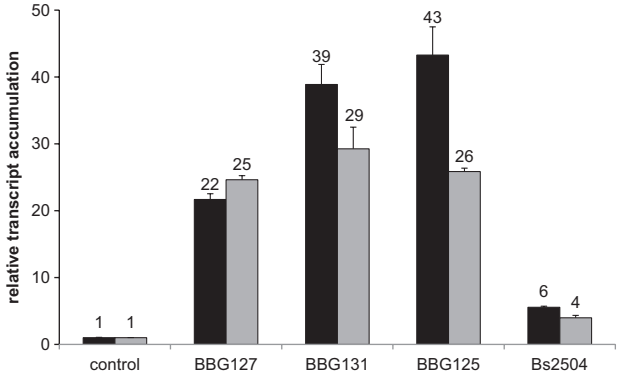

Fig. 7 Expression of the *chit4c* defence marker after perception of *Bacillus subtilis* strains. Transcript accumulation of the gene marker *chit4c* was monitored at 9 h (black bars) and 24 h (grey bars) after challenge with the indicated strains or $MgCl_2$ (control). Analyses were performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The level of transcripts was calculated using the standard curve method from duplicate data, with the grapevine *EF-1α* gene as the internal control. Results are expressed as relative transcript accumulation (fold increase) over the $MgCl_2$ control. Data presented are means of duplicate experiments ± standard deviation (SD).

DISCUSSION

The substantial structural diversity of LPs produced by *Bacillus* (but also *Pseudomonas* and other bacterial genera) suggests that these metabolites may have different modes of action related to their capacity to enhance plant protection (Raaijmakers *et al*., 2010). Iturins and fengycins, but not surfactins, are known to possess direct antifungal activities (Ongena and Jacques, 2008; Perez-Garcia *et al*., 2011). To date, only surfactins and fengycins have been described as ISR inducers (Falardeau *et al*., 2013; Ongena *et al*., 2007). Interestingly, whereas ISR to pathogens by LPs has been investigated in numerous studies, local defence responses after LP perception have been poorly documented (Falardeau *et al*., 2013). Here, we demonstrate, for the first time, elicitor properties for mycosubtilin, a member of the iturin family. Mycosubtilin is able to activate ion fluxes and to stimulate defence gene expression in grapevine. Mycosubtilin-triggered innate immune responses in this plant system are even stronger, in terms of intensity, than are those of surfactin and the fengycin member, plipastatin. The defence response signature is reminiscent of that observed with RLs, another amphiphilic MAMP produced by *Pseudomonas* and *Burkholderia* species (Varnier *et al*., 2009), especially as cell death could be observed above a concentration threshold. Plipastatin is clearly perceived by grapevine plant cells, as illustrated by a proton influx, but, surprisingly, this early signalling event is not accompanied by the induction of our defence gene markers. Fengycin family members have been shown to be involved as MAMPs in tomato and potato, but not in bean (Ongena *et al*., 2005b, 2007), suggesting a clear plant-dependent response for these molecules. Similarly, plant specificities in the

recognition of the typical MAMP flagellin from a beneficial rhizobacterium have recently been described (Trda *et al*., 2014). Surfactin displays an intermediate MAMP profile in grapevine, with a strong alkalinization response, but a weaker induction of defence genes, than mycosubtilin. Significant ROS production is only observed in surfactin-treated cells. In tobacco, the addition of surfactin, but not fengycin or iturin, induces defence-related early events, such as extracellular medium alkalinization and ROS production (Henry *et al*., 2011; Jourdan *et al*., 2009). Surfactin is also able to stimulate the defence enzymes PAL and LOX, and to modify the pattern of phenolics produced by tobacco-elicited cells (Jourdan *et al*., 2009). Like surfactin, synthetic ultrashort cationic LPs have also been described as potent inducers of medium alkalinization of tobacco suspension-cultured cells and are able to stimulate the expression of defence-related genes in cucumber and Arabidopsis seedlings (Brotman *et al*., 2009).

Interestingly, the defence gene signature observed in grapevine after treatment with the three LPs was not restricted to this plant. Challenge of *A. thaliana* with purified LPs also led to a differential activation of defence genes with a similar pattern. As in grapevine, mycosubtilin was the most active in the induction of defence gene expression, followed by surfactin. Plipastatin remained inactive. The strong activation of *PR1* by mycosubtilin and, to a lesser extent, by surfactin suggests that these two LPs activate the SA-dependent signalling pathway in *A. thaliana*. In grapevine, we observed a clear up-regulation of the JA-regulated gene marker *gluc* and the *Vv17.3* gene marker of the SA-dependent signalling pathway after challenge with mycosubtilin. Therefore, it is plausible that SA- and JA-dependent signalling pathways are involved in the activation of defence responses in grapevine plant cells perceiving this LP. Results obtained with surfactin are more difficult to interpret given the low level of gene expression, especially with these two markers. Nevertheless, a significant induction of *Vv17.3* (seven-fold over the control) was constantly monitored at 9 h with surfactin, suggesting that the SA signalling pathway may also be activated by this MAMP in grapevine. In agreement with our results, a recent study has demonstrated that surfactin is a major determinant for the stimulation of an immune response in melon plants and that this response is SA and JA dependent (Garcia-Gutierrez *et al*., 2013).

Interestingly, homologous desensitization experiments confirmed that surfactin did not display a refractory state in grapevine cells similar to tobacco cells (Henry *et al*., 2011). We also found that plipastatin did not exhibit a refractory state profile, suggesting that, like surfactin, recognition of the molecule could be independent of a specific receptor and may involve direct perception at the plasma membrane level by a lipid-driven process (Henry *et al*., 2011). Mycosubtilin has been shown to interact with cholesterol, ergosterol and phospholipid membrane models (Nasir and Besson, 2011, 2012; Nasir *et al*., 2010), but it is not known whether this LP could interfere with plant plasma membranes, especially by forming pores and inducing important modifications of the membrane permeability, as demonstrated for fungal membranes (Nasir and Besson, 2011). Physiological relevance of LP-driven elicitation was also confirmed by the use of K-252a, a potent inhibitor of protein kinases in animals and plants (Ruegg and Burgess, 1989). K-252a is known to cause significant changes in phosphorylated protein patterns within minutes after elicitation and to inhibit or greatly decrease alkalinization in tomato cells (Felix *et al*., 1991, 1993). The same effect on pH reduction was observed in our grapevine cell system after surfactin, mycosubtilin and plipastatin treatment, indicating that the alkalinization triggered by all these molecules is related to changes in the phosphorylation of proteins and is an active MAMP response.

Bacillus subtilis perception by Arabidopsis cells, although suppressing some defence responses at the root level (probably allowing colonization) (Lakshmanan *et al*., 2012), has also been shown to trigger transcriptomic changes in this plant organ with the up-regulation of several defence-related candidate genes (Lakshmanan *et al*., 2013). In addition, *B. subtilis* was able to induce systemic expression of defence genes (Rudrappa *et al*., 2010) or MTI-triggered responses, such as stomatal closure, in Arabidopsis leaves (Kumar *et al*., 2012). In tobacco and tomato, this bacterium is able to stimulate systemic defence reactions (Ongena *et al*., 2005a). *Bacillus subtilis* supernatants have also been shown to stimulate an alkalinization response and ROS production in tobacco cell suspensions (Cawoy *et al*., 2014; Jourdan *et al*., 2009). Here, we show that the model non-LP-producing strain BBG127 of *B. subtilis* is able to trigger an innate immune response in grapevine cells, involving early signalling perception and defence gene induction. These results are consistent with a recent study on grapevine using a natural *B. subtilis* strain originating from vineyards that induced ROS production and *trans*-resveratrol synthesis following bacterial perception (Verhagen *et al*., 2011). Here, we further demonstrate that the *B. subtilis*-triggered immune response does not require LPs as the non-LP-producing strain fully retains its ability to induce defence responses. Our results suggest that other MAMPs are probably involved in the process. In agreement with this hypothesis, the ability of *B. subtilis* to induce stomatal closure is lost in *fls2* Arabidopsis mutants, suggesting that flagellin is necessary for *B. subtilis*-triggered MTI in this plant (Kumar *et al*., 2012). Moreover, volatile compounds and, more particularly, 2,3-butanediol and acetoin, were also identified as determinants for elicitation by *Bacillus* spp. (Farag *et al*., 2013; Rudrappa *et al*., 2010; Ryu *et al*., 2004). A saturating threshold level of elicitation by several MAMPs may explain the similar profile of alkalinization observed in our conditions after challenge with the *Bacillus* strains producing or not producing LPs. However, our results on defence genes also show that the overproduction of surfactin or mycosubtilin by *B. subtilis* strains can enhance the

level of defence markers, especially at early time points. This indicates a potential role of these LPs in the potentiation of defence responses in grapevine following bacterial perception. Intriguingly, the plipastatin-overproducing strain exhibited a lower defence-inducing profile. Whether plipastatin may retain some effector properties suppressing plant defence responses remains to be investigated. Interestingly, a cell culture filtrate of *B. subtilis* strain B17 was found to suppress flg22-induced MAMP-activated root defence responses (Lakshmanan *et al*., 2012). Nevertheless, the effector(s) involved in the process have not been characterized to date.

We found that the three LPs were able to enhance grapevine leaf tolerance against the necrotrophic pathogen *Bo. cinerea*. However, plipastatin's protection was transient. This could be related to the fact that this LP exhibits antibiosis, but does not have significant elicitation properties. In contrast, surfactin does not exhibit any antifungal properties and we can therefore assume that the resistance associated with this compound is related to the activation of the innate immune response in grapevine. Mycosubtilin clearly displayed both anti-botrycidal and elicitation activities, and it is plausible that both are involved in *Bo. cinerea* restriction. Similar properties have been described for RLs (Varnier *et al*., 2009), chitosan (Aziz *et al*., 2006) and phosphites (Massoud *et al*., 2012). However, the importance of the innate immune response in the RL- and phosphate-induced resistances has been clearly revealed using signalling- and defence-defective Arabidopsis mutants (Massoud *et al*., 2012; Sanchez *et al*., 2012). *Bacillus subtilis* is known to be a very efficient ISR-inducing strain in many plants, such as cucumber, bean, tomato and tobacco (Ongena and Jacques, 2008; Perez-Garcia *et al*., 2011), mainly through the production of surfactins and, to a lesser extent, of plipastatin (Ongena *et al*., 2007). However, biocontrol activity of this bacterium on grapevine does not seem to be as effective. Only the mycosubtilin-overproducing strain of *B. subtilis* was able to slightly enhance grapevine protection against *Bo. cinerea*. These results are in agreement with a previous study showing that a natural *B. subtilis* originating from the vineyard was much less efficient than *Pantoea agglomerans*, *Acinetobacter lwoffii* and *Pseudomonas fluorescens* in triggering an ISR in grapevine (Verhagen *et al*., 2011). Interestingly, the plipastatin-overproducing strain used by Ongena *et al*. (2007) and in the present work also exhibited contrasting ISR properties towards *Bo. cinerea* infection in bean and tomato (Ongena *et al*., 2007). In our experiments, leaves from vitro-plantlets were immersed directly in bacterial solutions and, a few days later, were inoculated with *Bo. cinerea* conidia. In these conditions, we investigated local resistance induced by the bacterial strains and not a typical ISR. These two experimental procedures could account for the differences observed in the protection results. As iturins are involved as key factors in the direct antagonism of *B. subtilis* towards fungi, such as *Podosphaera fusca* (Romero *et al*., 2007), and because purified mycosubtilin is directly active against *Bo. cinerea*, it is likely that the antifungal properties of this compound are involved in the biocontrol of *Bo. cinerea* by *B. subtilis* in our plant system. It remains to be determined whether the eliciting properties of the compound are also important for *B. subtilis*-driven grapevine enhanced protection. Unfortunately, defence gene markers ('resistance marker') closely linked to pathogen resistance are not available in grapevine (Delaunois *et al*., 2014), making a direct correlation difficult to establish.

Altogether, our results demonstrate a high diversity in the defence responses triggered by *B. subtilis* LPs. Moreover, they highlight an interesting potential for these compounds in the protection of grapevine from *Bo. cinerea*. LPs act as MAMPs in grapevine, and the combination of elicitor activity with antifungal properties (in the case of plipastatin and mycosubtilin) may participate in a high level of plant protection (Fig. 8). Natural *B. subtilis* strains usually produce the three classes of LPs. How the combination of these LPs acts in grapevine protection is a key question that remains to be investigated. It is likely that the different LPs are necessary in their natural concentrations and combinations in relation to each other to be most effective in triggering innate immunity and as protective agents. However, natural *Bacillus* strains are known to produce variable concentrations of each LP, depending on the time of interaction with the plant or during the establishment of biofilms (Cawoy *et al*., 2014; Debois *et al*., 2014). It is therefore challenging to determine which LP is involved at a given time and for which effect. Further research is needed to clarify this.

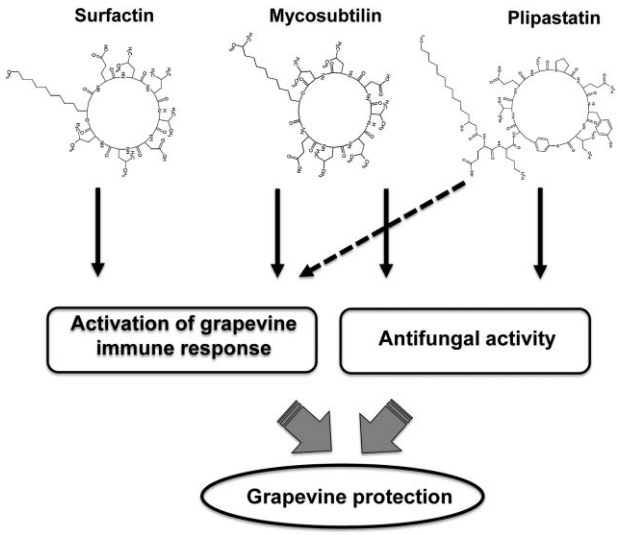

Fig. 8 Proposed model showing the mechanisms by which the lipopeptides (LPs) produced by *Bacillus subtilis* enhance grapevine protection.

EXPERIMENTAL PROCEDURES

Grapevine cell suspension culture and vitro-plantlets

Cell suspensions of 41B (*Vitis vinifera* L. cv. Chasselas × *V. berlandieri*) were cultured in Murashige–Skoog (MS) medium (pH 5.8) containing vitamins (×1.5), sucrose (30 g/L), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.2 mg/L) and 6-benzylaminopurine (BAP, 0.5 mg/L), and were propagated in the dark at 25 °C with shaking at 120 rpm. They were subcultured every 7 days for maintenance in the exponential phase. For the experiments, cells subcultured for 7 days were filtered, weighed and resuspended in derived MS medium for 3–4 days at a concentration of 8 g/100 mL for a total volume of 10 mL. Grapevine vitro-plantlets (*V. vinifera* L. cv. Chardonnay 7535) were grown in modified MS medium at 26 °C with a photoperiod of 16 h of light according to Bézier *et al*. (2002).

Microorganisms and LP treatments

Botrytis cinerea strain 630 cultures were initiated by transferring pieces of solid tomato/agar medium [tomato juice 25% (v/v), agar 3.75% (w/v)] containing mycelium to fresh solid tomato/agar medium and incubated at 21 °C. Conidia were collected from 2-week-old plates with 8 mL of growth culture medium ($MgSO_4$, 3 mM; KH_2PO_4, 12.8 mM; D-glucose, 22 mM; peptone, 4 g/L; citric acid, 10 mM; final pH 3.1). *Bacillus subtilis* strains BBG127 and BBG131 used in this study have been described previously by Coutte *et al*. (2010). Strain BBG125 was characterized by Bechet *et al*. (2013). Strain Bs2504 was characterized by Ongena *et al*. (2007). The strain BBG127 is unable to produce any LPs and was used as a negative control in our experiments. The strains BBG131, BBG125 and Bs2504 overproduce surfactin, mycosubtilin and plipastatin, respectively. LP production under controlled conditions in shaken flask cultures of these strains led to a production of 1540 ± 38.2 mg/L of surfactin by BBG131 (Coutte *et al*., 2010), 104 ± 9.57 mg/L of mycosubtilin by BBG125 (Bechet *et al*., 2013) and 452 mg/L of plipastatin by Bs2504 (Ongena *et al*., 2007). Strains of *B. subtilis* were grown at 37 °C with shaking at 130 rpm in Luria–Bertani (LB) medium. Before any inoculation treatment, overnight bacterial cultures were collected by centrifugation (4500 ***g***, 10 min) and washed with sterile $MgCl_2$ (10 mM). Final concentrations of bacteria in grapevine cell suspensions were expressed in cfu/mL. The three main LPs used in this study, surfactin, mycosubtilin (iturin family) and plipastatin (fengycin family), were produced and purified from the different strains of *B. subtilis* using an integrated bioprocess recently described by Coutte *et al*. (2013). LPs were resuspended in 0.1% dimethylsulphoxide (DMSO; Sigma-Aldrich, Saint-Quentin Fallavier, France) and were added directly to the grapevine cell suspension medium at the indicated concentrations.

Bioassays in cell cultures

Medium alkalinization measurements were performed according to Felix *et al*. (1993) using a standard pH-meter (Basic, Denver Instrument, Goettingen, Germany). In order to test the physiological relevance of the proton fluxes, medium alkalinization was also measured after treatment of the cell suspension with 1 μM of the protein kinase inhibitor K-252a (Sigma-Aldrich; diluted in DMSO 0.05%) according to Felix *et al*. (1993). The inhibitor was added to plant cell suspensions 5 min prior to LPs (50 μg/mL). For refractory state experiments, 50 μg/mL of LPs were added to the cells and a second addition of LPs was performed when the pH of the cells returned to baseline. Cell death and ROS quantifications were performed according to Bordiec *et al*. (2011).

RNA extraction and real-time PCR analysis

Cell suspension cultures treated with LPs and/or bacterial strains were filtered and frozen in liquid nitrogen. RNA extraction and qRT-PCR were performed as described previously by Le Henanff *et al*. (2013). The sequences of the defence gene primers used for qRT-PCR have been described previously by Bordiec *et al*. (2011).

Protection assays

Conidia of *Bo. cinerea* were collected from 2-week-old plates with 8 mL of growth culture medium ($MgSO_4$, 3 mM; KH_2PO_4, 12.8 mM; D-glucose, 22 mM; peptone, 4 g/L; citric acid, 10 mM; final pH 3.1), filtered to remove mycelia and counted. The suspension was adjusted to 5×10^5 conidia/mL of culture medium and placed under 150 rpm agitation at 21 °C for 6 h. Six-week-old vitro-plantlets were completely immersed in solutions of LPs at the indicated concentration or in water or DMSO 0.1% (controls) for 30 s, and were placed in growth chambers; 1, 2 or 4 days later, leaves were excised from grapevine vitro-plantlets and placed on wet (Whatman Velizy-Villacoublay, France) 3MM paper in plastic Petri dishes. A drop of 5 μL of the 1×10^5 conidia/mL solution was inoculated on the upper face of the leaves. Symptoms were scored 2 or 4 days after inoculation. Infected leaves were assigned to three different classes based on lesion surfaces: no lesion, lesions affecting less than 50% of the leaf surface and lesions affecting more than 50% of the leaf surface. Fisher's test was performed to show significant differences between treatments and water control.

Protection from *Bo. cinerea* in grapevine plants challenged by *B. subtilis* strains was investigated by immersing leaves from vitro-plantlets in bacterial solutions (10^7 cfu/mL) cultured as described above. Two days later, leaves were placed on Petri dishes and inoculated with *Bo. cinerea* conidia (10^5 conidia/mL). Symptoms were observed 2 days after inoculation and quantified as described above.

Antibiosis assay

Antibiosis assay on *Bo. cinerea* germination was conducted on TLC (thin layer chromatography) membranes. LPs were spotted onto a CCM membrane (10 μL for each LP at a concentration of 50 μg/mL) which was allowed to dry for 10 min. Freshly harvested conidia (10^5 conidia/mL) were mixed with Peptone/Tween agar medium (45 °C), poured on the CCM membrane and then cooled down to 20 °C for 20 min. Fungal growth was observed 4 days later.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Effect of K-252a on the extracellular alkalinization induced by lipopeptides (LPs). K-252a (1 μM) was added 5 min before treatment with LPs (50 μg/mL). Data presented are means of triplicate experiments ± standard deviation (SD).

Fig. S2 Differential activation of reactive oxygen species (ROS) production after perception of lipopeptides (LPs). Cell suspensions were treated with LPs at 50 μg/mL. ROS production after treatment with surfactin (filled circles), mycosubtilin (filled squares), plipastatin (filled diamonds) and dimethylsulphoxide (DMSO) 0.1% (open circles). Data presented are means of duplicate experiments ± standard deviation (SD).

Fig. S3 Cell death assays using Evans blue staining. Control cells were challenged with $MgCl_2$ or dimethylsulphoxide (DMSO) 0.1% as indicated. Surfactin, mycosubtilin and plipastatin were used at 50 μg/mL. Bacterial strains BBG127, BBG131, BBG125 and Bs2504 were used at 10^7 colony-forming units (cfu)/mL. Cell death was monitored 24 h post-treatment. In these experiments, 100% of dead cells corresponds to an optical density at 600 nm (OD_{600}) of 1.6. Data presented are means of triplicate experiments ± standard deviation (SD). 'b' indicates statistically significant differences compared with the control according to Tukey's multiple comparison test ($P < 0.05$).

Fig. S4 Arabidopsis defence gene expression in response to lipopeptides (LPs). Leaves from 5-week-old *Arabidopsis thaliana* col-0 plants were sprayed with LPs (0.5 mg/mL) or dimethylsulphoxide (DMSO) 0.1% (control) according to Sanchez *et al*. (2012). Transcript accumulation of *PR1*, *PDF1.2* and *PR4* genes was monitored 24 h post-treatment. Analyses were performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) according to Sanchez *et al*. (2012). Results are expressed as relative transcript accumulation (fold increase) over the control. Data presented are means of duplicate experiments ± standard deviation (SD).

Fig. S5 Protection from *Botrytis cinerea* in grapevine plants challenged by *Bacillus subtilis* strains. Leaves from vitro-plantlets were immersed in bacterial solutions at 10^7 colony-forming units (cfu)/mL. Two days later, leaves were placed on Petri dishes and inoculated with *Bo. cinerea* conidia (10^5 conidia/mL). Symptoms were scored 2 days after inoculation by defining three lesion surface classes: no lesion (white bars), lesions affecting less than 50% of the leaf surface (grey bars) and lesions affecting more than 50% of the leaf surface (black bars). Results are means of two independent experiments ($n = 20$). Asterisks indicate statistically significant differences in the percentage of leaves in the different classes compared with the $MgCl_2$ control (Fisher's test, $^{**}P = 0.005$).