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Insights into the molecular basis of biocontrol of *Brassica* pathogens by *Bacillus amyloliquefaciens* UCMB5113 lipopeptides

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● **Background and Aims** Certain micro-organisms can improve plant protection against pathogens. The protective effect may be direct, e.g. due to antibiotic compounds, or indirect, by priming of plant defence as induced systemic resistance (ISR). The plant growth-promoting rhizobacterium *Bacillus amyloliquefaciens* UCMB5113 shows potential for disease management of oilseed rape. To investigate the mode of action of this protection, especially in relation to jasmonic acid-dependent ISR, *Bacillus* UCMB5113 was tested with *Arabidopsis thaliana* mutants and several important fungal pathogens of *Brassica* species.
● **Methods** Secreted lipopeptide fractions from *Bacillus* UCMB5113, together with synthetic peptide mimics, were evaluated for their effects on fungal phytopathogens and *A. thaliana*. The structures of secreted lipopeptides were analysed using mass spectrometry. Plant mutants and reporter lines were used to identify signalling steps involved in disease suppression by lipopeptides.
● **Key Results** In plate tests Bacillus UCMB5113 and lipopeptide extracts suppressed growth of several fungal pathogens infecting Brassica plants. Separation of secreted lipopeptides using reversed-phase high-performance liquid chromatography revealed several fractions that inhibited fungal growth. Analysis by mass spectrometry identified the most potent compounds as novel linear forms of antifungal fengycins, with synthetic peptide mimics confirming the biological activity. Application of the lipopeptide extracts on Arabidopsis roots provided systemic protection against Alternaria brassicicola on leaves. Arabidopsis signalling mutants and PDF1.2 and VSP2 promoter-driven GUS lines indicated that the lipopeptide fraction involved jasmonic-acid-dependent host responses for suppression of fungal growth indicative of ISR.
● **Conclusions** The ability of *Bacillus* UCMB5113 to counteract pathogens using both antagonistic lipopeptides and through ISR provides a promising tool for sustainable crop production.

Key words: Antagonism, *Arabidopsis thaliana*, *Bacillus amyloliquefaciens*, beneficial bacteria, biocontrol, lipopeptide, rhizosphere.

INTRODUCTION

Plant exposure to biotic stresses such as microbial pathogens and insect pests hamper crop production. Although many chemical pesticides have been developed to control different biotic stress factors, insect pests and pathogens may develop resistance over time, following repeated pesticide use. Plant stress management can also be improved by breeding. As development of plant resistance based on genetic engineering approaches are currently unfavourable in the European Union, crop development is reliant upon conventional breeding, which can be slow, costly and assumes the presence of the desired trait in the available germplasm (Seifi *et al.*, 2013).

An alternative strategy to manage stress factors is based on the use of biological control agents (BCAs). BCAs are naturally occurring organisms that can counteract pathogens and pests attacking plants. BCAs are considered environmentally friendly tools that can produce healthier crops and improve yield

(Bhattacharyya and Jha, 2012). Plant growth-promoting rhizobacteria (PGPR) were first described due to their ability to support plant growth but many PGPR isolates were also found to serve as BCAs. Micro-organisms are prime candidates as BCAs because of their potential wide spectrum of antagonistic activity to different pathogens (Beneduzi *et al.*, 2012). Furthermore, many BCAs can also enhance disease protection by priming induced systemic resistance (ISR) in the host plant (Pieterse *et al.*, 2014).

Priming of enhanced defence refers to the sensitization of plants by certain non-pathogenic micro-organisms or chemicals that causes stronger and more rapid defence responses upon stress challenge (Conrath *et al.*, 2015). A number of bacteria and fungi as well as natural or synthetic chemicals have been shown to prime long-lasting plant defence to a variety of pathogens (Pozo and Azcón-Aguilar, 2007; Pieterse *et al.*, 2014). Several commercial products based on priming micro-organisms are available for control of diseases on various crops

(e.g. Bhattacharyya and Jha, 2012). Root colonization of the micro-organisms is a key step to initiate the beneficial interaction and in general the colonizers remain as epiphytes on the rhizoplane followed by embedding in a biofilm. ISR is a jasmonic acid (JA)-dependent process in which enhanced sensitivity to JA rather than increased JA levels seems to be the case (Pieterse *et al.*, 2002). In contrast, plant defence as systemic acquired resistance (SAR) mounted in response to successful recognition of biotrophic pathogens, is salicylic acid (SA)-dependent (Durrant and Dong, 2004). JA signalling is also involved in plant defence to necrotrophic pathogens and insect herbivores (Pieterse *et al.*, 2012). JA metabolism is complex and several different metabolites occur in plants depending on the stimulus that may fine tune the response to be more appropriate (Wasternack and Hause, 2013). In reality, however, numerous hormones and extensive cross-talk regulate defence responses, in which, for example, JA and SA can antagonize each other and JA responses are also modulated by other host factors such as ethylene and abscisic acid (ABA) (e.g. Shigenaga and Argueso, 2016). A number of *Arabidopsis* mutants and reporter lines have been developed to facilitate analysis of hormonal involvement in different processes, with, for example, activation of JA-dependent *VSP2* and *PDF1.2* indicating the involvement of MYC/ABA and ERF/ethylene, respectively (Pieterse *et al.*, 2012). While several steps leading to SAR are well characterized, the molecular mechanisms behind the priming of ISR are less well known. The SAR and ISR responses can generally be clearly distinguished with respect to involvement of key regulators such as NPR3 and NPR4 (SAR) or COI1, MYB72 and MYC2 (ISR) as well as activation of defence genes, such as pathogenesis-related (PR) genes (SAR) or *PDF1.2* and *VSP2* (ISR) (Pieterse *et al.*, 2014). However, both SAR and ISR depend on the redox-sensitive transcription cofactor NPR1 (Pieterse *et al.*, 2014; Conrath *et al.*, 2015). NPR1 is activated by SA and then translocated to the nucleus, functioning as coactivator of PR genes providing SAR, while during development of ISR, NPR1 seems to act in the cytosol although its exact role remains to be established.

Many *Bacillus* species have great potential as PGPR and BCAs because of their ability to enhance plant growth and antagonize phytopathogens (Kloepper *et al.*, 2004; Cawoy *et al.*, 2011). Several *Bacillus* products are available on the market for crop protection against various pathogens (Cawoy *et al.*, 2011). *Bacillus* species produce numerous secondary metabolites with antibiosis properties, especially cyclic lipopeptides (LPs) that are classified based on their amino acid composition, the structure of their peptide ring and the attached fatty acid (Ongena and Jacques, 2008). LPs are synthesized non-ribosomally by large multi-enzyme complexes, non-ribosomal peptide synthetases (NRPSs) (Ongena and Jacques, 2008). The NRPSs are complex structures in which each module incorporates a monomer and the enzymatic machinery functions as a templated pathway (Strieker *et al.*, 2010). The three main groups of LPs formed by members of the *Bacillus subtilis* and *Bacillus amyloliquefaciens* species are surfactins, iturins and fengycins. These LPs have been studied intensively because of their antagonistic activity toward phytopathogens (Ongena and Jacques, 2008). Surfactins are heptapeptides linked to a β-hydroxy fatty acid, acting as efficient biosurfactants with haemolytic activity, with antagonism to viruses and bacteria but with limited antifungal

activity. Iturins are also heptapeptides but linked to a β-amino fatty acid. These, by contrast, display pronounced antifungal activity but rather limited antiviral and bactericidal activities. Fengycins are decapeptides linked to a β-hydroxy fatty acid that show marked activity against filamentous fungi but with less haemolytic activity when compared to surfactins and iturins. The ability of *Bacillus* species to produce many LPs with complementing and synergistic activities make these bacteria promising tools for biotechnological application (Cochrane and Vederas, 2016).

The widely cultivated oil crop oilseed rape (*Brassica napus*) is exposed to many phytopathogens, many of which are controlled through agrochemical approaches, in the absence of appropriate resistant germplasm. A shift towards BCAs to control these common diseases would cause less environmental impact and allow promotion of more organic cultivation. In previous studies, several strains of *B. amyloliquefaciens* were shown to protect oilseed rape against several phytopathogens (Danielsson *et al.*, 2007; Sarosh *et al.*, 2009). In this context, it is important to increase information on the structural basis of the components involved in conferring such protection and their requirements, to further evaluate their potential practical application. One of the issues we wished to address was if the secreted compounds supported plant protection in a direct (microbial antagonism) or indirect (ISR) manner, or in both ways. In this study, therefore, an LP-enriched fraction secreted from the strain *Bacillus* UCMB5113 was evaluated for its antagonistic effects towards common *Brassica* fungal pathogens. The most interesting LPs were structurally characterized via mass spectrometry analysis and synthetic peptide mimics tested for determination of their effect. Furthermore, we studied the potential indirect protective effect of the secreted LP compounds on *Brassicaceae* plant defence using the model plant *Arabidopsis thaliana* and various mutants and reporter lines, enabling the involvement of common defence-related hormones in this response to be evaluated.

MATERIALS AND METHODS

Bacterial and fungal strains and growth conditions

The root colonizing *B. amyloliquefaciens* subsp. *plantarum* strain UCMB5113 (Reva *et al.*, 2004) was maintained on Luria–Bertani (LB) medium. The fungal strains *Alternaria brassicicola* 20297, *Alternaria brassicae* 980:3 and *Botrytis cinerea* 30158 were grown on potato-dextrose agar (PDA; Difco) at $22\,^{\circ}$C, 16/8-h light and dark photoperiod. *Verticillium longisporum* D11 and *Sclerotinia sclerotiorum* 13MM were also kept on PDA but grown in darkness. Spores were isolated from *A. brassicicola* cultures grown for 3–4 weeks by scraping off the surface layer into water followed by filtration through miracloth. Spore counts were determined using a haemocytometer, with a fresh spore suspension (5×10^5 mL^{-1}) used to inoculate plant leaves.

Growth inhibition test of phytopathogenic fungi by Bacillus UCMB5113

The phytopathogens were inoculated at the centre of a PDA plate and a single colony of *Bacillus* UCMB5113 was streaked

in the periphery of the Petri dish. The samples were incubated at 22 °C, 16/8 h light and dark photoperiod while *V. longisporum* and *S. sclerotiorum* were grown in darkness. Growth inhibition of the fungal pathogen strains was recorded 3–14 d later and enlarged images of the samples were measured (mm). The experiment was repeated at least three times with similar results obtained in each. The results of a representative experiment are shown.

Isolation of LPs from Bacillus *UCMB5113 exudate*

An LP fraction was prepared from *Bacillus* UCMB5113 exudate by a protocol described by Kim *et al.* (2004). *Bacillus* UCMB5113 was grown in LB medium for 5 d at 28 °C and 150 r.p.m. reaching a stationary phase. The bacterial culture was centrifuged at room temperature for 25 min at 1000 *g*. The supernatant was filtered through a 0·45-μm filter membrane and precipitated by adding 3 M HCl to pH 2 followed by incubation at 4 °C for 30 min and centrifugation at 10 000 *g* for 20 min at 4 °C. The precipitates were collected and dissolved in chloroform/methanol (2 : 1, v/v) and the solvents were then removed using a rotary vacuum evaporator. The pellet was dissolved in methanol and filtered through a 0·2-μm filter membrane creating the LP extract. Approximately 0·5 mg of crude lipopeptides per litre of original medium was obtained. Further, the LP extract was fractionated using a reversed-phase high-performance liquid chromatography (RP-HPLC) preparative column (nucleosil C18, 16 × 125 mm, 7-μm beads, 120-Å pore size, Knauer Gmbh, Berlin, Germany) with a flow rate of 5 mL min⁻¹. The eluants contained 0·1 % trifluoroacetic acid (TFA) in (A) water or (B) HPLC-grade acetonitrile. The starting concentration of B was 5 % followed by a linear gradient to 20 % B at 15 min with hold for 5 min, linear gradients to 50 % B at 70 min, 75 % B at 90 min and 85 % B at 100 min, hold to 110 min, a linear gradient to 95 % at 120 min and hold to 130 min before going back to 5 % B. The eluate was monitored at 215 nm and the major peak fractions were collected.

Fungal growth inhibition tests using the LP extract

The phytopathogens *A. brassicicola*, *A. brassicae*, *B. cinerea*, *S. sclerotiorum* and *V. longisporum* were grown to cover one-quarter of a Petri dish with PDA and then inoculated with 20 μL (50 ng) *Bacillus* LP extract at one spot on the periphery of the Petri dish and methanol as negative control on the opposite side. The plates were incubated at 22 °C, 16/8 h light and dark photoperiod except for *V. longisporum* and *S. sclerotiorum* that were grown in darkness. The peak fractions obtained from RP-HPLC were also examined for antagonistic activity against these phytopathogens in a similar way. The experiment was repeated at least three times with similar results and the results of a representative experiment are shown.

Thermal stability of LP extract compounds

The LP extract was incubated in a heating block at 55, 75 or 95 °C for 0, 12, 24, 48 and 72 h. Aliquots (50 μL, 100 ng) of the heat-exposed compounds were tested for antagonistic activity

towards *A. brassicicola* and effects recorded 7 d later. The experiment was repeated at least twice with similar results and the results of a representative experiment are shown.

Antifungal bioassay using leaf treatment

Leaves from 4-week-old *Arabidopsis thaliana* Col-0 plants were detached and dipped in 20 mL (2·5 ng mL⁻¹) of *Bacillus* LP extract (dissolved in 5 % methanol) and/or 5 % methanol and water as control. Leaves were then kept on top of a wet filter paper in a Petri dish. Three microlitres of *A. brassicicola* fungal spore suspensions was dropped on the leaves and the plates were incubated at 22 °C, 16/8 h light and dark photoperiod. Disease symptoms on the leaves were recorded 4 d after pathogen inoculation. The experiment was repeated at least twice with similar results. The results of a representative experiment are shown.

Mass spectrometry (MS) analysis of LPs

LP profiling was performed by reversed-phase ultrahigh-performance liquid chromatography-MS (UPLC; Waters, Acquity class H) coupled with a single quadrupole mass spectrometer (SQDetector, Waters, Acquity) on an Acquity UPLC BEH C18 (Waters) 2·1 × 50 mm, 1·7 μm column, which allowed detection of all three major LP families. Elution was initiated with 30 % acetonitrile (0·60 mL min⁻¹). After 2·43 min, the percentage of acetonitrile was raised to 95 % and held until 5·2 min. The column was then stabilized at an acetonitrile percentage of 30 % for 1·7 min. Compounds were identified on the basis of their retention times compared with authentic standards (98 % purity, Lipofabrik Society, Villeneuve d'Asc, France) and the masses detected in the SQDetector. Ionization and source conditions were set as follows: source temperature, 130 °C; desolvation temperature, 400 °C; nitrogen flow, 1000 L h⁻¹; cone voltage, 120 V.

Exact mass measurements and tandem MS experiments were performed with a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (9·4T Solarix; Bruker Daltonics, Bremen, Germany) equipped with an electro-spray ionization matrix-assisted laser desorption ionization (ESI/MALDI) dual ion source including SmartbeamII laser (355 nm). For MS/MS experiments, a MALDI time-of flight (TOF)/TOF mass spectrometer was used (UltraFlex II, Bruker Daltonics). The selection window in the LIFT cell was set to optimize the isolation (depending on the presence of adducts or other peaks close to the peak of interest). Several spectra were accumulated to improve the signal-to-noise ratio of the MS/MS spectrum and facilitate interpretation.

Fungal growth inhibition test using synthetic peptides

Synthetic peptides with the L-form of GluOrnTyrThrGlu ValProGlnTyrLeu with acetylated ('AcePEP') or myristoylated ('MyrPEP') amino terminus were supplied by ProteoGenix SAS (Schiltigheim, France). The fungus was grown as previously described and 200 ng of synthetic LP with fatty acid or acetyl group, LP extract or methanol as control was inoculated

in the periphery of the growth medium. Plates were incubated at 22 °C, 16/8 h light and dark photoperiod. Fungal growth inhibition was recorded after 5 d. The experiment was repeated at least twice and the result of a representative experiment is shown.

Effects of LP extract on fungal disease suppression in plants

Arabidopsis thaliana wild type Col-0 and mutants were employed in this study. These comprised *coi1-16* with JA receptor deficiency (Ellis and Turner, 2002), *sid2* with aberrant SA synthesis (Wildermuth *et al.*, 2001), *jar1* with inability to form the active jasmonyl–isoleucine conjugate (Staswick *et al.*, 1992), *myc2* defective in ISR signalling (Lorenzo *et al.*, 2004), *myb72* defective in ISR signalling (Van der Ent *et al.*, 2008) and *npr1* deficient in SAR response (Cao *et al.*, 1994). β-Glucuronidase (GUS) marker lines driven by *VSP2* and *PDF1.2* promoters were also used. Seeds were surface sterilized (1 min in ethanol, 5 min in 10 % bleach containing chlorine and tween 20, and 3 × 5 min in sterile water). The seeds were then germinated in Petri dishes containing half-strength MS medium (M0222, Duchefa) with 0·6 % agar, with incubation at 22 °C, 16/8 h light and dark photoperiod. One-week-old seedlings were transplanted to 10 × 10-cm square plates containing half-strength MS medium with 0·8 % agar. Each plate contained four seedlings with a distance of 2·5 cm between the plants. The roots were treated with 5 μL (2·5 ng mL^{-1}) LP extract and/or 5 % methanol and water. The plates were incubated at 22 °C, 16/8 h light and dark photoperiod and positioned vertically.

Two weeks after LP treatment, plants were inoculated with *A. brassicicola* spores (5 × 10^5 mL^{-1}) by adding 3 μL drops onto the surface of four leaves per plant as described (Thomma *et al.*, 1998). Seven days after challenge, disease severity was scored according to Van der Ent *et al.* (2008) and samples were collected for pathogen quantification. For each pathogen assay, inoculated leaves of three plants were pooled together and taken as one biological replicate, with three biological replicates evaluated. Fungal DNA was isolated from plant leaves using a GenJET genomic DNA purification kit (Thermo Fisher Scientific). SYBR GREEN-based quantitative real-time PCR (qPCR) was conducted as described previously (Fridborg *et al.*, 2013). DNA equivalent to 50 ng was used in a 20-μL PCR in a Bio-Rad iQ cycler with iQ SYBR Green Supermix (Fermentas). A 40-cycle two-step amplification protocol (10 s at 95 °C, 30 s at 60 °C) was used for all measurements. Primer sequences used were: forward primer 5′- GCATGTCCGCTCACCAATATC-3′, reverse primer 5′-GCCTGGGATCTTGGAATGC-3′ (*A. brassicicola* cutinase, ABU03393). As reference gene, *Ubiquitin 5* (At3g62270) from *Arabidopsis* was used and amplified by the primers 5′-CGATGGATCTGGAAAGGTTC-3′ and 5′-AGCTCCACAGGTTGCGTTAG-3′. The identities of the amplicons and the specificity of the reactions were verified by melting curve analysis. The data were analysed using the comparative C$_T$ method with PCR efficiency correction. The average and the standard error of three technical replicates of a pool of three plants and with three different biological samples are shown. The experiment was repeated at least once and the result of a representative experiment is shown.

Histochemical GUS reporter gene expression

To generate JA-responsive GUS reporter plants, promoter regions upstream of the translation start of *VSP2* or *PDF1.2* were amplified from genomic DNA. *PDF1.2* (AT5g44420) was amplified with forward primer TCgagctcAAAAATCTTTGGTGCTTG (*Sac*I restriction site) and reverse primer CGccatggGATGATTATTACTATTTTGTTTTC (*Nco*I) generating a 1541-bp fragment. *VSP2* (AT5g24770) was amplified using forward primer ATggtaccATTTTGTTGCTGCTTGGAC (*Kpn*I) and reverse primer GCaagcttGTTTTTTATGGTATGGTTTAT (*Hind*III) generating a 1472-bp fragment. The PCR fragments were subsequently digested with the respective restriction enzymes to replace the 2× 35S promoter in the GUS expression vector pJIT166. The pPro-GUS expression cassette were released from PJIT166 with *Sac*I + *Xho*I or *Kpn*I + *Xho*I digestion and cloned into the T-DNA vector pBINPLUS. The pBINPLUS plasmids containing the different constructs were transformed into *Agrobacterium tumefaciens* strain C58, followed by transformation into *Arabidopsis* using the floral-dip protocol (Davis *et al.*, 2009). A minimum of three homozygous T3 lines were used in each experiment. The resulting GUS transgenic lines (*VSP2:GUS* and *PDF1.2:GUS*) were grown on half-strength MS medium with 0·8 % agar for 1 week before being treated with LP extract, methanol or water and challenged with *A. brassicicola* 2 weeks later. Samples were collected 3 d after inoculation for expression analysis. The GUS staining solution contained 50 mM sodium phosphate buffer (pH 7·2), 10 % Triton X100, 5 mM ferro-cyanide, 5 mM ferri-cyanide and 2 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt). The samples were incubated in the GUS staining solution at 37 °C for maximum 24 h, and destained using 70 % ethanol for at least 24 h before photodocumentation.

Statistical analysis

If not stated otherwise, all calculations of mean and standard deviations were based on three or more samples, with *t*-tests or ANOVA Tukey HSD test conducted using the appropriate application in Minitab. Samples sharing a letter are not significantly different at the 5 % level.

RESULTS

Antifungal activity of B. amyloliquefaciens *UCMB5113*

The disease suppressive potential of *B. amyloliquefaciens* UCMB5113 was first assessed as growth inhibitory activity against several *Brassica* fungal pathogens grown on PDA medium. In all cases (*A. brassicicola*, *A. brassicae*, *B. cinerea*, *S. sclerotiorum* and *V. longisporum*), a clear zone of growth inhibition was observed in the configuration with *Bacillus* cells (Fig. 1A). Whilst the strongest growth inhibition was observed for *A. brassicicola*, *A. brassicae* and *V. longisporum*, the effect was statistically significant in all cases (Supplementary Data, Table S1).

Next, the activity of compounds secreted by *Bacillus* UCMB5113 grown in LB broth was tested after crude fractionation of the cell-free supernatant using acid precipitation. This allowed enrichment of many antibiotic compounds, notably
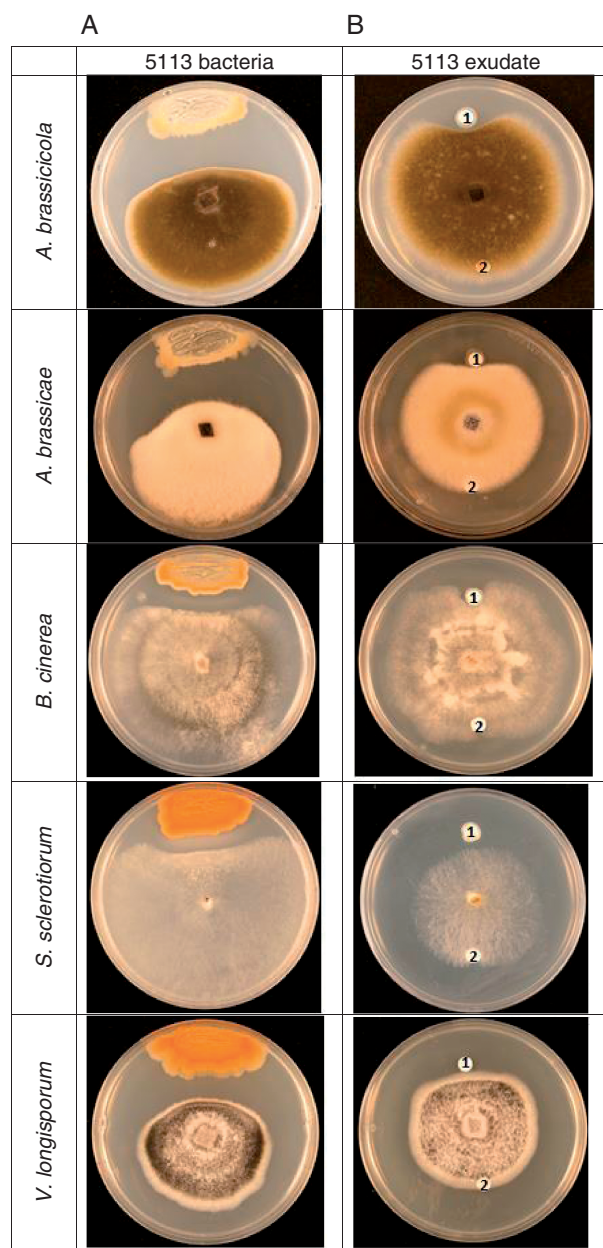
FIG. 1. Screening of antagonistic activity of *Bacillus amyloliquefaciens* UCMB5113 against fungal phytopathogens. (A) A single *B. amyloliquefaciens* UCMB5113 colony was streaked on one side of the PDA plate and fungal strains were inoculated at the centre. Growth inhibition was studied 3–14 d later. (B) LP extract was tested for antifungal activity to phytopathogens grown on PDA. The pathogen was inoculated at the centre of the plate and aliquots of the LP extract (1) or methanol (solvent control) (2) were added at the periphery of the dish. The plates were incubated at room temperature and growth was recorded after 3–14 d.

lipopeptides, from the growth medium. When this LP extract was tested for activity against phytopathogens, a significant zone of growth inhibition was observed for *A. brassicicola*, *A. brassicae* and *B. cinerea* (Fig. 1B). The antagonistic effect of the LP extract was weaker than for living bacteria but revealed consistent production and secretion of antifungal compounds occurring in this *Bacillus* strain.

The antifungal activity of the LP extract was tested on plants by treating leaves of *A. thaliana* Col-0 prior to inoculation with *A. brassicicola*. Plants treated with the LP extract showed fewer necrotic symptoms compared to the methanol- and water-treated control leaves, indicating spray application of LP extracts to be useful for plant protection (Supplementary Data, Fig. S1).

Antagonistic activity stability of the Bacillus LP extract

To assess the thermostability of the active compounds, LP extract was heated at 55, 75 and 95 °C for 0, 12, 24, 48 and 72 h. The compounds were shown to maintain activity after exposure to up to 95 °C for 72 hours, as shown by the retainment of antifungal activity (Supplementary Data, Fig. S2, Table S2).

Identification of the most active antifungal compounds formed by Bacillus UCMB5113

The semi-purified LP extract was further fractioned using preparative RP-HPLC. Fourteen major fractions collected (Fig. 2A) were tested for growth inhibition of *Brassica* phytopathogens. A number of the fractions showed antagonistic activity, although the relative sensitivity varied among the different pathogens (Fig. 2B) (Supplementary Data, Table S3). Fraction 9 was antagonistic to all pathogens and expressed the highest toxicity.

UPLC-ESI-MS analysis of fraction 9 showed one major peak together with several additional peaks of much lower intensities (Fig. 3A). In all, this fraction contained five different compounds with molecular ions detected at m/z 1396·8, 1435·8, 1467·8, 1481·6 and 1495·8. The last ion represented approx. 80 % of the total amount based on the peak area (Fig. 3B) with a molecular mass in the range of those corresponding to antifungal cyclical LPs of the fengycin family. However, it did not match any of the previously described forms. Further structural information on the main compound was obtained by tandem MS analyses. Fragmentation of cyclized fengycins secreted by *Bacilli* was quite poor, yielding only two main y-type ions (Supplementary Data, Fig. S3). Those fragments correspond to the peptide moiety and may be used as signature ions for discriminating between A, B, C and S variants. By contrast, many ions were generated upon fragmentation of the molecular species m/z 1495·8 (Fig. 3C), strongly suggesting that the peptide chain was linearized. Signature ions were detected at m/z 1126·57 and 1012·38 but did not match any known ions. However, those ions are typical for fengycins from the B1 family taking into account a mass shift of 18 Da due to ring opening (also visible on signature ions). The detection of most of the corresponding y-type, b-type and some internal fragments allowed unambiguous confirmation that the ion at m/z 1495·8 corresponded to the protonated form of the linear C_{15}-fengycin B1. This linear C_{15}-fengycin B1 was also detected in fraction 8, but at much lower relative amounts compared to fraction 9 (Supplementary Data, Fig. S3). This further supports the key role of this fengycin form for antifungal activity, since fraction 8 was also clearly less active at inhibiting the phytopathogens compared to fraction 9. MS/MS analyses revealed that the four other minor ions in fraction 9 also most probably corresponded
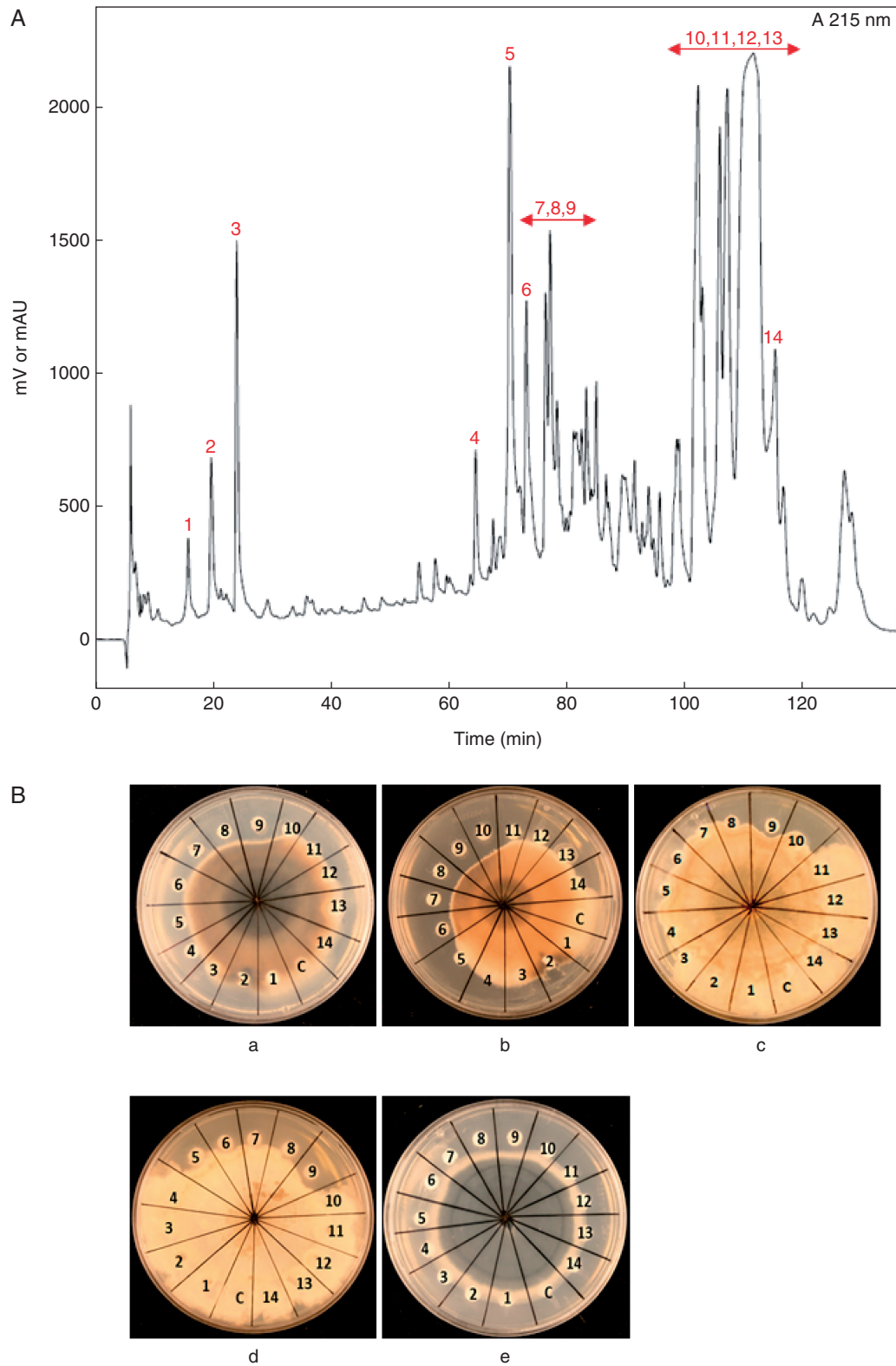
FIG. 2. Fractionation of *Bacillus amyloliquefaciens* UCMB5113 LP extract and antifungal tests. (A) RP-HPLC profile of LP extract obtained after acid precipitation of *B. amyloliquefaciens* UCMB5113 growth medium was obtained using a C_{18} reversed phase column using a water/acetonitrile/TFA gradient and the eluent monitored at 215 nm. (B) Growth inhibitory activity of the LP extract fractions isolated by RP-HPLC was tested on the phytopathogens (a) *A. brassicicola*, (b) *A. brassicae*, (c) *B. cinerea*, (d) *S. sclerotiorum* and (e) *V. longisporum*. HPLC fractions 1–14 or methanol as control (C) were added to different parts of the Petri dish and growth was studied 3–14 d later.
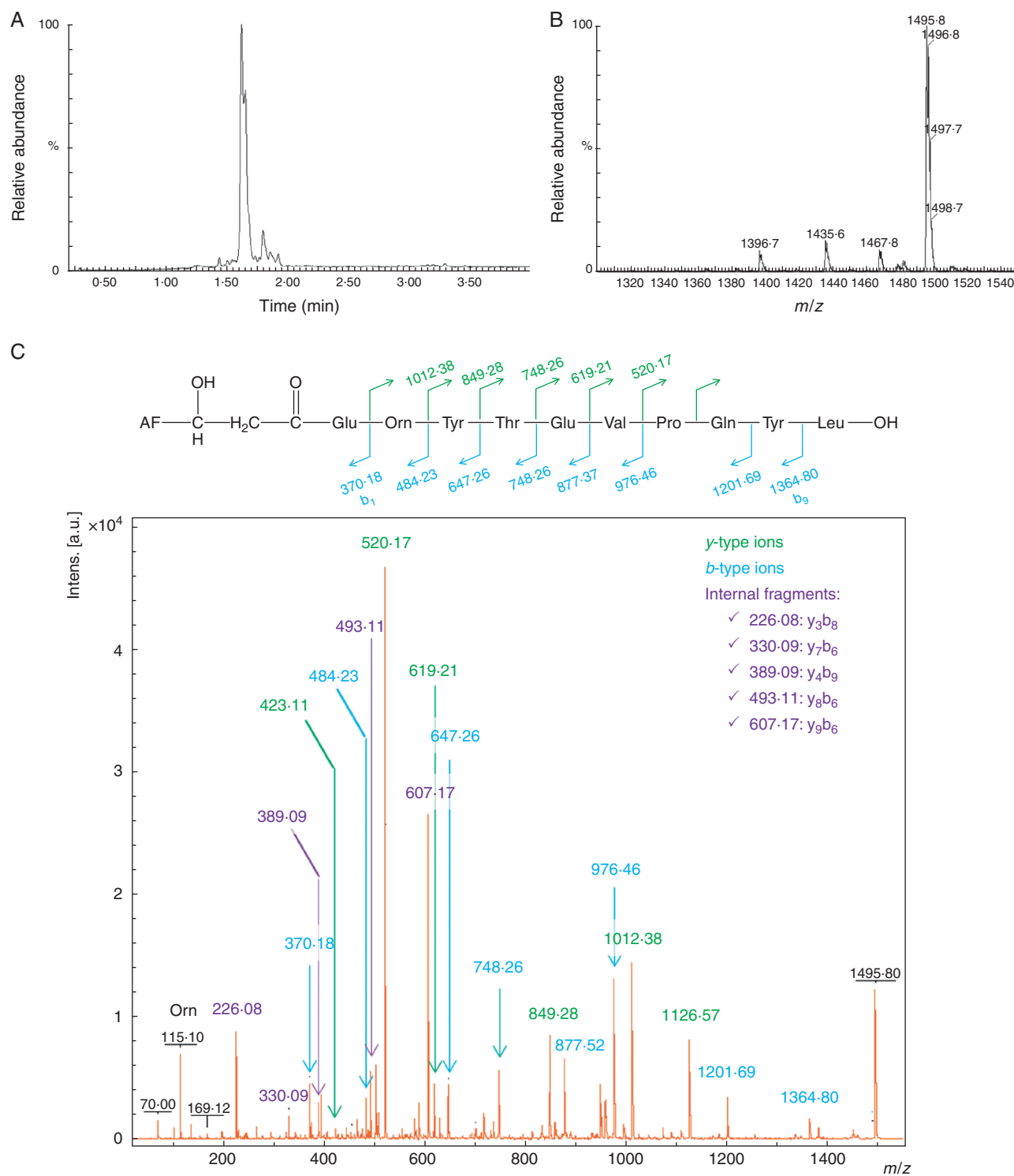
Fig. 3. Analysis of HPLC fraction 9 by UPLC-ESI-MS. (A) LC-MS chromatogram revealing one major peak eluting at 1·95 min. (B) MS spectrum showing that this peak mainly contained a compound with *m/z* 1495·8 that was further structurally characterized by MS-MS as illustrated in C. (C) Fragmentation pattern obtained and typical y- and b-type ions as well as internal fragments that were unambiguously assigned leading to the linear peptide sequence represented. Note that the analysis cannot distinguish Leu from Ile so Leu was arbitrarily chosen in the molecular structure. Further, the isomery of the fatty acid chain cannot be determined so the chain is written as a linear form for convenience.

to fengycin variants based on their mass and fragmentation patterns (Supplementary Data, Fig. S3) but their contribution to the antifungal activity was rather low, if any. As shown in Fig. S3, ions at m/z 1467·8 and 1481·6 were also present in fraction 7, which displayed almost no antifungal activity, while peaks at m/z 1396·8 and 1435·8 were detected in similar relative proportions in fraction 8 as in fraction 9.

Time-course LP profiling over the first days of culture for *Bacillus* UCMB5113 revealed that this strain also readily produced the iturin and surfactin families of LPs (Supplementary Data, Fig. S4) and showed that the linear C_{15}-fengycin B1 accumulated in the supernatant in a similar kinetic trend as compared to the cyclic fengycins (Supplementary Data, Fig. S5). This linear form was observed as early as 24 h after incubation and increased over time, suggesting that it was actually *de novo* co-produced together with the other forms. The majority of the fengycins were cyclic in nature and after 72 h the linear B1 form represented only 6 % of the total fengycins.

Effects of synthetic peptides on fungal growth

LP mimics of the major component of fraction 9 were synthesized to confirm the biological activity of the natural compound. For this, the synthetic LP EOYTGVPQYL with acetylated (AcePEP) or myristoylated (MyrPEP) N-terminus (Supplementary Data, Fig. S6) were compared to test the effect of a lipophilic fatty acid tail for bioactivity towards *A. brassicicola* and *V. longisporum*. MyrPEP but not AcePEP showed a zone of fungal growth inhibition such as that observed for the LP extract (Fig. 4A) (Supplementary Data, Table S4). Such differential activity was also observed by testing these synthetic peptides for systemic disease suppression on *Arabidopsis* plants grown on MS plates. Both macroscopic disease symptoms and qPCR data for quantification of the pathogen showed a significant suppression of *Alternaria* growth, but only for plants treated with MyrPEP (Fig. 4B, C).

Effect of Bacillus *UCMB5113 LP extract on plant systemic defence*

To test the ISR-triggering potential of the LP extract, *Arabidopsis* roots were treated prior to challenge with *A. brassicicola*. The *coi1-16* and *jar1* lines are defective in JA-induced transcriptional regulation, *sid2* plants cannot accumulate SA and activate an SAR, *myc2* and *myb72* lines are defective in transcription factors necessary to transmit the priming of ISR, while *npr1* plants display neither SAR nor ISR. The levels of fungal growth in plant leaves were analysed by qPCR specific for an *Alternaria* cutinase gene. Significantly lower levels of *Alternaria* were observed in wild type Col-0, *jar1* and *myc2* plants after LP treatment compared to solvent controls while *npr1* plants showed elevated levels (Fig. 5). The level of *Alternaria* was unaffected by the LP extracts in *coi1-16*, *sid2* and *myb72* plants.

Plant defence gene expression after treatment with Bacillus *UCMB5113 LP extract*

To further investigate the role of JA for the systemic defence responses against *A. brassicicola* in *Arabidopsis*, the expression
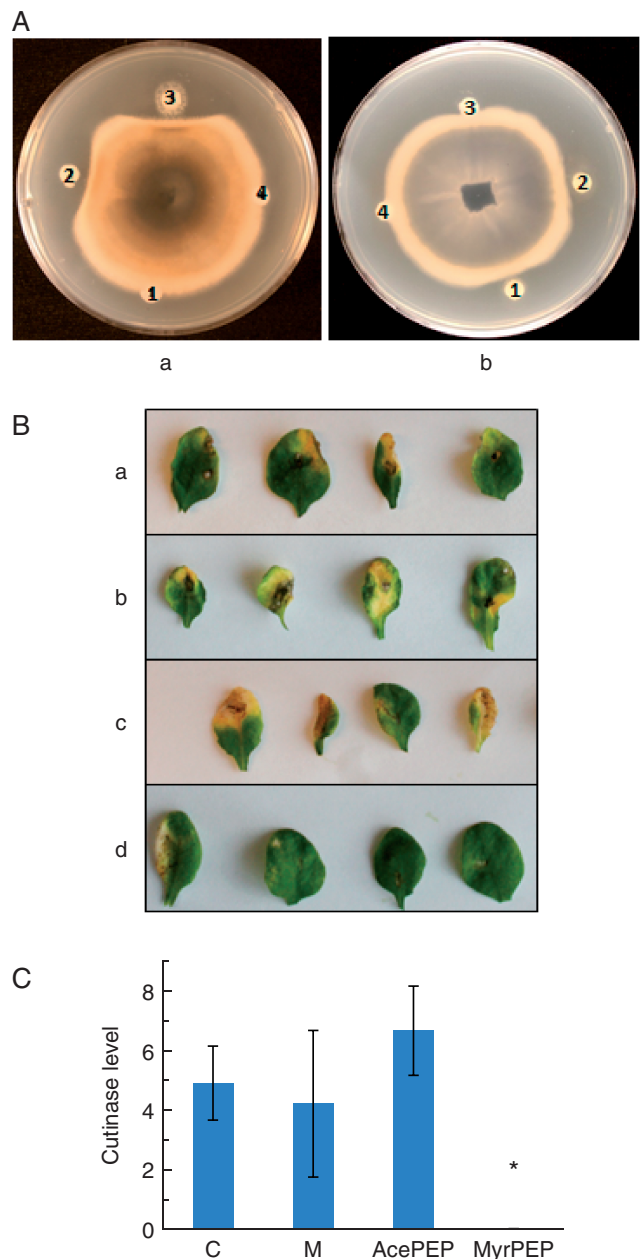

FIG. 4. Effects of synthetic peptides on growth of fungal pathogens. (A) Synthetic peptide with acetyl side chain (a1, b3), synthetic peptide with fatty acid side chain (a2, b2), *B. amyloliquefaciens* UCMB5113 LP extract (a3, b1) or methanol (a4, b4) was dropped on discs in the peripheral part of a PDA plate containing *A. brassicicola* or *V. longisporum* in the centre. Effects on fungal growth were recorded 5 d later. (B) Suppressive effect of synthetic peptides against *A. brassicicola* growth on *Arabidopsis thaliana* Col-0 leaves. One-week-old seedlings grown on MS media were treated with (a) water, (b) 5 % methanol, (c) synthetic peptide with acetyl side chain and (d) synthetic peptide with fatty acid side chain compounds. Two weeks after treatment, four leaves of each plant were inoculated with *A. brassicicola*. The presence of necrotic lesions was recorded 1 week after fungal inoculation. (C) Quantification of fungal level in plants treated with synthetic peptides. Five days after fungal inoculation, the level of *Alternaria* DNA on plants was quantified by qPCR where cutinase levels relative to ubiquitin5 expression are plotted. ANOVA was run to compare DNA levels among different treatments ($n = 4$). Plants were untreated ('C'), or treated with methanol as solvent control ('M'), synthetic peptide with acetyl side chain ('AcePEP') and synthetic peptide with fatty acid side chain ('MyrPEP').
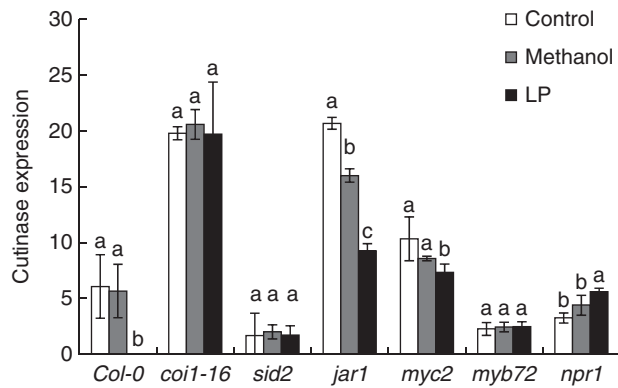
FIG. 5. qPCR analysis of *A. brassicicola* levels in *B. amyloliquefaciens* UCMB5113 primed *Arabidopsis* plants. One-week-old seedlings of *Arabidopsis* wildtype or the mutants *coi1-16*, *sid2*, *jar1*, *myc2*, *myb72* and *npr1* were tested. Roots of one-week-old seedlings grown on MS media were treated with water, 5 % methanol or an LP extract. Two weeks after treatment, four leaves of each plant were inoculated with *A. brassicicola*. Five days after fungal inoculation, the level of *Alternaria* DNA was quantified by qPCR. ANOVA was run to compare DNA levels among the treatments for each plant line where different letters indicate significant differences. Plants were untreated (white bar), treated with methanol as solvent control (grey bar) or treated with LP extract (black bar).

of the JA marker genes *VSP2* and *PDF1.2* were analysed after pre-treatment of roots with the LP extract. *VSP2* expression also depends on ABA and MYC2 while *PDF1.2* expression also needs ethylene as well as the ethylene-dependent transcription factors ERF1 and ORA59. *VSP2:GUS* and *PDF1.2:GUS* plants pretreated with *Bacillus* LP extract and subsequently challenged with fungal spores were stained histochemically. Plant roots treated with LP extract showed a significantly elevated systemic expression of *VSP2* in leaves and roots after fungal spore inoculation. This was not observed for plants treated with water and/or methanol (Fig. 6A–C). No distinct differences in the expression of *PDF1.2:GUS* were observed for either treatment (Fig. 6D–E).

DISCUSSION

Co-cultivation of *Bacillus* UCMB5113 with the phytopathogens *A. brassicicola*, *A. brassicae*, *B. cinerea*, *S. sclerotiorum* and *V. longisporum* resulted in growth suppression of all pathogens, demonstrating that the bacterium secretes antifungal compounds. The major bioactive molecules produced by this *Bacillus* species are LPs, which display a wide spectrum of activity. LPs are genetically and biochemically well characterized and important in plant colonization, direct antagonism to phytopathogens and stimulation of plant immunity (Souto *et al.*, 2004; Ongena *et al.*, 2007; Ongena and Jacques, 2008; Cawoy *et al.*, 2014; Farace *et al.*, 2014; Rahman *et al.*, 2015). An LP-enriched extract from *Bacillus* UCMB5113 culture broth retained antifungal activity as observed also when intact bacteria were used. Bioassays using *Arabidopsis* plants showed that treatment of leaves with a *Bacillus* UCMB5113 LP extract provided disease protection. Furthermore, treatment of plant roots with the LP extract resulted in disease suppression when challenged with *Alternaria*, indicating primed systemic defence as
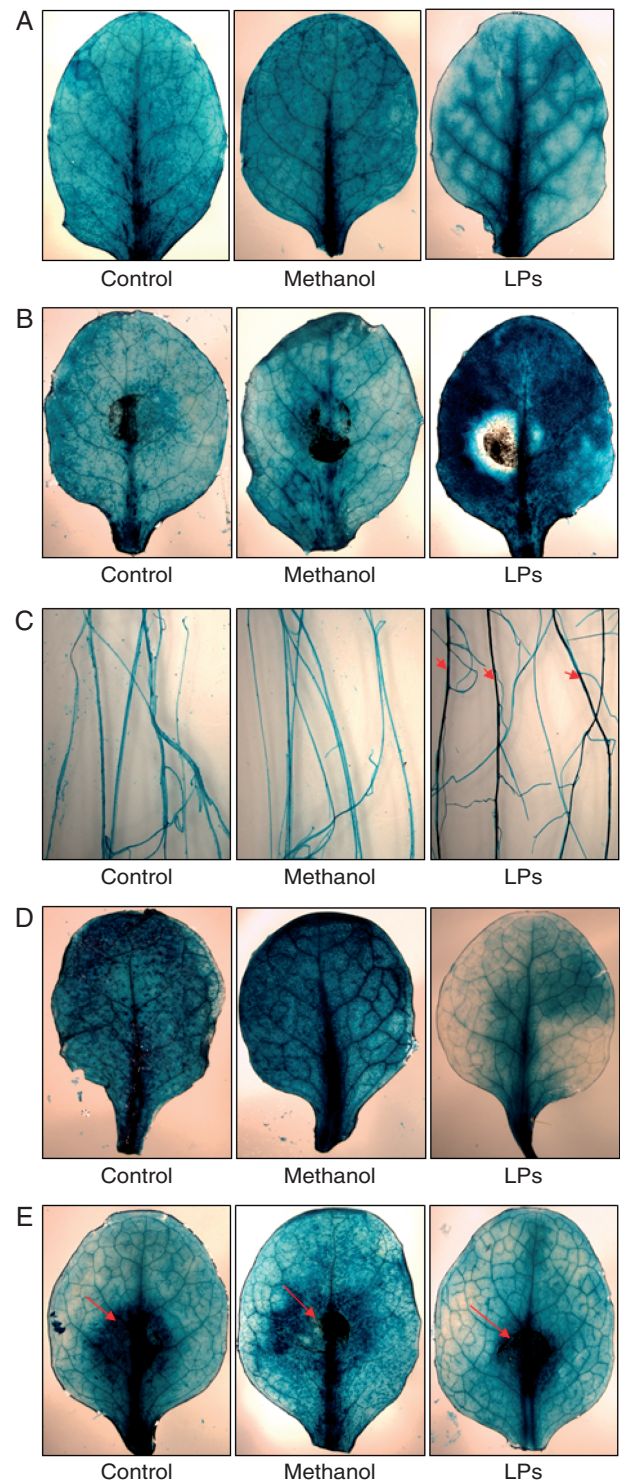
FIG. 6. Effects on JA reporter plants by LP extract and *A. brassicicola*. One-week-old GUS reporter plants with different root treatments by water, methanol or LP extract were challenged with *A. brassicicola* spores on leaves 2 weeks later. Three days after fungal inoculation, plants were stained for reporter gene GUS expression. (A) *VSP2:GUS* without fungal treatment; (B) *VSP2:GUS* with fungal treatment; (C) *VSP2:GUS* roots after fungal treatment; (D) *PDF1.2:GUS* without fungal treatments; (E) *PDF1.2:GUS* with fungal treatment. Arrows indicate site of fungal inoculation on leaves and the enhanced expression in roots.

ISR. Other studies have also demonstrated disease suppression by BCAs based on LPs (e.g. Tran *et al.*, 2007; Waewthongrak *et al.*, 2014). Signalling has mostly been studied in *Pseudomonas* strains (van Loon *et al.*, 1998; Verhagen *et al.*, 2010) for which the ISR concept was coined, although *Bacillus*-derived ISR has also been demonstrated recently (Falardeau *et al.*, 2013; Farace *et al.*, 2014; Rahman *et al.*, 2015). Analysis with signalling-compromised plants as well as JA-marker GUS reporter lines enabled evaluation of how plant hormones involved in different defence responses responded to *Bacillus* UCMB5113 LPs. Growth of *Alternaria* was suppressed by *Bacillus* UCMB5113 LPs in wildtype, *jar1* and *myc2* lines, while *coi1-16, myb72* and *sid2* plants did not respond and the *npr1* accession even displayed increased *Alternaria* levels after LP treatment. Priming of ISR by *Pseudomonas* acts locally in roots through MYB72 and systemically through MYC2 (Pieterse *et al.*, 2014). JAR1 produces the Ja–Ile conjugate that stimulates COI-dependent degradation of JAZ proteins allowing MYC2 to stimulate G-box containing promoters of JA-responsive genes (Kazan and Manners, 2013). The growth of *A. brassicicola* in plants is known to be affected by JA (e.g. Thomma *et al.*, 1998). Our data show that the LP-enriched extract suppressed *Alternaria* without the need for JA–Ile formation but could not rescue plants with compromised JAZ degradation and did not work very well in the absence of MYC2 (where redundancy may assuage the effect). Root treatment of *VSP2:GUS* plants with the LP extract followed by fungal inoculation resulted in distinct staining systemically in leaves and roots while fungal inoculation alone only raised local *VSP2* expression. In contrast, plants expressing *PDF1.2:GUS* showed low local expression around the fungal inoculation site on leaves both in the control and in the LP extract-treated samples. These results imply JA involvement in the effects observed by the LP extract but with no obvious dependence on ethylene while ABA may contribute to a reaction involving MYC to activate *VSP2* transcription. Involvement of ABA in plant defence has been demonstrated for other JA-dependent pathogens with a necrotrophic life style (Adie *et al.*, 2007). These results point to a complex signalling caused by the *Bacillus* UCMB5113 LPs involving JA.

MS analysis of the most active fractions from the *Bacillus* UCMB5113 LP extract allowed identification of novel linear forms of antifungal fengycins. Linear forms are unusual, as the standard form is a cyclic structure formed by the multicomplex fengycin biosynthetic apparatus. Many studies have shown the ability of *Bacillus* species to produce different cyclic fengycins (Vanittanakom *et al.*, 1986; Sun *et al.*, 2006; Deleu *et al.*, 2008; Eeman *et al.*, 2009; Malfanova *et al.*, 2012; Pathak *et al.*, 2012; Farace *et al.*, 2014; O'Connor *et al.*, 2014). However, one study (Pathak *et al.*, 2012) also provided data in support of the presence of linear forms of fengycin in LP extracts of the banyan tree endophyte *Bacillus subtilis* K1, but no biological activity was reported. That report also found linear fengycins upon direct analysis of cell suspensions, although at low levels. Our time-course analysis suggested strongly that the linear C_{15} fengycin B1 is co-produced by *Bacillus* UCMB5113 along with several other structural variants. pH increases slowly during growth of *Bacillus* UCMB5113 in LB, which could cause non-enzymatic hydrolysis of the bond involved in cyclization. However, *in vitro* tests where cyclic fengycins were incubated at higher pH did not result in ring opening (our unpublished data). In addition to NRPS templated pathways, non-templated reactions can occur where the arrangement of the peptide is more difficult to predict and various post-synthesis modifications may further change the structure (Giessen and Marahiel, 2012). The linear forms could thus be synthesized as such or result from subsequent modifications (Aleti *et al.*, 2015). The mechanisms forming these linear but highly active forms of fengycins warrant further investigation.

Use of synthetic peptide mimics of the identified *Bacillus* UCMB5113 linear fengycin showed that the fatty acid side chain is necessary for direct antagonistic activity to phytopathogens and priming of ISR in *Arabidopsis* plants since replacement with an acetyl group gave no significant inhibition of *A. brassicicola* and *V. longisporum* in plate assays or with plants. LPs can serve as membrane-disturbing biosurfactants providing a direct protective effect against phytopathogens (Ongena and Jacques, 2008). The membrane-disturbing properties of LPs imply that many kinds of micro-organisms can be targeted especially when a mixture of LPs is produced. Other surfactin and fengycin LPs have been shown to act as elicitors of ISR in plants (Ongena *et al.*, 2007; Cawoy *et al.*, 2014). Estimates of up to 10 % of the *B. amyloliquefaciens* genomes may be designated for production of secondary metabolites, providing a plethora of potentially antagonistic molecules (Chen *et al.*, 2009; Niazi *et al.*, 2014). In addition, *Bacillus* strains can interact with a wide range of plant species, providing potential protection to many different kinds of pathogens (e.g. Alvarez *et al.*, 2012; Garcia-Gutierrez *et al.*, 2013; Adam *et al.*, 2014; Kalai-Grami *et al.*, 2014).

Our results indicate that the LP extract from *Bacillus* UCMB5113 provides systemic resistance in plants and is dependent on JA, indicative of a primed ISR response. *Bacillus* UCMB5113 has the ability to produce many secondary metabolites, resulting in a complex cocktail of various antibiotics that can act directly and indirectly, supporting efficient plant protection to phytopathogens. Novel forms of such potentially antifungal linear fengycins may serve as a basis for the development of more powerful plant protective agents. The results obtained suggest that *Bacillus* UCMB5113 shows potential as a BCA to counteract *Brassica* phytopathogens. The LP compounds produced by this bacterium are stable under high temperatures, indicating long-term efficacy. Use of ecosystem services such as *Bacillus* UCMB5113 that could support plant protection through priming of ISR is an important step towards more sustainable crop production, reducing the need for chemical pesticides. Further studies of the mechanisms involved in beneficial plant–microbe interactions will advance our understanding of the complex molecular basis and improve the potential to identify requirements for successful interaction and priming.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic. oup.com/aob and consist of the following. Figure S1: suppressive effect of *B. amyloliquefaciens* UCMB5113 LP extract against *A. brassicicola* growth on *A. thaliana* Col-0 leaves. Figure S2: thermostability of *B. amyloliquefaciens* UCMB5113 LP extract in relation to antagonistic activity. Figure S3: MS

analyses of fengycins. Figure S4: UPLC-MS profiling of LPs present in the crude culture supernatant of *B. amyloliquefaciens* UCMB5113 after 48 h of culture. Figure S5: accumulation of a linear form of C_{15}-fengycin. Figure S6: structures of synthetic peptides. Table S1: fungal growth inhibition with bacteria and LP extracts. Table S2: heat stability analysis of LP extracts. Table S3: fungal growth inhibition with LP fractions after HPLC separation. Table S4: fungal growth inhibition by synthetic LP mimics.

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