Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*

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Received: *27 February 2007*
Accepted: *25 April 2007*

Summary

• *Pseudomonas* strains have shown promising results in biological control of late blight caused by *Phytophthora infestans*. However, the mechanism(s) and metabolites involved are in many cases poorly understood. Here, the role of the cyclic lipopeptide massetolide A of *Pseudomonas fluorescens* SS101 in biocontrol of tomato late blight was examined.

• *Pseudomonas fluorescens* SS101 was effective in preventing infection of tomato (*Lycopersicon esculentum*) leaves by *P. infestans* and significantly reduced the expansion of existing late blight lesions. Massetolide A was an important component of the activity of *P. fluorescens* SS101, since the *massA*-mutant was significantly less effective in biocontrol, and purified massetolide A provided significant control of *P. infestans*, both locally and systemically via induced resistance.

• Assays with *nahG* transgenic plants indicated that the systemic resistance response induced by SS101 or massetolide A was independent of salicylic acid signalling. Strain SS101 colonized the roots of tomato seedlings significantly better than its *massA*-mutant, indicating that massetolide A was an important trait in plant colonization.

• This study shows that the cyclic lipopeptide surfactant massetolide A is a metabolite with versatile functions in the ecology of *P. fluorescens* SS101 and in interactions with tomato plants and the late blight pathogen *P. infestans*.

Key words: biocontrol, colonization, induced systemic resistance, *Phytophthora infestans*, *Pseudomonas*, surfactants.

New Phytologist (2007) **doi**: 10.1111/j.1469-8137.2007.02138.x

Introduction

Oomycetes form a diverse group of eukaryotic, fungus-like microorganisms containing a wide range of economically important pathogens of plants, insects, fish and animals (Kamoun, 2003). Among the plant pathogenic Oomycetes, *Phytophthora infestans* is the most notorious, causing late blight of potato and tomato. In the disease cycle, zoospores are essential propagules in the preinfection process and a potential target to control *P. infestans* and other Oomycete pathogens (Donaldson & Deacon, 1993; Erwin & Robeiro, 1996; van West *et al.*, 2002). Late blight is traditionally controlled by a combination of cultural practices and chemical applications. To control late blight biologically, several antagonistic microorganisms have been tested for their activity against *P. infestans*, including nonpathogenic *Phytophthora cryptogea* (Stromberg & Brishammar, 1991) and endophytic microorganisms such as *Cellulomonas flavigena*, *Candida* sp., and *Cryptococcus* sp. (Lourenço Júnior *et al.*, 2006). Although some effective fungal antagonists were identified, bacterial antagonists have shown by far the most promising results to date. Bacteria with antagonistic activities against *P. infestans* are mainly found in the genera of *Pseudomonas* and *Bacillus* (Sanchez, 1998; Yan *et al.*, 2002; Daayf *et al.*, 2003; Kloepper

et al., 2004). In most of these studies, however, the mechanisms and metabolites involved in the biocontrol activity were not investigated in detail.

Several strains of *Pseudomonas fluorescens* were recently described that produce surface-active compounds, designated biosurfactants, with destructive effects on zoospores of *P. infestans* and other Oomycetes, including *Pythium* species (De Souza *et al.*, 2003; De Bruijn *et al.*, 2007). For *P. fluorescens* strain SS101, the biosurfactant was identified as massetolide A, a cyclic lipopeptide with a nine-amino-acid peptide ring linked to 3-hydroxydecanoic acid (De Souza *et al.*, 2003). Application of *P. fluorescens* SS101 to soil or bulbs effectively controls *Pythium* root rot of flowerbulb crops in both bioassays and small-scale field experiments (De Boer *et al.*, 2006). The role of massetolide A in the biocontrol activity of *P. fluorescens* SS101 against Oomycete pathogens, however, has not been resolved to date.

The present study aims at a comprehensive investigation of the potential of *P. fluorescens* SS101 to control late blight of tomato. The ability of strain SS101 to prevent infection and to control the development of existing infections of *P. infestans* was investigated in plant assays. The role of massetolide A in biocontrol of *P. infestans* was studied by comparing the activity of strain SS101 with that of its massetolide A-deficient mutant and purified massetolide A. The role of systemic resistance in tomato plants induced by strain SS101 or massetolide A was determined by physically separating the inducing agents from the late blight pathogen. Transgenic *nahG* tomato plants, which are unable to accumulate salicylic acid, were included to assess whether salicylic acid acts as a signal in the induced systemic resistance response. Finally, the role of massetolide A in plant colonization by *P. fluorescens* SS101 was investigated by comparing the population dynamics of wild-type strain SS101 on the surface and in the interior of tomato plants with that of its massetolide A-deficient mutant.

Materials and Methods

Microorganisms and growth conditions

Pseudomonas fluorescens strain SS101 was originally isolated from the rhizosphere of wheat grown in a soil suppressive to take-all disease (De Souza *et al.*, 2003). Biochemical analysis revealed that SS101 produces at least five cyclic lipopeptide surfactants. Massetolide A is the main cyclic lipopeptide produced by SS101 (De Souza *et al.*, 2003), and the other cyclic lipopeptides detected in cell-free culture supernatants are derivatives of massetolide A differing in amino acid composition of the peptide ring (I. de Bruijn *et al.*, unpublished). In this study, a spontaneous rifampicin-resistant derivative of SS101 was used. Mutant 10.24 was derived from the rifampicin-resistant derivative of SS101 by mutagenesis and has a single Tn*5* insertion in *massA*, the first nonribosomal peptide synthetase (NRPS) gene required for the biosynthesis of massetolide A (I. de Bruijn *et al.*, unpublished). Mutant 10.24 does not produce massetolide A, nor any of the other massetolide A derivatives produced by wild-type strain SS101. Mutant 10.24 is resistant to rifampicin (100 μg ml^{-1}) and kanamycin (100 μg ml^{-1}). For the bacterial inoculum used in the plant assays, strain SS101 and mutant 10.24 were grown on *Pseudomonas* agar (PSA) plates (Difco, Le Pont de Claix, France) at 25°C for 48 h. Bacterial cells were washed in sterile demineralized water before use. For treatment of tomato seeds, roots or leaves, washed cell suspensions of SS101 or 10.24 were diluted in sterile demineralized water to a final concentration of 10^9 CFU ml^{-1} ($OD\ 600_{nm} = 1$).

Phytophthora infestans strain 90128 (A2 mating type, race 1.3.4.6.7.8.10.11) was used in all bioassays. The strain was grown on rye sucrose agar (Latijnhouwers *et al.*, 2004) for 7–9 d in the dark at 18°C. To obtain zoospores, full-grown plates (9 cm diameter) were flooded with 20 ml of sterile distilled water and hyphae were fully submerged with a glass spreader. Flooded plates were placed in the cold (4°C) for 1–2 h, after which the suspension was gently filtered (50 μm mesh) to remove sporangia. Zoospore density was determined microscopically at 100 × magnification and adjusted to a final concentration of $3–4 \times 10^3$ swimming zoospores ml^{-1}.

Purification and detection of massetolide A

The cyclic lipopeptide massetolide A (molecular mass 1139 Da) was extracted from cell cultures of *P. fluorescens* SS101 as described by De Souza *et al.* (2003). In summary, strain SS101 was grown on PSA agar plates for 48 h at 25°C. Bacterial mats were suspended in sterile demineralized water and cells were pelleted by centrifugation at 5500 ***g*** for 20 min. The cell-free culture supernatant was collected, acidified with HCl to pH 2 and incubated for 1 h on ice to precipitate massetolide A. The precipitate was obtained by centrifugation (5500 ***g***, 30 min) and washed twice with acidified (pH 2) sterile demineralized water. The precipitate was dissolved in sterile demineralized water by adjusting the pH to 8 with 0.5 M NaOH, lyophilized and stored at –20°C. The precipitate was analysed by reverse-phase high-performance liquid chromatography (RP-HPLC) linked to a photodiode-array detector as described by De Souza *et al.* (2003) and De Bruijn *et al.* (2007). For extraction of massetolide A from tomato leaves, the protocol of Asaka & Shoda (1996) was used. The extraction efficiency was tested by spiking 1 mg of massetolide A (70% purity) to tomato leaves submersed in the solvents used for extraction (HPLC-grade acetonitrile and trifluoroacetic acid (0.1% (v/v)). The concentration of massetolide A was determined based on peak area (at 206 nm) using a six-point standard curve.

Plant cultivation and biocontrol assays

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Moneymaker Cf0) were sown in a tray containing a mixture of commercial

potting soil and quartz sand (3 : 2, w/w). The initial water content of the soil mixture was adjusted to 40% (v/w). The tray was covered with a transparent lid and kept in the climate chamber (20°C, 16 : 8 h day : night photoperiod) for 2 wk to stimulate germination. Seedlings were then transplanted to $7 \times 7 \times 8$ cm (L × W × H) black plastic PVC pots containing the same soil mixture and kept in the climate chamber at 20°C for 3 wk. For the biocontrol assays with *P. infestans*, plants were transferred to a 15°C growth chamber to create conditions favourable for infection and disease development. For the induced resistance assays, the *nahG* transgene, which is unable to accumulate salicylic acid (Gaffney *et al.*, 1993), was included. The *nahG* derivative was kindly provided by Dr Jan van Kan (Laboratory of Phytopathology, Wageningen University, the Netherlands). Before use, tomato seeds were surface-sterilized, dried in a flow cabinet, and sown and maintained as already explained.

Prevention of late blight infection of tomato leaves

To study the effects of *P. fluorescens* SS101 and massetolide A on late blight of tomato, two leaves located on the second branch from the stem base of 5-wk-old tomato plants were immersed in bacterial suspension (10^9 CFU ml^{-1}) for 1 min or in a solution of massetolide A in sterile demineralized water (pH 8). Leaves immersed in sterile demineralized water (pH 8) for 1 min served as a control. Treated tomato plants were transferred to trays covered with transparent lids. After incubation for 1 d in a growth chamber at 15°C, the lower side of each treated tomato leaf was inoculated with 3 µl droplets of a *P. infestans* zoospore suspension ($3–4 \times 10^3$ swimming zoospores ml^{-1}) or 3 µl droplets of sterile demineralized water (pathogen-free control). Two droplets were placed abaxially on each side of the leaf's midvein. Tomato plants were incubated in the growth chamber, and at several days after zoospore inoculation, disease incidence and lesion area were scored. Disease incidence was scored visually by counting the number of zoospore droplets that developed into a lesion. The area of the lesions was determined by an electronic marking gauge linked to the IBREXDLL software (IBR Prozessautomation) as described by Latijnhouwers *et al.* (2004). Each treatment had four or five replicates with one plant per replicate. For each plant, disease incidence and lesion area were assessed on two leaves, each treated with four zoospore droplets.

Effect of *P. fluorescens* SS101 and massetolide A on existing late blight lesions

Two tomato leaves in the second branch from the stem base of 5-wk-old tomato plants were inoculated with 3 µl droplets of a *P. infestans* zoospore suspension or sterile demineralized water (pathogen-free control). The droplets were placed abaxially on each side of the leaf's midvein. Tomato plants were then incubated at 15°C for 3–4 d to allow the late blight lesions to develop. The initial lesion area was determined as already described. Subsequently, the lesioned tomato leaves were dipped in bacterial suspension (10^9 CFU ml^{-1}) or in solutions with different concentrations of massetolide A for 1 min; sterile-demineralized water served as a control. Tomato plants were then transferred to the climate chamber (15°C) and the areas of the late blight lesions were measured again at 2 and 5 d after treatment with water (control), the bacterial suspension or massetolide A. The increase in lesion area was calculated by dividing the lesion area after treatment by the initial lesion area assessed before treatment with water, the bacteria or massetolide A.

Induced resistance assays

To determine the role of induced resistance as a mechanism in late blight control, two types of experiments were conducted to physically separate the inducing agents from the pathogen (Supplementary material, Fig. S1). In one series of experiments, the inducing agents were applied to the lower leaf and the pathogen inoculated on the upper leaves. Therefore, two adjacent leaves on the first branch from the stem base of 5-wk-old tomato plants were immersed in bacterial suspension or in a solution of massetolide A for 1 min. After incubation of the treated tomato plants for 24 h at 15°C, two adjacent leaves on the second branch from the stem base were inoculated with 3 µl droplets of a zoospore suspension of *P. infestans* as described earlier. In the second series of experiments, roots of 2-wk-old tomato seedlings were washed gently with running tap water to remove adhering soil, blotted dry with sterile paper tissue, and dipped in a bacterial suspension or in a solution of massetolide A for 10 min. Sterile demineralized water (pH 8) served as a control. The seedlings were then transplanted and maintained as explained above. Approximately 2 wk after treatment, the tomato leaves were challenge-inoculated with zoospores of *P. infestans*, as already described. Disease incidence and lesion area were assessed at different time points after pathogen inoculation. For each treatment, six replicates were used.

Effect of seed treatment on late blight incidence, severity, and sporangia formation

The efficacy of seed treatments to control *P. infestans* was investigated by immersing tomato seeds in bacterial suspensions (10^9 CFU ml^{-1}) or in a solution of massetolide A for 1 h. Immersing seeds in sterile demineralized water for 1 h served as a control. After treatment, seeds were dried in a flow cabinet and sown in the soil mixture described above. The final density of strain SS101 and mutant 10.24 on the tomato seeds was 1×10^6 CFU per seed, as determined by dilution-plating suspensions from seeds onto PSA medium supplemented with rifampicin. Tomato plants were maintained under the same growth conditions as indicated above. After 5 wk of

plant growth, tomato leaves were inoculated with zoospores of *P. infestans* as described earlier. Disease incidence and lesion area were assessed at different time points after pathogen inoculation. For each treatment, six replicates were used. Nine days after zoospore inoculation, tomato leaves were harvested and lesion areas determined as described earlier. The lesions were then excised from the leaves and transferred to 1.5 ml sterile tubes containing 1 ml of isotone II electrolytic buffer (Coulter Electronic Inc., Fullerton, CA, USA). The sporangia were released from the sporangiophores by vigorously shaking on a vortex mixer for 1 min. The density of the sporangia was determined microscopically at 100× magnification in 5 µl aliquots. Combined with the lesion area, sporangia formation per unit lesion area was calculated.

Plant colonization by introduced bacterial strains

Two-week-old tomato seedlings and 6-wk-old tomato plants raised from bacteria-treated seeds (described earlier) were used to study the role of massetolide A in plant colonization by *P. fluorescens* SS101. The parts of the seedlings and plants surveyed included roots, stems, cotyledons and true leaves. For each plant part, bacterial densities were determined for the surface and interior. To determine surface colonization, approx. 1 g (fresh weight) of roots was suspended in 5.0 ml of 0.01 M $MgSO_4$, vortexed for 1 min, sonicated for 1 min (Bransonic 12) and vortexed again for 15 s before dilution-plating. Surface colonization of stems, cotyledons and leaves was determined by suspending approximately 1 g of cotyledons, leaf or stem sections in 5.0 ml of 0.01 M $MgSO_4$ supplemented with 0.05% (v/v) Tween 80, and then vortexing vigorously for 1 min before dilution-plating. Suspensions were plated onto PSA agar plates supplemented with rifampicin (for strain SS101) and on plates supplemented with rifampicin and kanamycin (for mutant 10.24). Delvocid (DSM, Delft, the Netherlands) was added (100 µg ml^{-1}) to the agar plates to prevent fungal growth. Plates were incubated for 48–72 h at 25°C, after which bacterial colonies were counted and population densities calculated.

For assessment of colonization of the root interior, root sections were surface-sterilized with 10% H_2O_2 for 15 s, rinsed twice with ample sterile demineralized water and blotted dry on sterile paper tissue. Surface-sterilized roots were homogenized with a mortar and pestle in 5.0 ml of 0.01 M $MgSO_4$ and serial dilutions were plated onto selective PSA medium as described in an earlier section. The efficacy of surface sterilization was checked with additional samples by printing the surface-sterilized root sections onto selective PSA agar plates. To determine colonization of the interior of cotyledons, true leaves and stems, these tissues were surface-sterilized with 10% H_2O_2 for 15 s, blotted dry with sterile paper tissue and rinsed twice with ample sterile demineralized water. The efficacy of sterilization of leaf and stem surfaces was checked as described earlier. Surface-sterilized plant tissues were homogenized with a mortar and pestle in 5.0 ml of 0.01 M $MgSO_4$. Suspensions were dilution-plated onto selective PSA media. Plates were incubated for 48–72 h at 25°C, after which bacterial colonies were counted and population densities calculated.

Statistical analysis

All experiments described in this study were performed at least twice. Representative results are shown. Population densities of the applied bacterial strains were log_{10}-transformed before statistical analysis. Differences between treatments in disease incidence, lesion area, and population densities of the applied bacterial strains were analysed by ANOVA followed by Student's *t*-test ($P < 0.05$; SAS Institute, Cary, NC, USA). Normal distribution of the data and homogeneity of variances were tested before ANOVA.

Results

Preventing late blight infections of tomato leaves by *P. fluorescens* SS101

Application of cell suspensions of *P. fluorescens* SS101 to leaves of tomato plants 1 d before inoculation with zoospores of *P. infestans* substantially reduced disease incidence (Fig. 1a). Also *massA*-mutant 10.24 reduced disease incidence significantly but to a lesser extent than wild-type strain SS101. The area of the few lesions observed on leaves treated with strain SS101 was significantly smaller than that of the late blight lesions in the control treatment (Fig. 1b). The effect of mutant 10.24 on lesion area was intermediate. In the control treatment, disease severity (lesion area) increased exponentially over a period of 9 d after zoospore inoculation, whereas lesion area remained very low for leaves treated with strain SS101 (Fig. 1c). For mutant 10.24, disease progress was intermediate between the control and the treatment with strain SS101. The population densities of wild-type strain SS101 and mutant 10.24 on treated leaves at 10 d after zoospore inoculation were 8.3 and 8.4 log CFU g^{-1} leaf, respectively.

Effect of massetolide A on late blight infections of tomato leaves

To further investigate the role of massetolide A in preventing late blight disease of tomato, massetolide A was purified from cell-free culture supernatant of strain SS101. RP-HPLC analysis revealed that, based on peak area (206 nm), massetolide A makes up, on average, 70% (ranging from 65 to 74%) of the purified extract from strain SS101 (Fig. 2a1). The other 30% of the extract is composed, for the most part (> 95%), of four additional cyclic lipopeptides (retention times 14–20 min, Fig. 2a1), three of which were identified by liquid chromatography/mass spectrometry (LC-MS) and nuclear

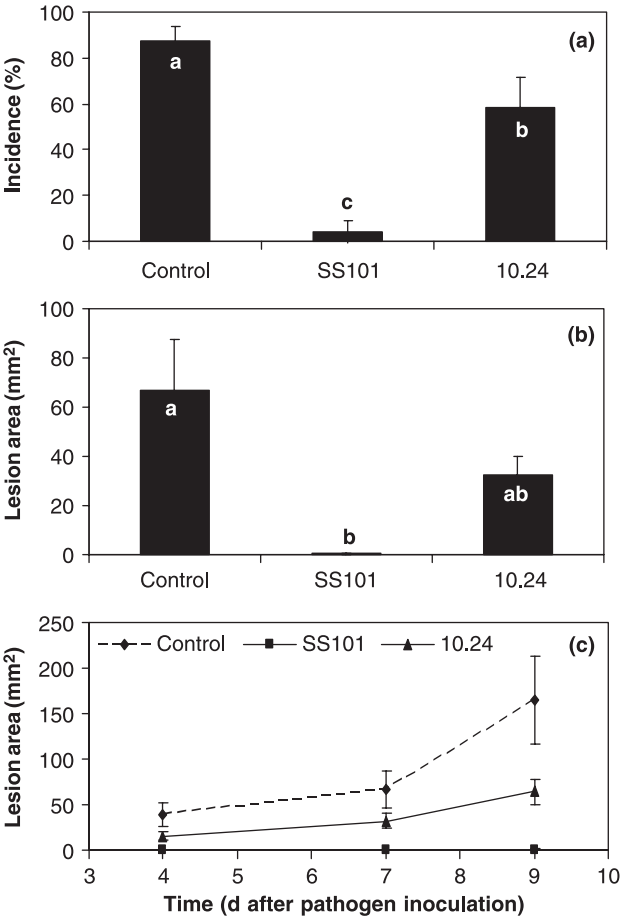

Fig. 1 Direct effect of *Pseudomonas fluorescens* SS101 and its massetolide A-deficient mutant 10.24 on tomato late blight. (a) Disease incidence refers to the percentage of zoospore droplets applied to tomato (*Lycopersicon esculentum*) leaves that lead to infection; (b) lesion area at 7 d after inoculation with zoospores of *Phytophthora infestans*; (c) disease severity (lesion area) at different time points after pathogen inoculation. Means ± SE of six replicates are given. Means with a different letter are significantly different ($P < 0.05$).

magnetic resonance (NMR) as derivatives of massetolide A (I. de Bruijn *et al.*, unpublished). The derivatives of massetolide A are most likely the result of the flexibility of the adenylation domains in amino acid selection and activation (Stachelhaus *et al.*, 1999). Massetolide A and its derivatives were absent in cell-free culture supernatant of *massA*-mutant 10.24 (Fig. 2a2).

The results of subsequent bioassays also showed that partially purified massetolide A significantly reduces disease incidence when applied to tomato leaves at a concentration of 100 μg ml^{-1}; no significant effects on disease incidence were observed at concentrations of 50 μg ml^{-1} (Fig. 2b1). Application of cell suspensions of strain SS101 was significantly more effective than application of massetolide A alone (Fig. 2b1), suggesting that, in addition to massetolide A, other bacterial traits are involved in late blight control by strain SS101. In the control treatment, lesion area increased exponentially over a period of 9 d after zoospore inoculation, whereas disease severity remained low for leaves treated with strain SS101 (Fig. 2b2); for leaves treated with different concentrations of massetolide A, disease progress and lesion areas at 9 d after pathogen inoculation (dpi) were intermediate between the control treatment and the SS101 treatment (Fig. 2b2). To provide further evidence for the role of massetolide A in the control of late blight of tomato, massetolide A was fractionated to purity and the structure was confirmed by LC-MS and NMR analyses (data not shown). The results of bioassays also showed that pure massetolide A significantly reduces disease incidence and lesion area when applied as a solution with a concentration of 100 μg ml^{-1} (equal to 88 μM) (Fig. 2c). No phytotoxic effects on the tomato leaves were observed after application of strain SS101, mutant 10.24, or massetolide A at the concentrations used.

High-performance liquid chromatography analysis of leaf extracts treated with a relatively high and easily detectable concentration (350 μM) of massetolide A revealed that, on average, 37% (± 7.8, $n = 3$) of the massetolide A is deposited on the tomato leaves directly after treatment. Based on the assumption that a similar fraction of massetolide A is deposited when leaves are treated with a solution of 44 μM (50 μg ml^{-1}) or 88 μM (100 μg ml^{-1}), the effective concentrations of massetolide A on the leaves are 16 and 32 μM, respectively. Given that massetolide A has zoosporicidal activity at concentrations of 22 μM or higher may explain, at least in part, the difference in biocontrol efficacy between the two concentrations of massetolide A used in the experiments (Fig. 2c). RP-HPLC analysis of extracts of tomato leaves harvested at 1 and 5 d after treatment did not allow *in situ* detection and quantification of massetolide A owing to interference of leaf-derived compounds that have similar retention times as massetolide A. Also, from leaves treated with cell suspensions of SS101, massetolide A could not be detected and quantified reliably because of background signals of compounds released from the tomato leaves during the extraction.

Effect of *P. fluorescens* SS101 and massetolide A on expansion of existing late blight lesions

To investigate if strain SS101 or massetolide A can reduce the development of existing late blight infections, tomato leaves with primary lesions of *P. infestans* were treated with cell suspensions of strain SS101, mutant 10.24, or with different concentrations of partially purified massetolide A. The results show that lesion area increased only threefold on leaves treated with strain SS101, whereas lesion area increased more than sevenfold in the control treatment (Fig. 3). On leaves treated with mutant 10.24, lesion area increased almost fivefold (Fig. 3b). Moreover, application of massetolide A to tomato leaves significantly reduced the growth of existing lesions in a concentration-dependent manner (Fig. 3b).

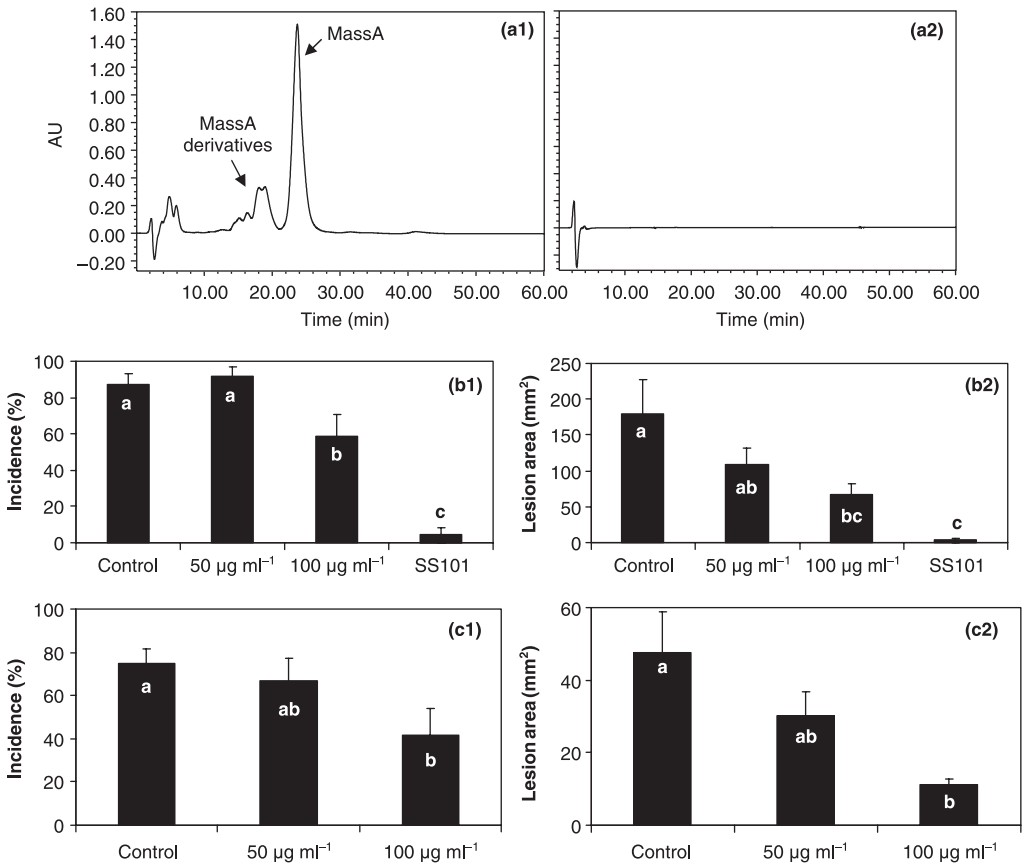

Fig. 2 (a1) Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram (206 nm) of the surfactant extract obtained from cell-free culture supernatants of *Pseudomonas fluorescens* SS101. The predominant peak with a retention time of 24 min was identified by liquid chromatography/mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) as massetolide A (MassA). The peaks with retention times ranging from 14 to 20 min represent at least four other cyclic lipopeptide surfactants, three of which were identified by LC-MS and NMR as derivatives of MassA. (a2) RP-HPLC chromatogram (206 nm) of the extract obtained from cell cultures of massetolide A-deficient mutant 10.24. (b, c) Direct effect of partially purified massetolide A and of HPLC-purified massetolide A on tomato late blight. Tomato (*Lycopersicon esculentum*) leaves were treated with strain SS101 or with different concentrations of partially purified massetolide A (b1, b2) or HPLC-purified massetolide A (c1, c2); 1 d later, leaves were inoculated with *Phytophthora infestans* zoospores. Disease incidence (b1, c1) and disease severity (lesion area) (b2, c2) at 9 d after pathogen inoculation. Means ± SE of six replicates are given. Means with different letters are significantly different ($P < 0.05$).

Induction of systemic resistance in tomato by *P. fluorescens* SS101 and massetolide A

To determine the role of induced resistance as a mechanism in late blight control by strain SS101 and massetolide A, two types of experiments were conducted to physically separate the inducing agents from the pathogen (Fig. S1). In one series of experiments, the inducing agents (bacterium or massetolide A) were applied to the lower leaf and the pathogen inoculated 1 d later on the upper leaves (Figs 4, 5). In the second series of experiments, the inducing agents were applied to roots of tomato seedlings and 2 wk later the tomato leaves were challenge-inoculated with *P. infestans* (Fig. S2). At the time disease severity was assessed, physical separation of strain SS101 and *P. infestans* was confirmed by dilution plating leaf suspensions onto agar media selective for the introduced bacterial strains. Both series of experiments showed that application of SS101 or massetolide A to leaves or roots significantly reduced lesion areas of tomato late blight, but did not reduce disease incidence (Fig. 4; Fig. S2). When applied to the lower leaf, mutant 10.24 was significantly less effective in reducing lesion area than wild-type SS101 or massetolide A (Fig. 4c). The results further showed that, also in the *nahG* transgene, lesion area was significantly reduced upon treatment of lower leaves with SS101 or massetolide A to values similar to that obtained in the wild-type progenitor tomato cv. Moneymaker (Fig. 5). These results suggest that induction of resistance in tomato against *P. infestans* by *P. fluorescens* SS101 or by massetolide A is independent of salicylic acid signalling.

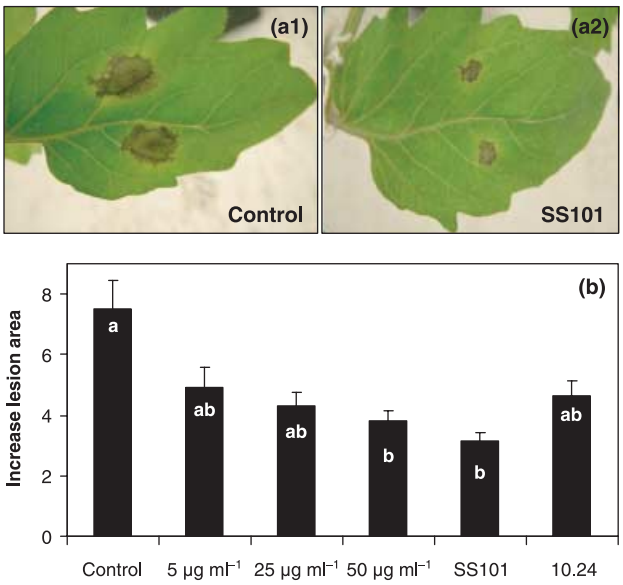

Fig. 3 Effect of *Pseudomonas fluorescens* SS101 on the growth of existing late blight lesions. Tomato (*Lycopersicon esculentum*) leaves were first inoculated with *Phytophthora infestans* zoospores. After the initial late blight lesions were formed, lesion areas were measured and leaves were subsequently treated with cell suspensions of *P. fluorescens* SS101, its massetolide A-deficient mutant 10.24 or with different concentrations of partially purified massetolide A. Five days later, lesion sizes were determined again and the increase in lesion area was calculated. (a2) Typical effect of *P. fluorescens* SS101 on the growth of existing lesions compared with the nontreated control (a1); (b) effect of SS101, 10.24 and different concentrations of massetolide A on the increase in lesion size. The means + SE of six replicates are shown. Means with a different letter are significantly different ($P < 0.05$).

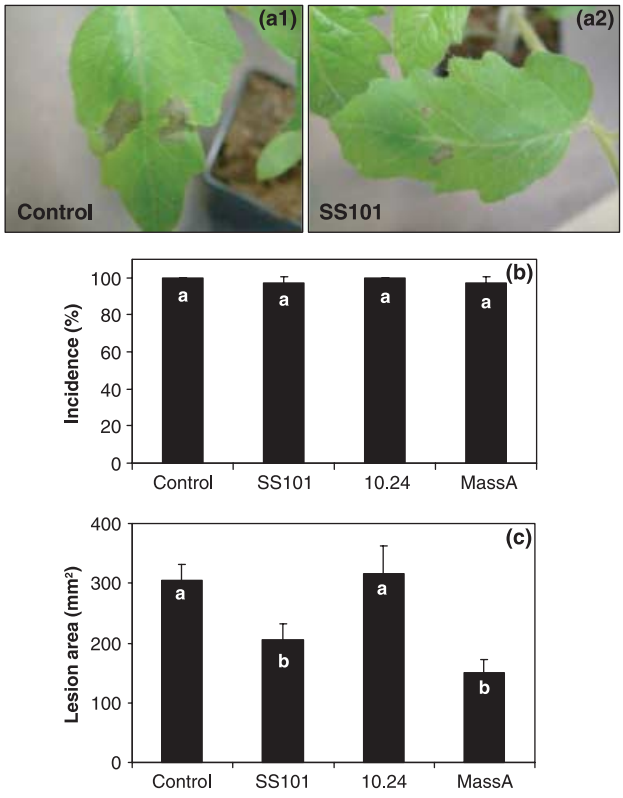

Fig. 4 Induced resistance in tomato against late blight by application of *Pseudomonas fluorescens* SS101 or massetolide A (MassA) to leaves. Twenty-four hours after treatment of the lower leaves of tomato (*Lycopersicon esculentum*) plants with strain SS101, mutant 10.24 or MassA (50 µg ml^{-1} (44 µM)), upper leaves were challenge-inoculated with zoospores of *Phytophthora infestans*. (a) Representative example of induced systemic resistance in tomato against late blight by strain SS101 applied to roots or leaves. At 7 d after pathogen inoculation, disease incidence (b) and lesion area (c) were determined. Means + SE of four replicates are given. Means with the same letter are not significantly different ($P < 0.05$).

Role of massetolide A in plant colonization by *P. fluorescens* SS101

The role of massetolide A in colonization of tomato plants was investigated by comparing the distribution and population dynamics of wild-type strain SS101 on the surface and in the interior of different plant parts with that of its *massA*-mutant 10.24. Wild-type strain SS101 and mutant 10.24 were applied separately to tomato seeds to a final density of 6.5 log CFU per seed each. When treated tomato seeds were sown in nonsterile potting soil, both SS101 and 10.24 colonized the surfaces of roots, stems and cotyledons of 14-d-old seedlings (Fig. 6a1). On the surfaces of roots and cotyledons of tomato seedlings, strain SS101 established significantly higher densities than mutant 10.24 (Fig. 6a1). Neither strain SS101 nor mutant 10.24 could be recovered from the interior tissue of roots and stem, but were present in the interior of cotelydons at a density of approximately 10^3–10^4 CFU g^{-1} (Fig. 6a2), which is approximately five- to 20-fold lower than the density found on the surface of the cotyledons (Fig. 6a1). After 44 d of plant growth, strain SS101 and mutant 10.24 were still detectable on the surfaces of roots and cotyledons, although their densities on the root surface had declined by approximately 1000-fold compared with their densities on roots of 14-d-old tomato seedlings (Fig. 6a1,b1). SS101 maintained its density on the surface of cotelydons to a value that was significantly higher than that of mutant 10.24 (Fig. 6b1). Strain SS101 and mutant 10.24 were not detectable on the surfaces and in the interior of stem, and on and in true leaves infested with *P. infestans* (Fig. 6b1, b2).

Effect of seed treatments on late blight incidence, lesion size and sporangia formation

In the same experiment used to study plant colonization from treated seeds (Fig. 6), the effects of strain SS101 and mutant 10.24 on late blight incidence, lesion area and sporangia formation were determined (Fig. 7). True leaves of 35-d-old tomato plants raised from bacteria-treated seeds were inoculated

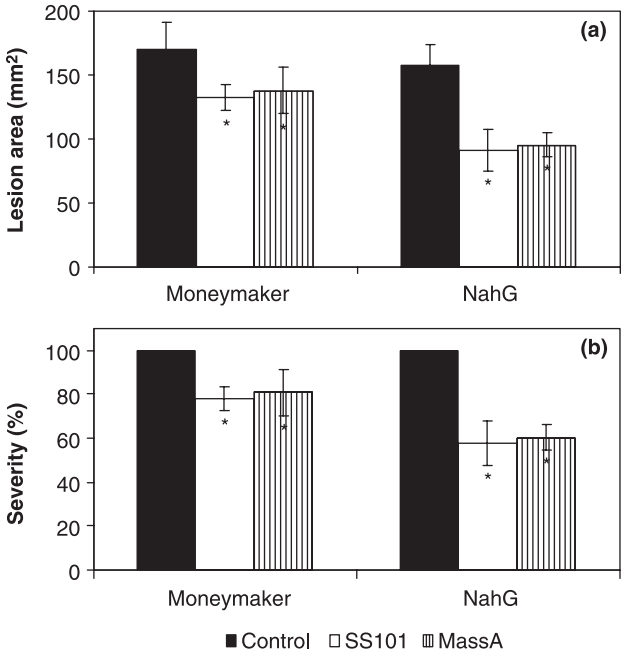

Fig. 5 Induced resistance in tomato against late blight by application of *Pseudomonas fluorescens* SS101 or massetolide A to leaves of tomato (*Lycopersicon esculentum*) cv. Moneymaker and its transgenic derivative *nahG*. Twenty-four hours after treatment of the lower leaves of the tomato plants with strain SS101 or massetolide A (MassA, 50 μg ml^{-1}), upper leaves were challenge-inoculated with zoospores of *Phytophthora infestans*. At 7 d after pathogen inoculation, lesion area (a) was determined. (b) The lesion areas in the SS101 and MassA treatments were expressed as a percentage of the lesion areas in the control treatments (set at 100%). Means ± SE of six replicates are given. An asterisk indicates a statistically significant reduction in disease severity ($P < 0.05$) relative to the control.

with *P. infestans* zoospores and disease incidence and severity assessed 9 d later. The results showed that treating tomato seeds with strain SS101 or mutant 10.24 did not reduce disease incidence (Fig. 7a). Strain SS101 significantly reduced lesion area, whereas mutant 10.24 reduced lesion areas to values that were intermediate between the control and the SS101 treatments (Fig. 7b). Strain SS101 significantly reduced sporangia formation per unit of lesion area, whereas mutant 10.24 gave an intermediate reduction (Fig. 7c). Given that the applied bacterial strains, SS101 and 10.24, could not be detected on the true leaves infested with zoospores of *P. infestans* (Fig. 6), these results indicate that the biocontrol effect of SS101 applied to tomato seeds is most likely mediated through systemic resistance elicited by the bacteria.

Discussion

This study shows that *P. fluorescens* strain SS101 not only prevented infection of tomato leaves by *P. infestans*, but also significantly reduced expansion of existing late blight infections and sporangia formation. This is in contrast to the results of previous studies on biocontrol of late blight, where the bacterial strains tested were effective mostly in preventing infections (Daayf *et al.*, 2003; Lourenço Júnior *et al.*, 2006). To date, biological control of plant diseases is mostly directed toward preventing infection of plants by pathogens, and only a few studies (Molina *et al.*, 2003) have addressed the effects of biocontrol agents on plants already infected by pathogenic bacteria, fungi or Oomycetes. Given that sporangia constitute an important primary and secondary inoculum source for *P. infestans*, the adverse effects of *P. fluorescens* strain SS101 on both lesion area and sporangia formation may lead to a reduction in disease development and epidemic progress of late blight of tomato.

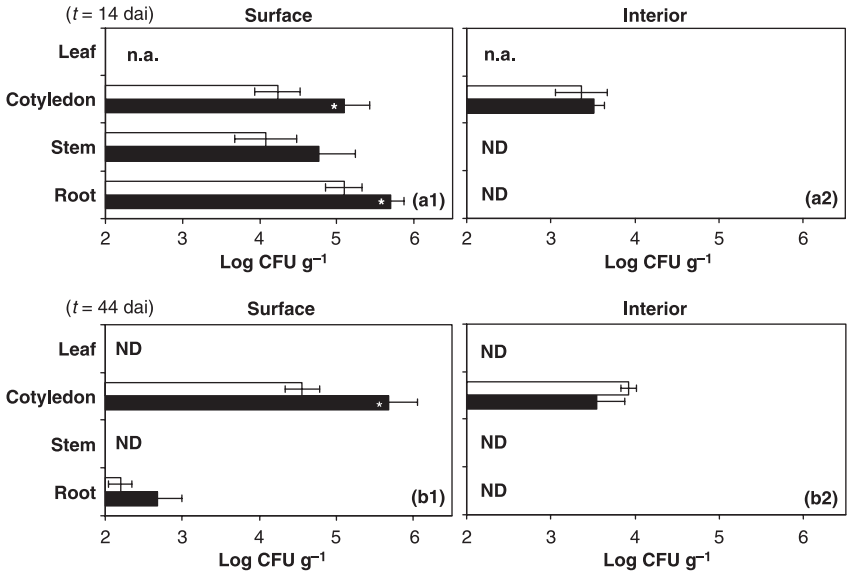

Fig. 6 Colonization of tomato (*Lycopersicon esculentum*) plants by *Pseudomonas fluorescens* SS101 and its massetolide A-deficient mutant 10.24. Tomato seeds were treated with SS101 (closed bars) or 10.24 (open bars) at a final density of 10^6 CFU per seed and sown in soil. After 14 d (a1, a2) and 44 d (b1, b2) of plant growth after inoculation (dai), population densities of the applied bacterial strains were determined on the surface and in the interior of roots, stem, cotelydons and on true leaves infested with *Phytophthora infestans*. The means ± SE of four replicates are shown. For each pair of bars, an asterisk indicates a significant difference ($P < 0.05$). n.a, not available; ND, not detectable (detection limit is log 2.0 CFU g^{-1}).

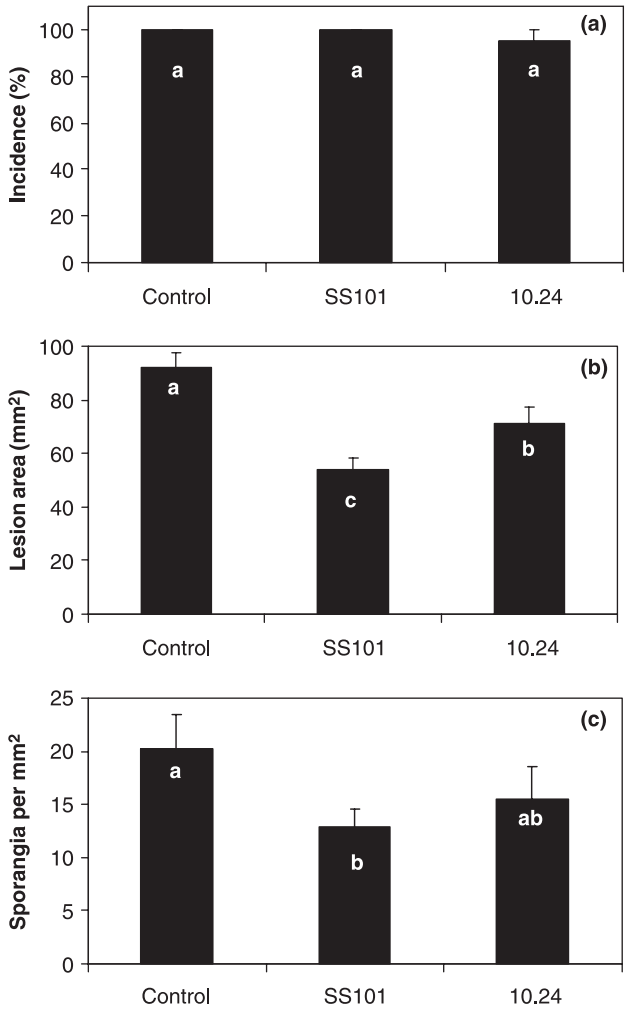

Fig. 7 Effect of seed treatments on late blight infection of tomato and on sporangia production by *Phytophthora infestans*. Tomato (*Lycopersicon esculentum*) seeds were treated with cell suspensions of *Pseudomonas fluorescens* SS101 or its massetolide A-deficient mutant 10.24 and sown in potting soil. After 5 wk of plant growth, tomato leaves were inoculated with zoospores of *P. infestans*. Disease incidence (a), lesion area (b) and the number of sporangia formed per unit of lesion area (c) were determined 9 d later. Means + SE of six replicates are shown. Means with a different letter are significantly different ($P < 0.05$).

The results also show that the cyclic lipopeptide surfactant massetolide A is an important component of the biocontrol activity of *P. fluorescens* SS101 against late blight of tomato. This conclusion is based on the observations that: (i) massetolide A-deficient mutant 10.24 was significantly less effective in biocontrol than the wild-type strain SS101 (Figs 1, 3, 4, 7); and (ii) application of purified massetolide A to tomato leaves and roots provided significant control of *P. infestans* (Figs 2–5, Fig. S2). Over the past decade, cyclic lipopeptides (CLPs) produced by *Pseudomonas* and *Bacillus* species have received considerable attention for their activity against a range of microorganisms, including mycoplasmas, trypanosomes, bacteria, fungi, viruses and Oomycetes (reviewed in Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006). In most of these studies, however, the antimicrobial effects of the CLPs were tested *in vitro* only and most biocontrol assays with plants did not include mutants deficient in CLP-biosynthesis. Work by Bais *et al.* (2004) was one of the first studies that included a mutant of *B. subtilis* strain 6051 defective in surfactin production and demonstrated that the wild-type strain was more effective in controlling root infection of Arabidopsis by *P. syringae* than the surfactin-deficient mutant. Another line of evidence that CLPs are important determinants of biocontrol activity was provided by Leclère *et al.* (2005), who showed that a derivative of the *B. subtilis* strain BBG100 that overproduces the CLP mycosubtilin showed increased activity against *Pythium* on tomato seedlings. The present study further extends these findings and provides, for the first time, evidence that the CLP massetolide A is an important component of the biocontrol activity of *P. fluorescens* strain SS101.

In several of the experiments described in this study, extractions were performed on tomato leaves treated with cell suspensions of strain SS101 to determine the concentrations of massetolide A produced *in situ* by the applied bacterial strain. Nielsen & Sørensen (2003) showed that on sugar beet seeds, *P. fluorescens* strains produce massetolide A-like CLPs at concentrations ranging from 0.2 to 0.6 μg per seed. In the analyses performed in our study, however, relatively low concentrations of massetolide A could not be quantified accurately because of interference of plant-derived compounds. Therefore, it is not clear from our study if the concentrations of purified massetolide A applied to tomato leaves (Fig. 2) are representative of the massetolide A concentrations produced by strain SS101 *in situ*. To improve detection and quantification of massetolide A, antibody-based detection will be explored in future studies. Immunological detection has been successfully adopted for *in situ* detection of syringopeptins: the competitive ELISA assay appeared to be approx. 100 times more sensitive than HPLC analysis and did not require extraction of plant material with organic solvents (Fogliano *et al.*, 1999). Antibodies will also be highly instrumental to study the localization, fate and stability of the massetolide A applied to plant tissues or produced by *P. fluorescens* SS101 *in situ*.

Previous studies by De Souza *et al.* (2003) have shown that massetolide A disrupts zoospore membranes at concentrations of 25 μg ml^{-1} and higher, leading to lysis of entire zoospore populations within 1 min of exposure. This zoosporicidal activity may explain, at least in part, the direct protection of tomato leaves against infection by zoospores of *P. infestans* (Figs 1, 2), but does not explain the suppressive effects of SS101 or massetolide A on lesion growth and sporangia formation. Subsequent assays in which strain SS101 or massetolide A were physically separated from the pathogen (Figs 4, 5, Fig. S2) demonstrated that induction of systemic resistance in tomato against late blight constitutes a main mode of the indirect activity against late blight. This was confirmed in

assays with tomato plants raised from seeds treated with strain SS101 (Fig. 7). The observation that mutant 10.24 also reduced disease severity significantly but, in general, to a lesser extent than wild-type strain SS101, indicates that bacterial determinants other than massetolide A also play a role in induced systemic resistance in tomato by strain SS101.

Induced systemic resistance (ISR) is a common phenomenon among multiple strains of antagonistic bacteria representing various genera, including *Pseudomonas* and *Bacillus* (van Loon *et al.*, 1998; Kloepper *et al.*, 2004). Bacterial determinants shown to be involved in induction of resistance in plants by *Pseudomonas* and *Bacillus* strains include lipopolysaccharides (Leeman *et al.*, 1995), flagellin (Gomez-Gomez & Boller, 2002; Zipfel *et al.*, 2004; Meziane *et al.*, 2005), siderophores (Leeman *et al.*, 1996), salicylic acid (De Meyer & Höfte, 1997), pyocyanin (De Vleesschauwer *et al.*, 2006), an N-alkylated benzylamine derivative (Ongena *et al.*, 2005b), 2,4 diacetylphloroglucinol (Lavicoli *et al.*, 2003), the volatiles 2,3-butanediol and acetoin (Ryu *et al.*, 2004), and *N*-acylhomoserine lactones (Schuhegger *et al.*, 2006). Han *et al.* (2006) suggested that possibly multiple other bacterial determinants, different from those identified so far, are involved in the induction of systemic resistance. The results of our study show, for the first time, that the cyclic lipopeptide massetolide A is a bacterial determinant of induced resistance in tomato by a saprophytic *P. fluorescens* strain. Studies by Kováts *et al.* (1991), Yan *et al.* (2002) and Doke *et al.* (1987) suggested that the induced defence responses in tomato and potato against *P. infestans* operate in part by adverse effects on encystment or attachment of zoospores or sporangia to the plant surface. The results of our study, however, showed that the systemic protection induced by strain SS101 or massetolide A did not reduce initial infection of tomato leaves by zoospores of *P. infestans*, but limited growth of the pathogen in the leaves leading to smaller lesions and reduced sporangia formation (Fig. 7, Fig. S2). Whether structurally different CLPs produced by strain SS101 or other *Pseudomonas* species also induce resistance in plants against *P. infestans* or other pathogens is as yet unknown and is currently being investigated. Work by Ongena *et al.* (2005a) suggested that fengycins, CLPs produced by *Bacillus subtilis*, could be involved in eliciting induced resistance, whereas the structurally different CLP mycosubtilin most likely does not have resistance-inducing activities (Leclère *et al.*, 2005). It should be emphasized, however, that the capacity of specific bacterial determinants to induce resistance may be highly dependent on the host–pathogen system tested, as was demonstrated by Meziane *et al.* (2005) and De Vleesschauwer *et al.* (2006).

In many cases, signal transduction in rhizobacteria-mediated ISR has been shown to be independent of salicylic acid (SA), and dependent upon ethylene (ET) and jasmonic acid (JA) (Pieterse *et al.*, 1998; Ton *et al.*, 2001; Yan *et al.*, 2002). Our results with *nahG*, the transgenic derivative of cv. Moneymaker, suggest also that the systemic resistance induced in roots or leaves by *P. fluorescens* SS101 or massetolide A is independent of SA (Fig. 5). Bioassays with *Def-1*, a JA-deficient mutant (Howe *et al.*, 1996), and with *Never Ripe*, a mutant deficient in ethylene signalling (Lanahan *et al.*, 1994), gave inconclusive results (data not shown). Future studies, involving northern and microarray-based analyses, will be necessary for a more comprehensive identification of the signalling pathways, including ET and JA, involved in the resistance responses induced by CLPs.

Cyclic lipopeptide surfactants not only have zoosporicidal, antimicrobial and ISR-eliciting activities, but have been postulated to play other important roles for the producing microorganisms, including attachment and detachment to surfaces, biofilm formation, and colonization of plant tissue (Lindow & Brandl, 2003; Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006). Several studies have shown that CLPs produced by *Pseudomonas* species are important in motility on soft agar media (Andersen *et al.*, 2003; Roongsawang *et al.*, 2003; De Bruijn *et al.*, 2007). The involvement of CLPs in bacterial motility may provide an advantage in colonization of plant tissue, in translocation from an inoculum source to new and more nutrient-rich niches on the plant surface, and in containment of plant pathogens (Andersen *et al.*, 2003). The results of our study showed that wild-type strain SS101, when applied to seeds, established significantly higher densities on roots and cotelydons of tomato seedlings than its CLP-deficient mutant 10.24 (Fig. 6). The presence of the introduced bacterial strains on and in cotelydons, but not on and in true leaves of tomato plants, is most likely the result of passive colonization/contamination of the cotyledons during germination of the bacteria-treated seeds and subsequent seedling emergence (Raaijmakers *et al.*, 1995). These results indicate that massetolide A contributes to colonization of tomato plants by *P. fluorescens* SS101 and extend the findings of Nielsen *et al.* (2005), who showed that the CLP amphisin produced by *Pseudomonas* sp. strain DSS73 is an important trait in colonization of sugar beet seeds and roots.

In conclusion, the results of this study showed that the CLP surfactant massetolide A is a metabolite with versatile functions in the ecology of producing strain *P. fluorescens* SS101 and with potential as a supplementary measure in the control of late blight.

Acknowledgements

The first and second authors of this paper contributed equally to the initiation and setup of this study. This work is sponsored by the Vietnamese Ministry of Education and Training (MOET) through project 322, by the German Academic Exchange Service (DAAD) and by the Dutch Graduate School of Experimental Plant Sciences (EPS). We thank Evelyn van Heese for her input in the plant assays, Dr Teris van Beek and Pieter de Waard from Wageningen University (the Netherlands) for

the LC-MS and NMR analyses, and Dr Renier van den Hoorn for the schematic drawings of the tomato plants. We are grateful to Prof. Pierre de Wit and Dr Jan van Kan for critically reading the manuscipt and their valuable suggestions and discussions.

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Schematic presentation of two experimental setups used to determine the role of *Pseudomonas fluorescens* SS101, mutant 10.24 or massetolide A in induction of systemic resistance in tomato against *Phytophthora infestans*. (a) 24 h after treatment of the lower leaves of the tomato plants with wild-type strain SS101, mutant 10.24, or massetolide (MassA, 50 μg ml^{-1}), upper leaves were challenge-inoculated with zoospores of *P. infestans*. Disease severity (lesion area) was determined 4 and 7 d after pathogen inoculation. (b) Roots of tomato seedlings were treated with strain SS101, mutant 10.24 or massetolide A (50 μg ml^{-1}), transplanted and grown for 2 wk under controlled conditions; then leaves were challenge-inoculated with zoospores of *P. infestans* and disease severity was determined 4 and 7 d after pathogen inoculation.

Fig. S2 Induced resistance in tomato against late blight by application of *Pseudomonas fluorescens* SS101 or massetolide A to roots of tomato seedlings. Two weeks after root treatment, tomato leaves were challenge-inoculated with zoospores of *Phytophthora infestans*. Results of two experiments are presented. (a1, a2) Results from Expt 1 (comparison of wild-type SS101 and massetolide A-deficient mutant 10.24); (b1, b2) results from Expt 2 (comparison of wild-type SS101 and partially purified massetolide A (50 μg ml^{-1})). Means + SE of six replicates are given. Means with the same letter are not significantly different ($P < 0.05$).

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