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# Characterization of Cichopeptins, New Phytotoxic Cyclic Lipodepsipeptides Produced by *Pseudomonas cichorii* SF1-54, and Their Role in Bacterial Midrib Rot Disease of Lettuce

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**Abstract**

The lettuce midrib rot pathogen *Pseudomonas cichorii* SF1-54 produces seven bioactive compounds with biosurfactant properties. Two compounds exhibited necrosis-inducing activity on chicory leaves. The two phytotoxic compounds, named cichopeptin A and B, are related cyclic lipopeptides composed of an unsaturated C12-fatty acid chain linked to the N-terminus of a 22 amino-acid peptide moiety. Cichopeptin B differs from cichopeptin A only in the last C-terminal amino acid residue, which is Val instead of Leu/Ile. Based on peptide sequence similarity, cichopeptins are new cyclic lipopeptides related to corpeptin, produced by the tomato pathogen *Pseudomonas corrugata*. Production of cichopeptin is stimulated by glycine betaine but not by choline, an upstream precursor of glycine betaine. Furthermore, a gene cluster encoding cichopeptin synthethases, *cipABCDEF*, is responsible for cichopeptin biosynthesis. A *cipA*-deletion mutant exhibited significantly less virulence and rotten midribs than the parental strain upon spray inoculation on lettuce. However, the parental and mutant strains multiplied in lettuce leaves at a similar rate. These results demonstrate that cichopeptins contribute to virulence of *P. cichorii* SF1-54 on lettuce.

## Introduction

Some plant pathogenic *Pseudomonas* spp., such as *Pseudomonas syringae* pv. *syringae*, *Pseudomonas fuscovaginae* and *Pseudomonas corrugata* produce necrosis-inducing lipopeptide phytotoxins (Flamand et al., 1996; Bender et al., 1999; Catara, 2007) which belong to either the syringomycin group or the phytotoxic lipodepsipeptide group including tolaasin and syringopeptin-like compounds (Raaijmakers et al., 2006; Gross and Loper, 2009). The members of the syringomycin group are cyclic depsinonapeptides attached to a β-hydroxy fatty acid tail. Tolaasin and syringopeptin-like compounds are cyclic lipodepsipeptides with a longer and more hydrophobic peptide chain and a higher molecular weight (Gross and Loper, 2009). These two groups of cyclic lipopeptides (CLPs) cause cellular lysis by formation of transmembrane pores in the plasma membranes of host cells that lead to disruption of the membrane electrical potential (Brodey et al., 1991; Hutchison et al., 1995; Coraiola et al., 2008). Although these CLPs can induce necrosis on plants, phytotoxic CLPs contribute to virulence but are not essential for pathogenicity of plant pathogenic pseudomonads (Bender et al., 1999; Scholz-Schroeder et al., 2001).

Biosynthesis of CLPs is accomplished via nonribosomal biosynthetic pathways through large multienzyme complexes, nonribosomal peptide synthethases (NRPSs), which are encoded by large gene clusters. NRPSs have a modular structure in which each module is responsible for the incorporation of one amino acid in the peptide chain. A module consists of a minimal set of three domains: an adenylation domain (A), a thiolation domain or peptidyl carrier protein domain (T or PCP) and a condensation domain (C) (Bender et al., 1999; Raaijmakers et al., 2010). Based on the specificity code in the sequence of the A-domains of the NRPS modules, the amino acid sequence of the synthesized CLP can be predicted *in silico* (Stachelhaus et al., 1999; Challis et al., 2000; de Bruijn et al., 2007; Gross et al., 2007).

*Pseudomonas cichorii* is an important plant pathogenic bacterium, with a broad host range and a worldwide distribution. *P. cichorii* causes necrotic leaf and stem lesions on many economically important hosts including lettuce, celery, chrysanthemum, tomato, coffee and soybean (Jagger, 1914; Wilk and Dye, 1974; Jones et al., 1984; Bradbury, 1986; Yu and Lee, 2012). In Belgium, *P. cichorii* causes midrib rot disease of greenhouse-grown butterhead lettuces (Cottyn et al., 2009; Cottyn et al., 2011; Pauwelyn et al., 2011). Symptom development on lettuce leaves is strongly associated with apoptosis-like programmed cell death (Kiba et al., 2006; Kiba et al., 2009). Intriguingly, the type three secretion system is essential for pathogenicity of *P. cichorii* on eggplant, but not on lettuce (Hojo et al., 2008; Kajihara et al., 2012). Production of phytotoxic substances by *P. cichorii* has been described (Shirakawa and Ozaki, 1993; Hu et al., 1998; Lazzaroni et al., 2003; Pauwelyn et al., 2013), but their role in pathogenicity of *P. cichorii* on lettuce is not clear.

*P. cichorii* SF1-54, which was isolated from diseased butterhead lettuce in Belgium, can produce seven bioactive compounds (named A to G) with biosurfactant properties (Pauwelyn et al., 2013). These bioactive compounds differ in inhibition spectra against different microorganisms and in the ability to cause necrosis on chicory leaves (summarized in supplementary Table S1). Compounds D and E, which have been well identified, characterized, and renamed cichofactins A and B, are new linear lipopeptides of the syringafactin family and are involved in virulence and surface motility, while compounds B and C appear to be related to pseudomycins (Pauwelyn et al., 2013). Compounds F and G are phytotoxic molecules and induce necrosis on chicory leaves. Moreover, they have antimicrobial activity against *Bacillus megaterium* but not against *Rhodotorula mucilaginosa*. In this study, the structure of the phytotoxic compounds F and G produced by *P. cichorii* SF1-54 was elucidated. A dual approach, which combined chemical characterization by high performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-

MS) and mining the NRPS encoding genes in the SF1-54 genome, was used. Furthermore, a mutant deficient in production of cichopeptins was constructed to study the involvement of the phytotoxins in virulence of *P. cichorii* SF1-54. Our results demonstrate that the phytotoxic CLPs are important virulence factors for *P. cichorii* SF1-54 on lettuce. Interestingly, we also found that production of phytotoxic CLPs by *P. cichorii* SF1-54 is linked to glycine betaine metabolism.

## Results

### Chemical analysis of phytotoxic lipopeptides produced by *P. cichorii* SF1-54 (cichopeptins)

Compounds F and G were purified by reverse-phase high performance liquid chromatography (RP-HPLC). Based on their phytotoxicity and antimicrobial activity against *B. megaterium* but not against *R. mucilaginosa* (supplementary Table S1), compounds F and G were expected to be new phytotoxic lipodepsipeptides because corpeptin and syringopeptin have similar properties (Lavermicocca et al., 1997; Emanuele et al., 1998).

To structurally characterize the phytotoxic metabolites, HPLC-purified compounds F and G were first submitted to total acid hydrolysis and subsequent HPLC separation of derivatized amino acids. With this methodology, it was not possible to establish a precise quantification of the various amino acids but 10 different residues with high relative amounts of Ala and Val, more than one Gly, one residue of Ser, Thr, Leu and Ile as well as three unidentified amino acids were detected in the purified sample of the molecule.These first results confirm the peptidic nature of compounds F and G which were further purified by HPLC-ESI-MS and characterized by ESI-MS-MS. The mass spectrum of purified extract of compounds F and G showed two major peaks at m/z 1026.1 and 1033.1, both doubly charged protonated forms ($[M+2H]^{2+}$) like syringopeptins (Monti et al., 2001; Grgurina et al., 2005).

Based on the *m/z* of the doubly charged protonated ions, the molecular weights of compounds F and G were calculated as 2052.4 and 2066.2 (exact mass measurements), respectively. Further characterization of the two molecules was obtained by selecting each ion and fragmenting it with Argon. Identification of b and y" ions reveals very similar fragmentation patterns of the $[M+H]^+$ ions corresponding to compounds F and G (supplementary Fig. S1 and Table S2).

The first b-ion fragments of compounds F and G were identical and compatible with 2,3-dehydroaminobutyric acid (dhAbu) and a fatty acid moiety, which is expected to be a $C_{12:1}$-OH residue upon calculation. Thus, the molecular weight difference of 14 between compounds F (MW 2052.4) and G (MW 2066.2) can be attributed to a difference in the peptide chain rather than in the fatty acid chain. The first twelve b ions were the same in the fragmentation spectra of compounds F and G indicating a structural identity of the N-terminal part of both molecules which includes the fatty acid moiety linked to at least twelve amino acid residues. Moreover, the masses of the y"-ion fragments obtained from compound G are always 14 Da higher than those of compound F (supplementary Table S2 and Fig. S1). This difference was maintained until the smallest y"-ions that could be detected at *m/z* 459.26 for compound F and at *m/z* 473.27 for compound G. This indicated that the amino acid substitution explaining the 14 Da difference between compounds F and G should occur within the first five residues (see below) at the C-terminal end. Further fragmentation of this part of the molecules was not obtained, most probably due to the fact that those residues are involved in the formation of an internal cycle as occurring in many CLPs (Raaijmakers et al., 2006; 2010). Nevertheless, based on those ESI-MS fragmentation data, the putative amino acid sequence of the first seventeen amino acids for both compounds F and G could be determined as dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile-Gly-Ala-Val-Ala-Val-dhAbu. Additionally, according to the spectrophotometric characteristics of both compounds (UV-

visible spectrum obtained in diode array-coupled HPLC analysis, data not shown), no aromatic amino acids are present in the peptide backbone. The proposed peptide structure of the fatty acid chain and the first seventeen amino acids of the peptide chain, with their site of cleavage yielding b- and y"-type ions, are presented in supplementary Fig. S2.

The complete peptide sequences of the two lipopeptides F and G could not be fully elucidated from these MS-MS analyses due to the high stability of the cyclic sub-structure involving the last five amino-acids. Multiple attempts to open the ring by chemical break down of the lactone bond (KOH 2M at 37°C for up to 4 hours; triethylamine 14.28%, pH = 9, 37°C, 4 hours or room temperature overnight) were not successful.

However, based on molecular weights, on amino acid composition and on previously reported structures of *Pseudomonas* lipopeptides, the remaining part at the C-terminal region of compound G could be composed of five amino acid residues, which could be Ala, Leu (or Ile), Ser, *a*Thr and an unknown amino acid. This suggests compounds F and G are structural relatives of corpeptins (Emanuele et al., 1998). Therefore, we renamed compounds G and F cichopeptins A and B, respectively, and next wanted to use an *in silico* approach to determine full peptide sequences.

**Sequence analysis of cichopeptin synthetases-encoding genes**

Since genes responsible for biosynthesis of phytotoxic CLPs in *P. cichorii* have not been reported, we performed genome mining based on the genes of syringopeptin synthetases (Scholz-Schroeder et al., 2003; Feil et al., 2005). First, the genome sequence of *P. cichorii* SF1-54 (Pauwelyn et al., 2013) was compared with the genomes of *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 (Buell et al., 2003; Feil et al., 2005) using the MAUVE program (Darling et al., 2010). Then, BlastN searches using the syringopeptin synthetase gene cluster of *P. syringae* pv. *syringae* B301D (*sypA*, *sypB* and *sypC*:

AF286216.2) (Scholz-Schroeder et al., 2003) as a query against the SF1-54 genome were performed. This resulted in the identification of a large NRPS gene cluster, spanning a region of 73,057 bp in the *P. cichorii* SF1-54 genome. The sequence, including the NRPS gene cluster and flanking regions, has been submitted to the Genbank database under accession number KJ513094. The structural organization of this NRPS gene cluster and its flanking genes is represented in Fig. 1.

Six NRPS-encoding open reading frames (ORFs) were identified in this gene cluster by the program Glimmer (Delcher et al., 1999). They are referred to as *cipA, cipB, cipC, cipD, cipE* and *cipF.* These six genes, which are 14.71, 9.64, 4.62, 13.01, 9.42 and 21.53 kb in size, respectively, encode NRPS modules with specific condensation (C), adenylation (A), and thiolation (T) domains (Fig. 1). These genes constitute together 22 NRPS modules, which confirms that the lipopeptide produced by *P. cichorii* SF1-54, is composed of 22 amino acids. Hence, the results of sequence analysis strongly suggest that the *cip* gene cluster is responsible for cichopeptin biosynthesis. The *cip* gene cluster exhibits two typical features of NRPS genes responsible for biosynthesis of lipopeptides: the presence of an N-terminal C domain in the first module (C-starter) and a tandem of thioesterase domains at the end of the *cip* cluster. Moreover, Blast analysis of cichopeptin synthetases identified that the recently released genome sequence of *P. cichorii* JBC1 has a very similar gene cluster (PCH70_25300, PCH70_25310, and PCH70_25320, the accession number CP007039.1) encoding hypothetical peptide synthetases. At the amino acid sequence level, cichopeptin synthetases showed 99% protein identity with these hypothetical peptide synthetases of *P. cichorii* JBC1.

**Analysis of cichopeptin synthetases**

To predict the peptide sequence of cichopeptins from genome sequencing data, bioinformatics analysis of the A-domain of each module of cichopeptin synthetases was

performed. The putative peptide sequence of cichopeptins is Thr/dhAbu-Pro-Ala-Ala-Ala-Ala-Val-Thr/dhAbu-Gly-Val-Ile-Gly-Ala-Val-Ala-Val-Thr/dhAbu-Thr/dhAbu-Ala-Dab-Ser-Leu/Ile/Val (supplementary Table S3). According to the biochemically determined peptide sequence of cichopeptins, the A-domains of the first, eighth, and seventeenth modules are responsible for specific recognition of dhAbu, and the eighteenth module recognizes *a*Thr. The prediction is in full agreement with the peptide sequence determined by mass spectrometry. Furthermore, it confirms that the unidentified C-terminal region of cichopeptins consists of five amino acid residues. Interestingly, the last module CipF is predicted to recognize three amino acids, Leu, Ile, and Val. The difference in molecular weight between Leu/Ile and Val is 14, and that is exactly in agreement with the molecular weight difference between cichopeptin A and B. The peptide sequences of the last five amino acid residues are *a*Thr-Ala-Dab-Ser-Leu/Ile (cichopeptin A) and *a*Thr-Ala-Dab-Ser-Val (cichopeptin B). The calculated masses of the C-terminal fragments of cichopeptins A and B are 18 Da higher than the masses determined by HPLC-ESI-MS (supplementary Table S2), indicating that one water molecule is lost for cyclization, confirming that cichopeptins A and B are CLPs with a C-terminal pentapeptide ring.

In *Pseudomonas*, dual condensation/epimerization (C/E) domains are responsible for generating D-amino acids into lipopeptides (Balibar et al., 2005; Rausch et al., 2007). As Gly and dhAbu are non-chiral monomers, the D/L configuration of other amino acid residues of cichopeptins was investigated by detection of specific signatures via weblogos within DownSeq of each C-domain (Caradec et al., 2014). According to the signatures, the C-domains of modules 3-8, 11-12, 14-17, and 22 are identified as dual C/E domains. Therefore, the peptidic structures of cichopeptins A and B are dhAbu_D-Pro_D-Ala_D-Ala_D-Ala_D-Ala_D-Val_-dhAbu_Gly_D-Val_D-Ile_Gly_D-Ala_D-Val_D-Ala_D-Val_dhAbu_D-*a*Thr_L-Ala_L-Dab_D-Ser_L-Leu/Ile and dhAbu_D-Pro_D-Ala_D-Ala_D-Ala_D-Ala_D-

Val_dhAbu_Gly_D-Val_D-Ile_Gly_D-Ala_D-Val_D-Ala_D-Val_dhAbu_D-*a*Thr_L-Ala_L-Dab_D-Ser_L-Val, respectively. The locations of dual C/E domains of cichopeptin synthetases are identical to those of syringopeptin synthetases according to *in silico* analysis. Furthermore, the specific signature detection confirms that the first C domain is a C-starter allowing the condensation with the lipidic moiety.

**Analysis of the *cip* flanking sequences**

Immediately downstream of the *cip* gene cluster encoding the cichopeptin synthetases, an ABC transporter system (99% protein identity with *P. cichorii* JBC) as well as a LuxR transcriptional regulator (100% and 57% protein identity with *P. cichorii* JBC1 and *P. syringae* pv. *japonica* M301072PT, respectively) have been identified in the genome of *P. cichorii* SF1-54 (illustrated in Fig. 1). The peptide sequences of the two ABC-transporter components showed 72% protein identity with the macrolide efflux protein encoded by *macA* of the orfamide gene cluster of *Pseudomonas fluorescens* Pf-5 (PFL_2148) and 81% protein identity with the macrolide efflux protein encoded by *macB* of the syringopeptin gene cluster of *P. syringae* pv. *syringae* B728a (Psyr_2618), respectively. Upstream of the cichopeptin synthetase genes, three genes are present that encode proteins exhibiting amino acid homology of 100% to proteins encoded by PCH70_25250, PCH70_25260, and of 99% to PCH70_25270-encoded protein of *P. cichorii* JBC1. These three proteins also showed homology of 65% to PseA, 60% to PseB and 72% to PseC. The PseABC efflux system is a tripartite resistance-nodulation-cell division (RND) transporter system, identified at the left border of the *syr-syp* genomic island of *P. syringae* pv. *syringae* B301D with an important role in secretion of syringomycin and syringopeptin (Kang and Gross, 2005). The gene located between the cichopeptin biosynthesis genes and the genes encoding the PseABC

efflux system shows 56% amino acid homology with a putative membrane protein of *P. corrugata*.

**Cichopeptin production is stimulated by glycine betaine**

Culture filtrate obtained from still $SRM_{AF}$ medium (Mo and Gross, 1991) showed clear phytotoxicity and contained cichopeptin concentrations of 21 ±16 µg/liter based on calculated data from nine different cultures. Culture filtrates obtained from shaken cultures did not show phytotoxicity. Production of cichopeptins was much lower than that of cichofactins (Pauwelyn et al., 2013) and was easily influenced by culture conditions, revealing that $SRM_{AF}$ medium is not optimal for *P. cichorii* to produce cichopeptins. Moreover, we were intrigued by the fact that cichopeptins contain two molecules of glycine, and that such high content in glycine is not common in *Pseudomonas* lipopeptides (Raaijmakers et al., 2006; Gross and Loper, 2009).

Glycine is a downstream product of choline metabolism (supplementary Fig. S3). Choline and glycine betaine are potentially abundant in plant hosts including lettuce (de Zwart et al., 2003; Zeisel et al., 2003; Chen et al., 2013). Genes involved in choline uptake and metabolism are present in the genome of *P. cichorii* SF1-54 (supplementary Table S4). Further analysis revealed that *P. cichorii* SF1-54$Nal^R$ can utilize choline and its breakdown product glycine betaine, but not glycine as the sole carbon source supplied to minimal medium under well-aerated conditions (supplementary Fig. S4A). We further tested the effect of choline and glycine betaine on *P. cichorii* SF1-54$Nal^R$ growth in $SRM_{AF}$ medium under static culture conditions, which were used for lipopeptide production. Various concentrations of choline and glycine betaine were tested, but only glycine betaine could be utilized by *P. cichorii* under static culture conditions in $SRM_{AF}$ medium (supplementary Fig. S4B). Among all conditions tested, *P. cichorii* exhibited the best growth in $SRM_{AF}$ medium containing 10

mM glycine betaine till 72 h incubation (supplementary Fig. S4B). In contrast, addition of choline caused an inhibitory effect on *P. cichorii* growth under static culture conditions (supplementary Fig. S4B). Thus, we decided to examine the effect of glycine betaine on cichopeptin production by *P. cichorii* SF1-54Nal$^R$. Supplementation of glycine betaine at a final concentration of 10 mM in $SRM_{AF}$ medium greatly enhanced cichopeptin production by *P. cichorii* SF1-54Nal$^R$ (1.3 and 3.0 fold increases for cichopeptins A and B, respectively, Figs. 2A-C). Production of those lipopeptides also reached similar levels by supplementation of glycine betaine at a higher (1% or 85 mM) concentration (supplementary Fig. S5). Addition of 1 mM glycine betaine could not stimulate cichopeptin production (Figs. 2A-C). In addition, supplementation of glycine betaine also enhanced production of cichofactins and compound A but not the production of the pseudomycin-like compounds B and C (Fig. 2A).

The filtrate obtained from *P. cichorii* SF1-54Nal$^R$ culture grown in $SRM_{AF}$ medium in the presence of 10 mM glycine betaine induced the strongest necrosis on chicory leaves among all treatments (Figs. 2D-I). The results are in accordance to the results of cichopeptin production determined by HPLC-ESI-MS analysis (Figs. 2A-C).

**_In planta_ fate of cichopeptins produced by _P. cichorii_ SF1-54**

Lettuce tissue inoculated with *P. cichorii* SF1-54 was analyzed using HPLC-ESI-MS to determine lipopeptide production of *P. cichorii* SF1-54 *in planta*. Cichopeptins were detected at 24 h post infiltration in amounts of 4.1±1.5 ng/g leaf fresh weight while at 48 h post inoculation, no cichopeptin was detected in infected lettuce tissue. In addition to cichopeptins, cichofactins, which are involved in swarming motility of *P. cichorii*, were detected at each time point. In addition, no trace of compounds A, B and C corresponding to other lipopeptides could be detected in infected lettuce tissue (Fig. 3).

Subsequently, we infiltrated lettuce leaves with a culture filtrate of *P. cichorii* SF1-54 to further study the distribution/stability of lipopeptides in lettuce tissue. The concentration of cichopeptins gradually decreased from 4.4±1.9 to 3.5±0.1 ng/g leaf fresh weight over 48 h after infiltration, while the concentration of cichofactins slightly reduced from 6.4±0.4 to 5.7±0.7 $\times 10^7$ peak area/g leaf fresh weight during the same time course.

**Biological role of cichopeptins produced by *P. cichorii* SF1-54**

To investigate the biological role of cichopeptins and their importance in pathogenicity for *P. cichorii* SF1-54, we constructed a cichopeptin-deficient mutant SF1-54-Δ*cipA* by deleting a 8,071 bp fragment in the cichopeptin synthetase A-encoding gene *cipA* (indicated in Fig. 1). Cells of both SF1-54NalR and mutant SF1-54-*ΔcipA* , suspended into a droplet of water on parafilm, caused immediate drop collapse (data not shown), indicating that the mutant still had biosurfactant activity. HPLC-ESI-MS analysis confirmed that SF1-54-*ΔcipA* no longer produced cichopeptins (compounds F and G in the chromatogram), but that deletion of the cichopeptin biosynthesis gene did not influence the production of cichofactins A and B (i.e. compounds D and E in the chromatogram, which are no longer produced by the cichofactin mutant SF1-54-*ΔcifAB* ), pseudomycin-like compounds B and C, and uncharacterized compound A (Fig. 4A).

As shown in Fig. 4B, the culture filtrate of SF1-54-Δ*cipA* still caused discoloration of chicory tissue but the symptoms were somewhat different from the necrosis induced by the culture filtrates of SF1-54Nal$^R$ and SF1-54-Δ*cifAB*. Furthermore, induction of discoloration and necrosis on chicory leaves by injection with a cell suspension of SF1-54-Δ*cipA* was reduced in comparison to the leaves inoculated with SF1-54Nal$^R$ and the cichofactin-deficient mutant SF1-54-Δ*cifAB* (Fig. 4C).

Moreover, SF1-54-Δ*cipA* exhibited significantly smaller inhibition zones against *B. megaterium* than the parental strain and SF1-54-Δ*cifAB* (Fig. 5A). Compared to the parental strain, growth inhibition of *B. megaterium* by SF1-54-Δ*cipA* was reduced by 45%. Parental strain and mutant did not differ in inhibitory activity against *Geotrichum candidum* and *R. mucilaginosa*.

*P. cichorii* SF1-54Nal$^R$ swarmed outwards from the inoculation point to cover almost the entire plate containing soft LB medium (0.5% agar) within 24 h. Swarming behavior of the cichopeptin-deficient mutant was identical to the parental strain, while the cichofactin mutant completely lost this ability, indicating that cichofactins, but not cichopeptins, contribute to swarming activity of *P. cichorii* SF1-54 (Figs. 5B and 5C).

When lettuce leaves were infiltrated with strains SF1-54Nal$^R$ or SF1-54Δ*cifAB*, cell collapse was observed in almost all of the infiltrated areas one day post inoculation (supplementary Fig. S6). When lettuce leaves were infiltrated with SF1-54Δ*cipA*, symptom development was significantly slower than in the other two treatments ($P < 0.05$). The cichopeptin-deficient strains mainly caused brown necrosis on lettuce leaves one day post inoculation, while cell collapse was clearly observed in the infiltrated areas two days post inoculation (supplementary Fig. S6).

To investigate the effect of cichopeptins on the virulence of *P. cichorii* SF1-54, greenhouse-grown butterhead lettuces were spray-inoculated with *P. cichorii* SF1-54Nal$^R$ and its cichopeptin-deficient mutant in greenhouse experiments. The cichofactin mutant strain SF1-54-Δ*cifAB* was also included. Lettuce plants inoculated with the cichopeptin-deficient mutant SF1-54-Δ*cipA* exhibited a significantly lower number of rotten midribs than lettuce plants inoculated with the parental strain (Fig. 6A). However, plants inoculated with the cichopeptin mutant SF1-54-Δ*cipA* or the cichofactin mutant SF1-54-Δ*cifAB* did not differ in number of rotten midribs. When lettuce midribs were inoculated with SF1-54Nal$^R$ and its

lipopeptide-deficient mutants, all strains grew vigorously and reached similar population densities (~$1\times10^8$ CFU/$cm^2$) in the inoculated midribs at 1, 2 and 3 days post inoculation (Fig. 6B).

## Discussion

### Structure and bioactivity of cichopeptins

Cichopeptin A and B are two structurally related cyclic lipopeptides composed of an unsaturated C12-fatty acid chain linked to the N-terminus of a 22 amino-acid peptide moiety. The difference between cichopeptins A and B occurs in the last amino acid residue, which is Leu/Ile and Val for cichopeptins A and B, respectively. Because the production of cichopeptin B was lower than that of cichopeptin A when *P. cichorii* SF1-54 was cultured in $SRM_{AF}$ medium, it is suggested that the A-domain of the last module of CipF protein recognizes Leu/Ile more specifically and/or frequently than Val during the synthesis of cichopeptins. Like other phytotoxic CLPs (Raaijmakers et al., 2006; Gross and Loper, 2009), cichopeptin contains several non-canonical amino acid residues, Dab and dhAbu, one *a*Thr and several Ala and Val (Fig. 7). Cichopeptins are structurally related to corpeptin B (Emanuele et al., 1998). The C-terminal pentapeptide rings of cichopeptins and corpeptin B are almost identical, but cichopeptins contain $Ala_6$, $Gly_9$, $Gly_{12}$ and $Val_{14}$ instead of $Val_6$, $Hse_9$, $dhAbu_{12}$ and $Ala_{14}$ in corpeptin B (Fig. 7). The fatty acid chain of corpeptin B is a cis-3-hydroxy-5-dodecenoate (Emanuele et al., 1998). The configuration of the fatty acid chain in cichopeptins is not clear since we could not elucidate the exact position of the double bond.

Although structurally related, the bioactivities of cichopeptins and corpeptins are slightly different. Cichopeptins and corpeptins show antimicrobial activity against *B. megaterium* but not against *R. mucilaginosa* (Emanuele et al., 1998; Pauwelyn et al., 2013). Syringopeptins also have the same properties (Lavermicocca et al., 1997). However,

phytotoxic lipodepsipeptides showed different levels of phytotoxicity. Chicory leaves treated with purified cichopeptins A or B exhibited clear necrotic symptoms, but no chlorosis was observed (Pauwelyn et al., 2013). Infiltration of crude culture filtrate or semipurified extract of *P. cichorii* SF1-54 in tobacco leaves also caused necrosis without symptoms of chlorosis (data not shown). In contrast, both corpeptins A and B are chlorosis-inducing cyclic lipodepsipeptides and only corpeptin A can cause necrosis of tobacco leaves (Emanuele et al., 1998). Moreover, fuscopeptin and syringopeptin, two other long lipodepsipeptides with internal cyclization at the C-terminal end, can induce necrosis of plant leaves (Iacobellis et al., 1992; Ballio et al., 1996). Thus, it is suggested that necrosis or chlorosis caused by phytotoxic lipodepsipeptides could be related to these structural traits.

**Cichopeptin production by *P. cichorii* is stimulated by glycine betaine**

We hypothesized that the choline metabolic pathway could be involved in biosynthesis of cichopeptins because the peptide backbone of cichopeptin contains two residues of glycine, a downstream product of choline metabolism. The choline catabolic pathway, which is conserved in three model *Pseudomonas syringae* strains, *P. syringae* pv. *phaseolicola* 1448a, *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *tomato* DC3000 (Chen and Beattie, 2007, 2008; Chen et al., 2013), is also present in *P. cichorii* SF1-54 (supplementary Fig. S5 and Table S4). *P. syringae* can sensitively detect and scavenge choline (the detection limit is 0.1 μM), which is presumably utilized for nutrition or osmoprotection (Chen and Beattie, 2007, 2008; Chen et al., 2013). In *P. syringae* pv. *syringae* B728a, it has been shown that glycine betaine functions both in nutrition and as an intracellular signal modulating compatible solute synthesis under hyperosmotic stress conditions (Li et al., 2013). Although *P. cichorii* can utilize either choline chloride or glycine betaine as the sole carbon source under well-aerated culture conditions, supplementation of

choline chloride and glycine betaine showed opposite effects on *P. cichorii* growth under static culture conditions for lipopeptide production. Addition of choline chloride in $SRM_{AF}$ medium showed an inhibitory effect on *P. cichorii* growth when grown statically. In *E. coli* and *Pseudomonas* choline is oxidized to glycine betaine via two enzymes, choline dehydrogenase (BetA) and betaine aldehyde dehydrogenase (BetB). The corresponding *bet* genes require aerobic conditions for their expression (Landfald and Strom, 1986; Eshoo, 1988; Wargo, 2013) and the growth pattern of static *P. cichorii* cultures in choline-containing $SRM_{AF}$ medium is similar to that of *E. coli* cultured in choline-containing medium under either fermentative or anaerobic conditions (Landfald and Strom, 1986). Furthermore, betaine aldehyde dehydrogenase is an $NAD^+$-dependent dehydrogenase (Landfald and Strom, 1986; Velasco-Garcia et al., 2000), suggesting that choline catabolism competes for available $NAD^+$ with glycolysis and the TCA cycle. A pronounced competition for $NAD^+$ may occur under static culture conditions, which were used for lipopeptide production by *P. cichorii* in this study. A possible competition for $NAD^+$ may also explain the repression of *P. cichorii* growth when statically grown in choline-containing $SRM_{AF}$ medium compared to *P. cichorii* growth in $SRM_{AF}$ medium. On the other hand, inability of choline utilization due to catabolite repression seems less likely because succinate rather than glucose affects choline utilization by *P. aeruginosa* and *P. syringae* via catabolite repression (Diab et al., 2006; Li et al., 2013). Succinate is not a component of $SRM_{AF}$ medium.

Compared to glucose and choline, glycine betaine is a relatively poor carbon source for *P. cichorii* when glycine betaine is the sole carbon source (supplementary Fig. S4A). Surprisingly, however, supplementation of 10 mM glycine betaine significantly enhanced bacterial growth under culture conditions for lipopeptide production and greatly stimulated cichopeptin production by *P. cichorii* SF1-54 (Figs. 2A-C). Thus, cichopeptin formation appears to be linked to the uptake and catabolism of glycine betaine from the environment.

Furthermore, the higher production observed for both cichopeptins upon addition of glycine betaine in the culture medium may result from an increased rate of the TCA cycle resulting from glycine betaine metabolism. The influence of the TCA cycle and amino acid production on lipopeptide production has already been suggested by de Bruijn and Raaijmakers (2009). They demonstrated that addition of some amino acid residues to the medium partially rescued massetolide production and swarming motility in a *clpP* (caseinolytic protease) mutant of strain *P. fluorescens* SS101. It was also suggested that some amino acids may induce NRPS gene transcription (de Bruijn and Raaijmakers, 2009), which is also possible for the expression of NRPS-encoding genes in *P. cichorii*.

Cichopeptins were detected in infected lettuce tissue at 24 h post inoculation but not at 48 h post inoculation. In the model pathosystem *Arabidopsis thaliana* and *P. syringae* pv. *tomato* DC3000, the level of glycine betaine but not choline was significantly elevated in DC3000-challenged Arabidopsis at 12 h post inoculation (Ward et al., 2010), but the biological impact of this accumulation was not clear. We speculate that *P. cichorii* may trigger accumulation of glycine betaine in lettuce leaves during an early stage of infection. Initially produced cichopeptins (at a relatively low level) may cause membrane disruption by interacting with phospholipid molecules as described for other cyclic lipodepsipeptides (Brodey et al., 1991; Coraiola et al., 2008), resulting in an increased glycine betaine release. The released glycine betaine may be sensed and metabolized by *P. cichorii* for further growth and cichopeptin production.

Another hypothesis is that glycine betaine may play a regulatory role *in P. cichorii* SF1-54. According to a study about the interaction between *P. syringae* pv. *syringae* B728a and its native host bean, water was more limited in the leaf interior (apoplast) than on the leaf surface (Yu et al., 2013). Water limitation in the apoplastic sites may result from low water content (e.g. rapid evapotranspiration) and/or from a high concentration of solutes which is

caused by nutrient leakage and secretion in the mesophyll (Yu et al., 2013). Moreover, genes involved in uptake and catabolism of glycine betaine and in biosynthesis and transport of phytotoxic CLPs syringomycin and syringopeptin in *P. syringae* pv. *syringae* B728a were more strongly induced in apoplastic sites than in epiphytic sites (Yu et al., 2013). Addition of 10 mM glycine betaine to $SRM_{AF}$ medium may result in a relatively high-osmolality environment, mimicking apoplastic conditions. Further study is necessary to verify whether cichopeptin production is stimulated by increasing osmolality.

**_In situ_ persistence of _P. cichorii_-produced lipopeptides**

Our data show the difference in persistence between cichofactin and cichopeptin in infected leaves. When lettuce leaves were infiltrated with *P. cichorii*, severe cell collapse was observed in almost all of the infiltrated areas at 24 h post inoculation (supplementary Fig. S6). At the same time point, cichopeptins were clearly detected in infected lettuce tissue (Fig. 3). However, no cichopeptin was detected at 48 h post inoculation, indicating the importance of cichopeptins at a relatively early stage during pathogenesis of lettuce midrib rot caused by *P. cichorii*. *In situ* cichopeptin production by *P. cichorii* SF1-54 may be associated with viable lettuce cells because cichopeptin could not detected in completely necrotic lettuce cells. Similar phenomena have been observed in CLP-producing *P. fluorescens* strains, which exhibit an early phase of CLP production on germinating seeds rather than in bulk soil (Nielsen et al., 2000; Koch et al., 2002; Nielsen and Sorensen, 2003). Additionally, it has been shown that germinating sugar beet seeds release unknown compounds that stimulate an early phase production of amphisin and tensin by *P. fluorescens* strains (Nielsen et al., 2000; Koch et al., 2002; Nielsen and Sorensen, 2003). Thus, we suggest that unknown stimulants associated with viable lettuce cells may trigger *in situ* cichopeptin production by *P. cichorii* SF1-54. On the other hand, cichofactins can be detected up to five days post inoculation,

suggesting that cichofactin production is not related to viability of lettuce cells. The results are in agreement with our previous study demonstrating that *P. cichorii* SF1-54 can consistently produce cichofactins under various culture conditions (Pauwelyn et al., 2013). The phenomenon of *in situ* cichofactin production is also reflected in their involvement in the *in planta* spread of *P. cichorii* (Pauwelyn et al., 2013).

Furthermore, it was reported that syringomycin produced in plant tissue would be rapidly metabolized into other substances or irreversibly bound (Gross and DeVay, 1977). When lettuce leaves were infiltrated with the culture filtrate of *P. cichorii* SF1-54, our data showed that cichofactin was quite stable in lettuce tissue. The concentration of cichopeptin in treated lettuce tissue decreased gradually but not dramatically, indicating that cichopeptin is not prone to degradation or metabolism by lettuce cells. However, we cannot exclude the possibility that cichopeptins might be more susceptible to metabolic degradation in necrotic lettuce tissues than cichofactins during plant pathogenesis. It has been shown that *P. cichorii* triggers apoptosis-like programmed cell death in lettuce which is highly associated with generation of reactive oxygen species, *de novo* protein synthesis, etc. (Kiba et al., 2006; Kiba et al., 2009). Reactive oxygen species are able to cause oxidative damage to biomolecules (Halliwell, 2006). Alternatively, cichopeptin could be degraded by *P. cichorii* SF1-54 or other microorganisms in infected lettuce leaves. It has been suggested that lipopeptides produced by *Pseudomonas* and *Bacillus* are degraded by other microorganisms or the residual producers in nonsterile soil (Asaka and Shoda, 1996; Nielsen and Sorensen, 2003; Raaijmakers et al., 2010). Apparently, *P. cichorii*-infected lettuce tissue is a complex environment like nonsterile soil. Further investigations are needed to study the *in situ* persistence of *P. cichorii*-produced lipopeptides, especially in complex environments.

**Cichopeptins are important virulence factors of *P. cichorii***

It proved to be very difficult to construct a *cipA*-deletion mutant of *P. cichorii* SF1-54. We tried three different deletion constructs; two constructs from different locations of *cipA* and one construct from *cipF*, and only the deletion construct described in this study was successfully maintained in *E. coli*. The other two deletion constructs had lethal effects on *E. coli,* a phenomenon that did not occur when we constructed a cichofactin biosynthesis mutant in our previous study (Pauwelyn et al., 2013). In addition, we obtained *cipA* mutants showing unexpected phenotypes (e.g. loss of cichofactin production) and several attempts were needed to obtain a cichopeptin-deficient mutant, which was not affected in the production of cichofactins and the other lipopeptides.

Our data demonstrated that cichopeptins are important for virulence of *P. cichorii* SF1-54 on lettuce. Compared with the parental strain, discolored but not necrotic symptoms were observed on chicory leaves inoculated with the culture filtrate of a *cipA*-deleted mutant (Fig. 4C). Thus, cichopeptin is an important necrosis-inducing factor in culture filtrate. Infiltration with the *cipA*-deletion mutant, however, still caused necrotic symptoms on chicory and lettuce leaves which cannot visually be differentiated from necrosis induced by the parental strain (Fig. 4 and supplementary Fig. S6). The type three secretion system may be one of the candidate virulence factors for the cichopeptin mutant of *P. cichorii* SF1-54 to cause symptoms on chicory and lettuce. It has been shown that the type three secretion system is not essential for virulence of *P. cichorii* on lettuce (Hojo et al., 2008; Kajihara et al., 2012; Pauwelyn, 2012). Thus, it is possible that both cichopeptin and the type three secretion system contribute to *P. cichorii*-induced necrosis in lettuce. We are currently investigating the roles of lipopeptides and type three secretion system in interactions between host plants and *P. cichorii* SF1-54.

The cichopeptin-deficient mutant of *P. cichorii* SF1-54 exhibited significantly weaker virulence to butterhead lettuce than the parental strain but SF1-54-Δ*cipA* was as virulent as

the cichofactin-deficient mutant (Fig. 6A), indicating that both phytotoxic cichopeptins and motility-related cichofactins affect virulence of *P. cichorii* SF1-54 on lettuce. It should be noticed that production of cichopeptin was not related to bacterial growth *in planta* (Fig. 6B). Collectively, cichopeptin contributes to induction of necrotic symptoms on lettuces and is a major virulence factor of *P. cichorii* SF1-54 like corpeptin and syringopeptin in the case of *P. corrugata* and *P. mediterranea* (Emanuele et al., 1998; Licciardello et al., 2009; Licciardello et al., 2012) and *P. syringae* pv. *syringae* B301D (Scholz-Schroeder et al., 2001), respectively.

In conclusion, we characterized cichopeptins, the phytotoxic CLPs produced by *P. cichorii* SF1-54. Our results demonstrated that cichopeptins contribute to virulence of *P. cichorii* SF1-54 on lettuce and play an important role at the early stages of lettuce infection. We also found that production of cichopeptins by *P. cichorii* SF1-54 is stimulated in the presence of glycine betaine, a compound that can be used for nutrition or functions in signaling osmotic stress.

## Materials and Methods

### Microorganisms and culture conditions

Microorganisms used in this study are listed in Table 1. *P. cichorii* SF1-54, its mutants and *E. coli* strains were stored at −80°C in Luria-Bertani broth (Sambrook and Russel, 2001) with 20% glycerol for long-term storage. *R. mucilaginosa* MUCL 30397, *G. candidum* MUCL 28959 and *B. megaterium* LMG 7127 were used as indicator organisms for bioassays of antimicrobial activities. *B. megaterium* and *R. mucilaginosa* were maintained at −80°C in LB broth and in potato dextrose broth (PDB, Difco, Erembodegem, Belgium) with 20% glycerol, respectively. *G. candidum* was maintained on potato dextrose agar (PDA, Difco) slants. *Pseudomonas* strains were routinely grown on Pseudomonas Agar F (PAF, Difco) or $SRM_{AF}$ (Mo and Gross, 1991) medium for production of lipopeptides. *B. megaterium* and *E.*

*coli* strains were grown on LB medium at 28°C and 37°C, respectively. *R. mucilaginosa* and *G. candidum* were cultured on PDA at 28°C. *Saccharomyces cerevisiae* was grown on yeast-extract-peptone-dextrose medium at 30°C (Shanks et al., 2006). Antibiotics were added to the growth media (if required) at the following concentrations: 100 µg/ml gentamycin and 10 µg/ml nalidixic acid.

**Chemical analysis of *P. cichorii* SF1-54 lipopeptides**

Phytotoxic compounds F and G were purified from the culture filtrate of *P. cichorii* SF1-54 by RP-HPLC according to the method of Pauwelyn et al (2013). Purified compounds were used for chemical characterization and structural determination.

*Amino acid analysis*

For amino acid analysis, HPLC-purified compounds were collected in glass tubes and dried using vacuum speed (Speed-Vac Plus SC110A; Savant, Thermo Fisher Scientific, Bremen, Germany). The samples were hydrolyzed at 115°C for 6 h in 1.5 ml of 6 N HCl. After drying in a speedvac (Savant Speed-Vac Plus SC110A) and dissolving in 100 µl 0.1 N HCl, the samples were analyzed on a ZORBAX Eclipse AAA column (Agilent) using a Agilent 1100 series HPLC (Hewlett Packard, Diegem, Belgium) by strictly following the instructions of the manufacturer. Briefly, the hydrolysed samples and amino acid standard solutions (purchased from Sigma-Aldrich, Diegem, Belgium) were automatically derivatized in the autosampler with o-phthalaldehyde (OPA) for most amino acids and 9-fluorenylmethyl-chloroformate (FMOC) for lysine, hydroxyproline and proline residues. After derivatization, 0.5 µl of each sample was injected with a flow rate of 2 ml/min and detected at 338 nm (OPA) and 262 nm (FMOC). Amino acids in the lipopeptide hydrolysis products were identified and

their relative amounts in the samples were estimated based on the amino acid standards analyzed simultaneously.

*Mass spectrometry*

Analysis of lipopeptides produced by *P. cichorii* SF1-54 was carried out on a LC-ESI-MS (reverse phase HPLC Waters Alliance 2695/diode array detector, in tandem with a Single Quad Waters SQD Mass spectrometer; Waters, Zellik, Belgium) on a XTerra MS C18 Column (3.5 µm, 4.6×150 mm, Waters). Putative lipopeptides of *P. cichorii* SF1-54 were eluted with a gradient of acetonitrile acidified with 0.1% formic acid in water acidified with 0.1% formic acid at a constant flow rate 0.5 ml/min at 40°C. Compounds were monitored in the positive ion mode and in-source settings in the SQD were as follows: source temp: 130°C; desolvation temp: 280°C; nitrogen flow: 600 liters/h; cone voltage: 100V. Similar settings were used for lipopeptide fragmentation, except the cone voltage which was 75V instead of 100V. Both instruments were controlled with the MassLynx software (Waters). Exact mass measurements and tandem mass spectrometry experiments were performed with a FT-ICR mass spectrometer (9.4T Solarix; Bruker Daltonics, Bremen, Germany) equipped with an ESI/MALDI dual ion source including SmartbeamII laser (wavelength 355 nm). Identification of lipopeptides was performed based on mass accuracy, isotopic pattern simulation and tandem mass spectrometry. For MS/MS experiments, selection window in the quadrupole was set to 5 Da and fragmentation was performed in the hexapole, using Ar as collision gas and an optimized collision energy of 50 V.

**Identification and bioinformatics analysis of the NRPS genes**

Putative NRPS encoding regions were found by comparison of the genome sequence of *P. cichorii* SF1-54 (Pauwelyn et al., 2013) with the genomes of *P. syringae* pv. *tomato*

DC3000 and *P. syringae* pv. *syringae* B728a (Buell et al., 2003; Feil et al., 2005) using the program MAUVE (Darling et al., 2010). Gap-filling in the putative NRPS encoding gene regions was performed by way of PCR and Sanger sequencing of PCR products (Agowa sequencing, Germany). Open reading frames (ORF) were identified using the program Glimmer (Delcher et al., 1999), available on the NCBI website. The catalytic domains present in the NRPS genes were identified using the NRPS/PKS analysis website (Bachmann and Ravel, 2009) (http://nrps.igs.umaryland.edu/nrps) and the NRPS-PKS website (Ansari et al., 2004) (http://www.nii.res.in/nrps-pks.html). The specificity prediction of the A-domain was conducted by using the web-based software NRPSpredictor2 (Rausch et al., 2005; Rottig et al., 2011) (http://nrps.informatik.uni-tuebingen.de/). The D/L configurations of amino acid residues of nonribosomal peptide synthetases were investigated by phylogenetic analysis of the C-domains via Phylogeny.fr website (Dereeper et al., 2008) and detection of specific signatures occuring in the DownSeq regions of each C-domain (Caradec et al., 2014). Genes adjacent to the putative NRPS genes were compared with the sequences available in GenBank using the program BlastX. Multiple alignments and phylogeny tree design were performed using ClustalW2 tool available at the EBI site (www.ebi.ac.uk).

**Growth conditions for enhanced production of cichopeptins**

Choline chloride, glycine, or glycine betaine (all purchased from Sigma-Aldrich, Diegem, Belgium) as the sole carbon source for *P. cichorii* growth were tested. *P. cichorii* SF1-54Nal$^R$ was cultured in 5 ml of the standard minimal medium (Meyer and Abdallah, 1978) containing 1% of each compound at 28°C with shaking at 150 rpm. Bacterial growth was measured at 620 nm.

$SRM_{AF}$ medium supplemented with various concentrations of choline chloride or glycine betaine was tested for *P. cichorii* growth under static conditions. In a sterile 24-well

plate, one ml of $SRM_{AF}$ medium in absence or in presence of choline chloride or glycine betaine was added in a well and a bacterial suspension of *P. cichorii* SF1-54Nal$^R$ was added in culture media to a final concentration of $1\times10^8$ CFU/ml. The plate was sealed with parafilm and incubated at room temperature as the conditions for lipopeptide production. Bacterial growth was measured at 620 nm.

$SRM_{AF}$ medium supplemented with various concentrations of glycine betaine was tested for production of cichopeptin. A bacterial suspension of *P. cichorii* SF1-54Nal$^R$ was added in culture media (25 ml in a 100-ml flask) to a final concentration of $1\times10^8$ CFU/ml. Bacterial cultures were incubated at room temperature for 6 days without shaking. After six-day incubation, the culture supernatants were collected and filtrated through 0.22-μm filters. The cell-free culture filtrates were used for assays of antimicrobial activity and phytotoxicity and HPLC-ESI-MS analysis. Three independent cultures of each treatment were analyzed and each culture was assayed in triplicate.

**_In planta_ detection of lipopeptides produced by _P. cichorii_ SF1-54**

Lettuce leaves were infiltrated with a bacterial suspension of *P. cichorii* strain SF1-54Nal$^R$ ($1\times10^6$ CFU/ml) or a filter-sterilized culture filtrate obtained from *P. cichorii* SF1-54Nal$^R$ culture grown in $SRM_{AF}$ medium. The lettuce leaves were placed on upside down Petri dishes into plastic infection trays filled with wetted sterile cotton. The infection trays were covered with a plastic lid to obtain high relative humidity and incubated at 25°C. At 24 and 48 h post inoculation, symptomatic tissue was excised from the lettuce leaves and ground in liquid nitrogen. After thawing, one gram of ground symptomatic leaf tissue was suspended in 3 ml of 50% acetonitrile, shaken for 1 h at room temperature, and then centrifuged (5 min at 10,000 rpm). Lipopeptides in the supernatants of extractions were concentrated with $C_{18}$

solid-phase extraction cartridges ($C_{18}$ SPE Maxi-Clean cartridges; Alltech, Grace, Lokeren, Belgium) and analyzed with LC-ESI-MS.

**Construction of the cichopeptin-deficient *P. cichorii* SF1-54 mutant**

A cichopeptin-deficient mutant of *P. cichorii* SF1-54Nal$^R$ was constructed by an *in vivo* cloning technique with the yeast *S. cerevisiae* InvSc1 (Hoang et al., 1998; Shanks et al., 2006). To construct deletion plasmid pMQ30-Δ*cipA*, primers CipA-F1 and CipA-R1 were used to amplify the upstream fragment, and primers CipA-F2 and CipA-R2 for the downstream fragment (Table 1). The cichopeptin-deficient mutant contained a deletion of 8,071 bp in the cichopeptin encoding genes, *cipA*. Deletion of the *cipA*-fragment was confirmed by PCR and the mutant was characterized phenotypically.

**Biological roles of cichopeptin in *P. cichorii* SF1-54**

Phytotoxic activity was tested on chicory or lettuce leaves by injection of 300 µl bacterial suspension [$1.0\times10^8$ CFU/ml in 50 mM phosphate buffer (PB, pH 7.0)] or culture filtrate with a needleless syringe used through the abaxial surface. The infiltrated leaves were incubated at 100% humidity and 25°C for 24 h and appearance of necrotic lesions was scored at 24 h after injection. PB, non-inoculated $SRM_{AF}$ medium or $SRM_{AF}$ medium supplemented with different carbon sources were used as negative control treatment.

Antimicrobial activity was tested against *B. megaterium, G. candidum* and *R. pilimanae* using the protocol described by Pauwelyn *et al*. (Pauwelyn et al., 2013). Briefly, bacterial suspensions of *P. cichorii* strains ($1\times10^8$ CFU/ml) were spotted on PDA plates in five replicates, allowing growth at 25°C for 5 days. The areas of colony growth were marked and the colonies removed with a sterile swab. The plates were exposed to chloroform vapors for 20 min to kill the remaining bacterial cells, followed by dissipation of the vapors for an

additional 40 min. The plate was then sprayed with a spore/cell suspension at a concentration about $10^6$ cells or spores/ml of the indicator microorganism. After 24-48 h incubation at 28°C, inhibition of the indicator microorganism was scored.

Swarming motility of *P. cichorii* strains was assayed on soft LB plates (0.5% agar). Bacteria of a fully grown colony on PAF-medium were applied in the centre of the soft LB agar plates with a sterile toothpick and the plates were subsequently incubated at 28°C up to 72 h after inoculation.

To test the virulence in detached butterhead leaves, the leaves were inoculated by infiltration with 100 µl of a bacterial suspension of each *P. cichorii* strain ($5\times10^5$ CFU/ml). On one leaf, four sites were inoculated with the same strain. Infiltrated leaves were incubated in humid boxes at 28 °C. Each strain was assayed in triplicate and the experiment was carried out twice. Disease symptoms were evaluated on the inoculated leaves one and two days post inoculation on a 0 – 3 scale: 0, no disease symptoms; 1, discoloration or small spot of the infiltrated tissue; 2, brown necrosis of the infiltrated tissue; and 3, collapse of the infiltrated tissue. The symptoms of disease scores are shown in supplementary Fig. S6. Data were analyzed by the Kruskal-Wallis and Mann-Whitney non-parametric tests ($P = 0.05$) in SPSS 21.0 for Windows.

Pathogenicity of the lipopeptide-deficient mutants was tested at Inagro, Rumbeke, Belgium, according to the methods described in our previous studies (Pauwelyn et al., 2011; Pauwelyn et al., 2013). Butterhead lettuce plants cv. Hofnar (Rijk Zwaan, De Lier, the Netherlands) at head formation stage were inoculated with *P. cichorii* strains. Tap water without bacteria was used as the un-inoculated treatment. Each treatment was assayed in three replicates with 24 plants per replicate. At harvest, disease severity was assessed by giving each plant a score ranging from 0 to 4, where 0 = healthy plant, 1 = little black spots on the leaf periphery of the inner crop leaves, 2 = infection of small side ribs or black spots or stripes

on the midrib, 3 = one, two or three rotten midribs, and 4 = four or more rotten midribs. Disease score of all plants was calculated as previously described (Pauwelyn et al., 2011; Pauwelyn et al., 2013). All data were statistically analysed using the software package SPSS 21.0 for Windows. As the data did not meet the conditions of normality and homogeneity of variance, non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P = 0.05$) were performed.

To test the ability of *P. cichorii* SF1-54 and its mutants to grow in lettuce midribs, the midrib of the inner lettuce leaves was inoculated with 1.0 ml bacterial suspension ($1\times10^5$ CFU/ml). The inoculated leaves were incubated at 100% humidity at 25°C. Nine leaf discs (1 cm diameter) were excised from the midribs of three lettuce leaves at 0, 1, 2, and 3 days post inoculation and macerated in 7.0 ml sterile 50 mM potassium phosphate buffer (PB, pH 7.0). Tenfold serial dilutions from the macerate were prepared with PB and 100 µl aliquots of each dilution were spread on three PAF plates with 50 µg/ml nalidixic acid. Colonies were counted 2 days after incubation at 28°C to estimate population densities. The experiment was repeated twice. All data were statistically analyzed using the software package SPSS 21.0 for Windows. As the data did not meet the conditions of normality and homogeneity of variance, non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P = 0.05$) were performed.

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## AUTHORS' CONTRIBUTION

C.J.H., E.P., M.O. and M.H. conceived and designed the experiments. C.J.H. and E.P. carried out most of the experiments and wrote the manuscript. M.O. and D. D. did the chemical characterizations; V.L. and P.J. carried out and helped with the bioinformatics analysis; P.B. coordinated the greenhouse experiments; M.H. and M.O. were involved in data analysis and revised the manuscript critically. All authors have approved the final version.

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## Figure legends

**Figure 1.** Schematic representation of the gene cluster involved in biosynthesis of cichopeptin and the surrounding open reading frames in the *P. cichorii* SF1-54 genome. The module and domain organization is indicated below the *cipA*, *cipB*, *cipC*, *cipD*, *cipE* and *cipF* genes. Predicted amino acids specificity is presented below each module and all amino acids are identified by standard three-letter biochemical notation. Abbreviation of nonstandard amino acids: Dab, 2,4-diaminobutyric acid; dhAbu, 2,3-dehydroaminobutyric acid. The region between arrowheads was deleted in the cichopeptin-deficient mutant.

**Figure 2.** Effect of glycine betaine (betaine) on cichopeptin production by *Pseudomonas cichorii*. *P. cichorii* SF1-54Nal$^R$ was cultured in $SRM_{AF}$ medium with or without betaine (1 or 10 mM) and the culture filtrates were analyzed by reverse phase ultra-performance liquid chromatography coupled with electrospray ionization mass spectrometry (UPLC-ESI-MS) and by phytotoxicity assay. **A,** Chromatogram of semipurified extracts of SF1-54Nal$^R$. **B,** Extracted ion chromatogram of semipurified extracts of SF1-54Nal$^R$ using masses of the most abundant ions of compounds F and G (respectively at *m/z* 1027 and m/z 1034). Y axes of LC-MS traces are linked at the same scale for comparison of lipopeptide production. Compounds F and G are cichopeptins B and A, respectively. Two peaks labeled F at different retention times correspond to compounds with the same mass but which probably slightly differ in the position/stereoisomery of some amino acids resulting in distinct chromatographical behaviors. **C,** Quantification of relative production of cichopeptins by SF1-54Nal$^R$ based on UPLC-ESI-MS data. Values represent the mean ± standard deviation of three independent cultures. Bars indicated with the asterisk are significantly different based on Fisher's Least Significant Difference test ($P < 0.05$). **D-I,** Assays of phytotoxicity of SF1-54Nal$^R$ culture filtrates on

witloof chicory leaves. Chicory leaves were inoculated with **D,** $SRM_{AF}$ medium; **E,** crude culture filtrate of SF1-54Nal$^R$ grown in $SRM_{AF}$ medium; **F,** $SRM_{AF}$ medium + 10 mM betaine; **G,** crude culture filtrate of SF1-54Nal$^R$ grown in $SRM_{AF}$ medium supplemented with 10 mM betaine; **H,** $SRM_{AF}$ medium + 1 mM betaine; **I,** crude culture filtrate of SF1-54Nal$^R$ grown in $SRM_{AF}$ medium supplemented with 1 mM betaine. Photographs were taken at two days post treatment.

**Figure 3.** Liquid chromatography electrospray ionization mass spectrometry detection of *Pseudomonas cichorii* SF1-54 lipopeptides in **A,** semipurified extract of *P. cichorii* SF1-54Nal$^R$ culture filtrate; **B,** extract of lettuce tissue infected with *P. cichorii* SF1-54Nal$^R$; and **C,** extract of mock-inoculated lettuce tissue. Lipopeptides were extracted at 24 h post inoculation and analyzed. Y axes of LC-MS traces are linked at the same scale. Detected lipopeptides in the chromatograms are indicated with a letter. D and E, cichofactins. F and G, cichopeptins. Peaks detected in panel C do not correspond to lipopeptides produced by *P. cichorii*.

**Figure 4. A,** Chromatograms of the semipurified extracts of *Pseudomonas cichorii* SF1-54Nal$^R$ and its lipopeptide-deficient mutants. Seven bioactive compounds in the semipurified culture extracts were identified by electrospray ionization mass spectrometry analysis. Seven bioactive compounds are indicated with a letter. D and E, cichofactins. F and G, cichopeptins.. In addition to the various forms of cichopeptin B (see legend of Fig 2), two cichopeptin A forms (peaks labeled G) were also detected at different retention times. They correspond to compounds differing by +1 mu for the doubly charged molecular ion (+2 mu for b fragment ions, data not shown) which is most probably explained by a saturated form of the acyl chain in the minor form. Y axes of LC-MS traces are linked at the same scale for comparison of lipopeptide production. Symptoms caused by *Pseudomonas cichorii* SF1-54Nal$^R$ and its

lipopeptide-deficient mutants on chicory**. B and C**, Chicory leaves were inoculated with bacterial culture filtrates **(B)** or bacterial suspensions **(C)**. Photographs were taken at one day post inoculation (B) and two days post inoculation (C). Each treatment has been repeated three times and a picture representative of each of the treatments is shown.

**Figure 5.** Phenotypic characterization of lipopeptide-deficient mutants of P. cichorii SF1-54NalR. **A,** Antimicrobial activities of P. cichorii SF1-54NalR and its mutants against three indicator microorganisms. Values represent the mean ± SD of five repetitions. **B,** Swarming activity of P. cichorii strains. Values represent the mean ± SD of two experiments with four repetitions per experiment. **C,** Swarming behavior of P. cichorii SF1-54 and its mutants on Luria-Bertani soft agar (0.5% [wt/vol]) plates. Bars indicated with asterisks are statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

**Figure 6.** Effect of cichopeptins on virulence of P. cichorii SF1-54. **A,** Greenhouse-grown butterhead lettuces were inoculated with suspensions of the wildtype or cichopeptin-deficient strains (1×106 CFU/ml) after head formation of lettuce. The cichofactin-deficient mutant of P. cichorii SF1-54 was also included in the pathogenicity assay. Tap water was used as the un-inoculated control. Virulence is represented as mean number of rotten midribs. Bars indicated with the same letter are not statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$). **B,** Population sizes of P. cichorii strains in lettuce. Values represent the mean standard deviation of three independent experiments. No significant difference was observed among population sizes of three P. cichorii strains in lettuce.

**Figure 7.** Structures of cichopeptin, corpeptin, fuscopeptin and syringopeptin SP22. The fatty acid chain of each cyclic lipopeptide is indicated. For cichopeptins, the general formula of the

fatty acid chain is presented because the exact location of the double bond was not elucidated. Abbreviation of nonstandard amino acids: Dab, 2,4-diaminobutyric acid; dhAbu, 2,3-dehydroaminobutyric acid; Hse, homoserine; aThr, allothreonine. The four amino acids that differ between cichopeptin and corpeptin are indicated in bold.

**Table 1.** Strains, plasmids and primers used in this study

| Strain, plasmid or primer | Genotype or description [a] | Source/Reference |
|---|---|---|
| Strains | | |
| *Pseudomonas cichorii* | | |
| SF1-54 | Natural isolate from infected greenhouse butterhead lettuce in Belgium | Cottyn et al., 2011 |
| SF1-54Nal$^R$ | A spontaneous nalidixic acid resistant mutant of *P. cichorii* SF1-54 | Pauwelyn et al., 2013 |
| SF1-54Δ*cifAB* | A cichofactin-deficient mutant of SF1-54Nal$^R$ | Pauwelyn et al., 2013 |
| SF1-54Δ*cipA* | A *cipA*-deletion mutant | This study |
| *Escherichia coli* S17-1 | *thi pro hsd*R *rec*A::RP4-2-Tc$^R$::Mu Km$^R$::Tn7(λ*pir*), Sm$^R$ | Simon et al., 1983 |
| *Bacillus megaterium* LMG 7127 | An indicator strain for lipopeptides | |
| *Rhodolotula mucilaginosa* MUCL 30397 | An indicator strain for lipopeptides | |
| *Geotrichum candidum* MUCL 28959 | An indicator strain for lipopeptides | |
| *Saccharomyces cerevisiae* InvSc1 | *MAT*a/*MAT*α *leu*2/*leu*2 *trp*1-289/*trp*1-289 *ura*3-52/*ura*3-52 *his*3-Δ1/*his*3-Δ1 | Invitrogen |
| Plasmids | | |
| pMQ30 | 7.6 kb mobilizable suicide vector used for gene replacements in *Pseudomonas* spp.; *Sac*B, URA3, Gm$^R$ | Shanks et al., 2006 |
| pMQ30-*cipA* | pMQ30 containing two ca. 1 kb fragments of the cichopeptin biosynthesis *cipA* gene to obtain a 8.1 kb deletion in the gene | This study |
| Primers (5'→3') | | |
| CipA-F1 | *CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGAT*GTTGAATCGTCAGGCCAATCG | This study |
| CipA-R1 | *GCACCTTCGCCACCATTCAG*GTAGATCTCACCCGCAACACCC | This study |
| CipA-F2 | *GGGTGTTGCGGGTGAGATCTAC*CTGAATGGTGGCGAAGGTGC | This study |
| CipA-R2 | *GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTG*CCGACACCAACTCCAACGTCG | This study |

[a] Primer extensions are in italic font. Tc$^r$, Km$^r$, Sm$^r$, and Gm$^r$ indicate resistant to tetracycline, kanamycin, streptomycin, and gentamycin, respectively.

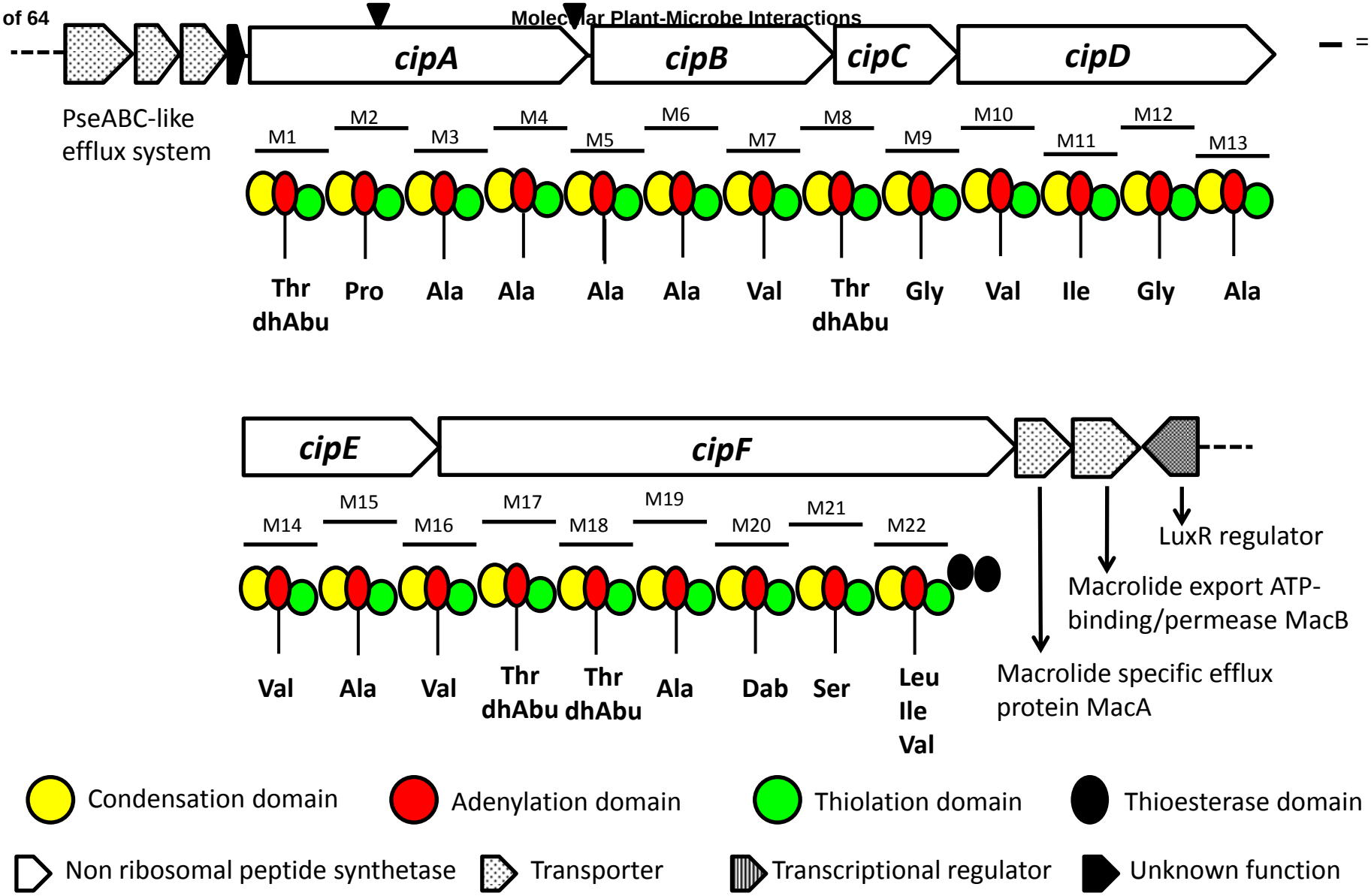



**Figure 1.** Schematic representation of the gene cluster involved in biosynthesis of cichopeptin and the surrounding open reading frames in the *P. cichorii* SF1-54 genome. The module and domain organization is indicated below the *cipA, cipB*, *cipC, cipD, cipE* and *cipF* genes. Predicted amino acids specificity is presented below each module and all amino acids are identified by standard three-letter biochemical notation. Abbreviation of nonstandard amino acids: Dab, 2,4-diaminobutyric acid; dhAbu, 2,3-dehydroaminobutyric acid. The region between arrowheads was deleted in the cichopeptin-deficient mutant.

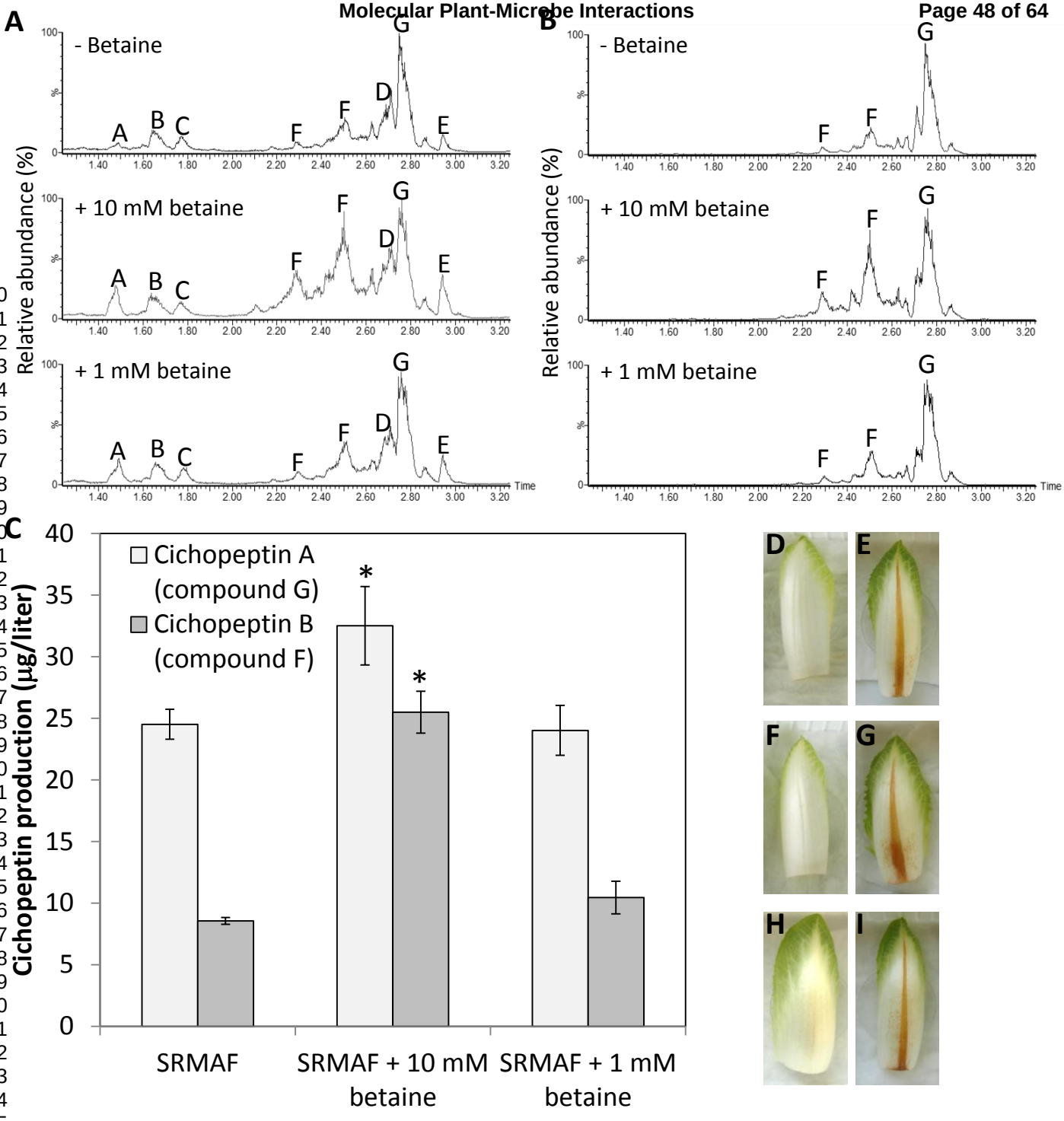



**Figure 2.** Effect of glycine betaine (betaine) on cichopeptin production by *Pseudomonas cichorii*. *P. cichorii* SF1-54Nal$^R$ was cultured in $SRM_{AF}$ medium with or without betaine (1 or 10 mM) and the culture filtrates were analyzed by reverse phase ultra-performance liquid chromatography coupled with electrospray ionization mass spectrometry (UPLC-ESI-MS) and by phytotoxicity assay. **A,** Chromatogram of semipurified extracts of SF1-54Nal$^R$. **B,** Extracted ion chromatogram of semipurified extracts of SF1-54Nal$^R$ using masses of the most abundant ions of compounds F and G (respectively at *m/z* 1027 and *m/z* 1034). Y axes of LC-MS traces are linked at the same scale for comparison of lipopeptide production. Compounds F and G are cichopeptins B and A, respectively. Two peaks labeled F at different retention times correspond to compounds with the same mass but which probably slightly differ in the position/stereoisomery of some amino acids resulting in distinct chromatographical behaviors. **C,** Quantification of relative production of cichopeptins by SF1-54Nal$^R$ based on UPLC-ESI-MS data. Values represent the mean ± standard deviation of three independent cultures. Bars indicated with the asterisk are significantly different based on Fisher's Least Significant Difference test ($P < 0,05$). **D-I,** Assays of phytotoxicity of SF1-54Nal$^R$ culture filtrates on witloof chicory leaves. Chicory leaves were inoculated with **D,** $SRM_{AF}$ medium; **E,** crude culture filtrate of SF1-54Nal$^R$ grown in $SRM_{AF}$ medium; **F,** $SRM_{AF}$ medium + 10 mM betaine; **G,** crude culture filtrate of SF1-54Nal$^R$ grown in $SRM_{AF}$ medium supplemented with 10 mM betaine; **H,** $SRM_{AF}$ medium + 1 mM betaine; **I,** crude culture filtrate of SF1-54Nal$^R$ grown in $SRM_{AF}$ medium supplemented with 1 mM betaine. Photographs were taken at two days post treatment.

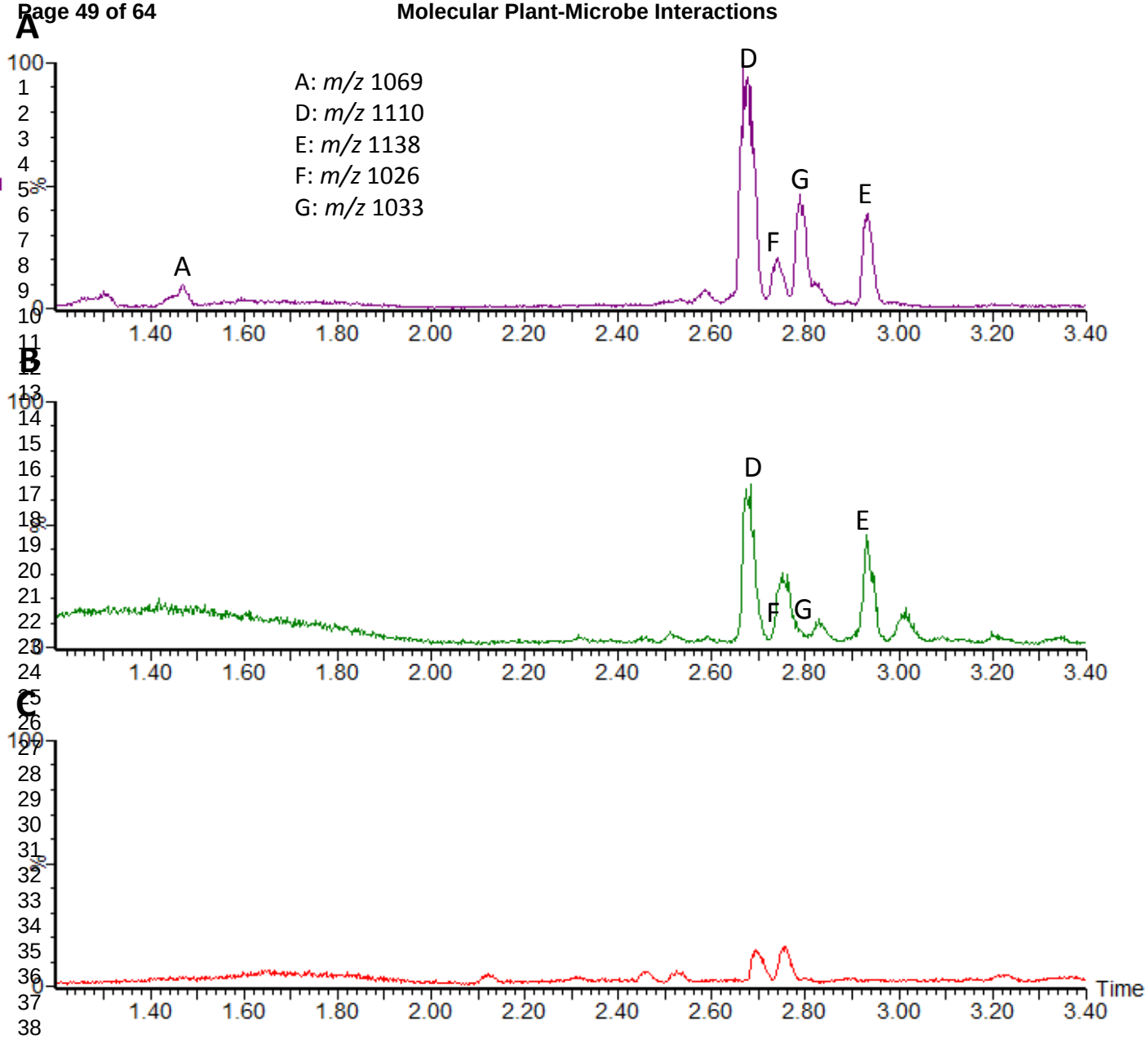



**Figure 3.** Liquid chromatography electrospray ionization mass spectrometry detection of *Pseudomonas cichorii* SF1-54 lipopeptides in **A,** semipurified extract of *P. cichorii* SF1-54Nal$^{R}$ culture filtrate; **B,** extract of lettuce tissue infected with *P. cichorii* SF1-54Nal$^{R}$; and **C,** extract of mock-inoculated lettuce tissue. Lipopeptides were extracted at 24 h post inoculation and analyzed. Detected lipopeptides in the chromatograms are indicated with a letter. D and E, cichofactins. F and G, cichopeptins. Peaks detected in panel C do not correspond to lipopeptides produced by *P. cichorii*.

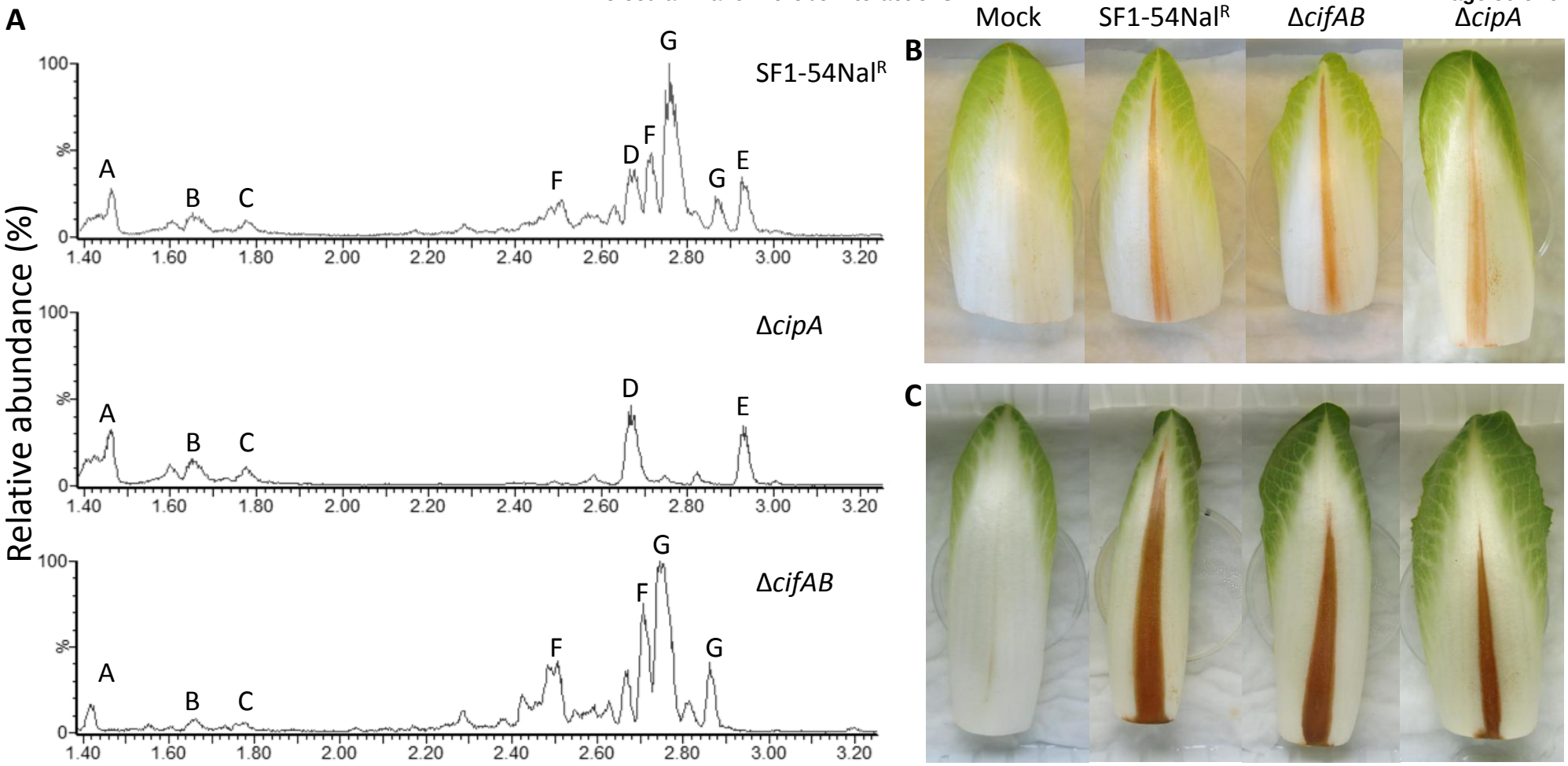
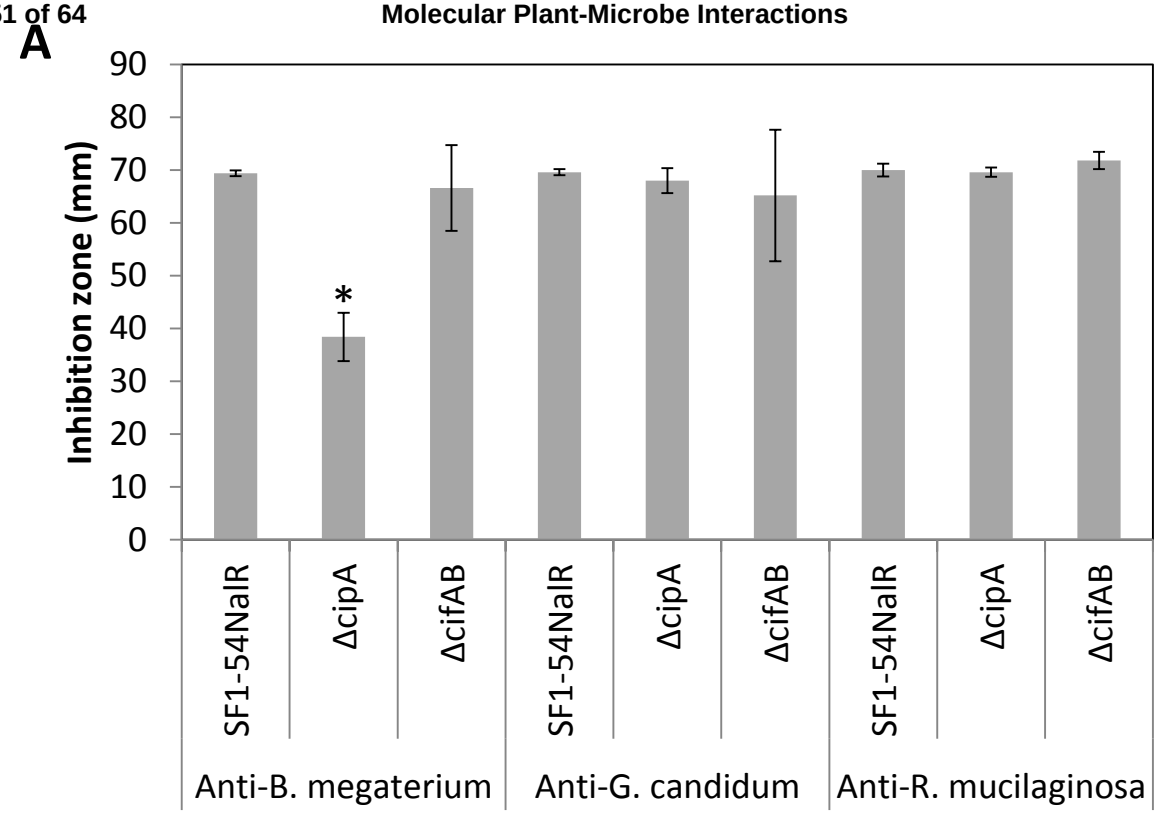
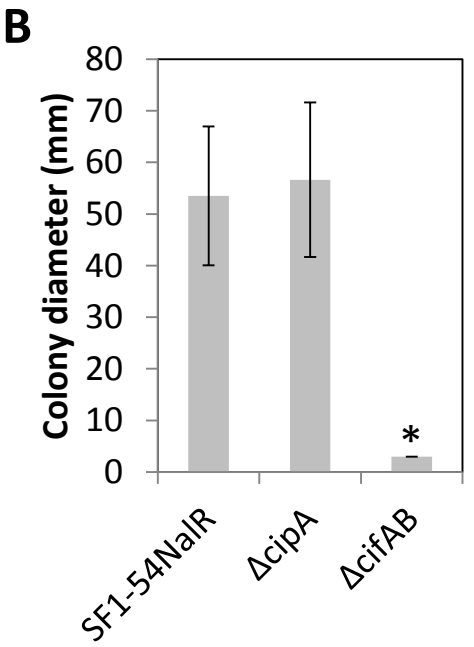



**Figure 4. A,** Chromatograms of the semipurified extracts of *Pseudomonas cichorii* SF1-54Nal$^R$ and its lipopeptide-deficient mutants. Seven bioactive compounds in the semipurified culture extracts were identified by electrospray ionization mass spectrometry analysis. Seven bioactive compounds are indicated with a letter. D and E, cichofactins. F and G, cichopeptins. In addition to the various forms of cichopeptin B (see legend of Fig 2), two cichopeptin A forms (peaks labeled G) were also detected at different retention times. They correspond to compounds differing by +1 mu for the doubly charged molecular ion (+2 mu for b fragment ions, data not shown) which is most probably explained by a saturated form of the acyl chain in the minor form. Y axes of LC-MS traces are linked at the same scale for comparison of lipopeptide production. Symptoms caused by *Pseudomonas cichorii* SF1-54Nal$^R$ and its lipopeptide-deficient mutants on chicory. **B and C,** Chicory leaves were inoculated with bacterial culture filtrates **(B)** or bacterial suspensions **(C)**. Photographs were taken at one day post inoculation **(B)** and two days post inoculation **(C)**. Each treatment has been repeated three times and a picture representative of each of the treatments is shown.



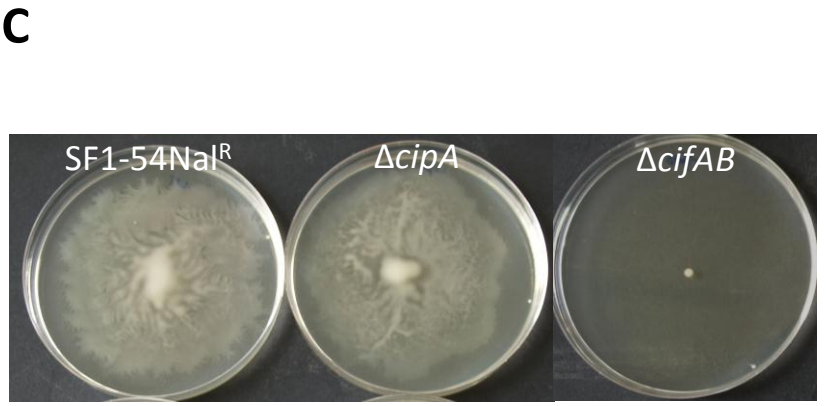




**Figure 5 .** Phenotypic characterization of lipopeptide-deficient mutants of *P. cichorii* SF1-54Nal$^R$. **A,** Antimicrobial activities of *P. cichorii* SF1-54Nal$^R$ and its mutants against three indicator microorganisms. Values represent the mean ± SD of five repetitions. **B,** Swarming activity of *P. cichorii* strains. Values represent the mean ± SD of two experiments with four repetitions per experiment. **C,** Swarming behavior of *P. cichorii* SF1-54 and its mutants on Luria-Bertani soft agar (0.5% [wt/vol]) plates. Bars indicated with asterisks are statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

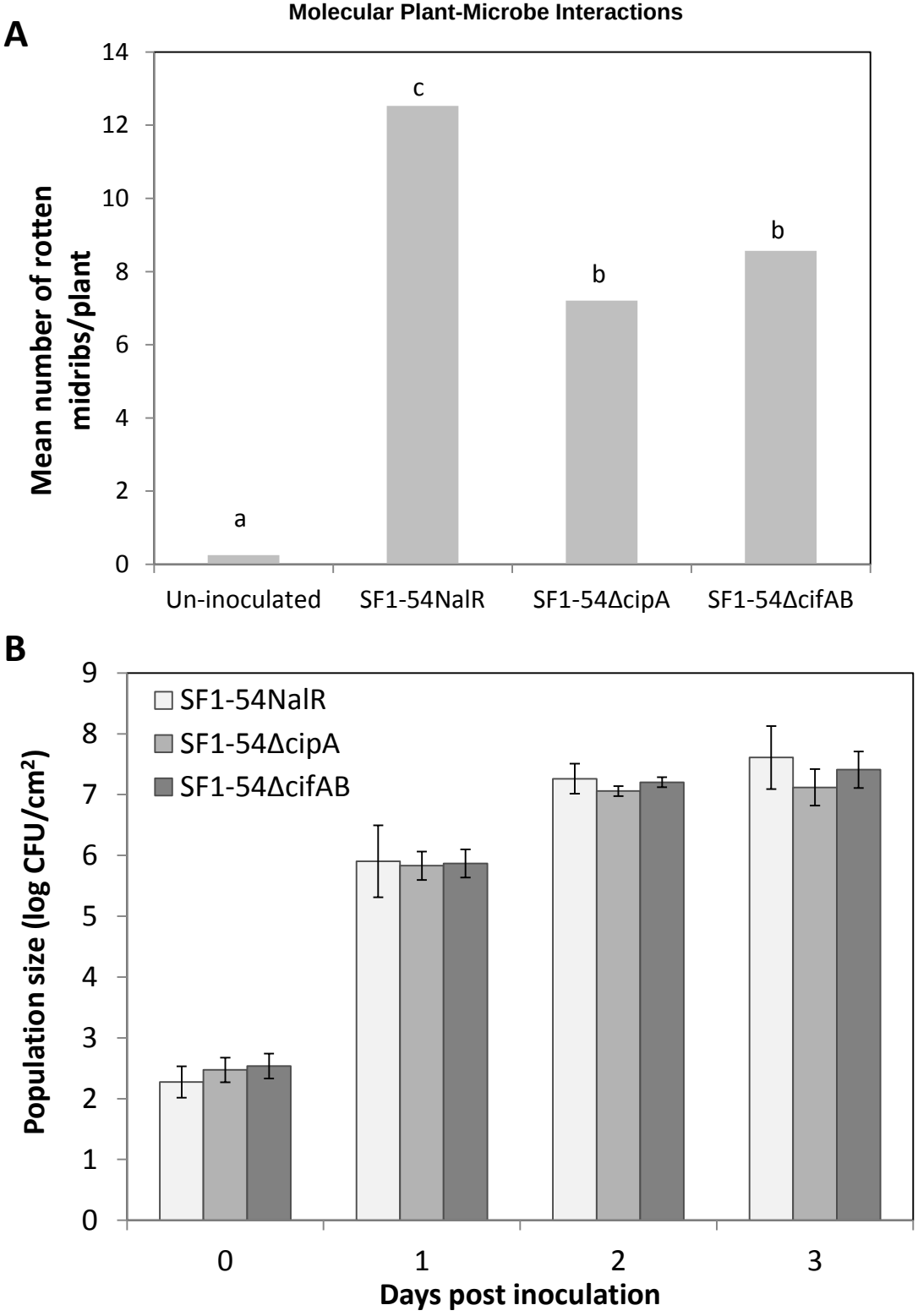



**Figure 6.** Effect of cichopeptins on virulence of *P. cichorii* SF1-54. **A,** Greenhouse-grown butterhead lettuces were inoculated with suspensions of the wildtype or cichopeptin-deficient strains ($1\times10^6$ CFU/ml) after head formation of lettuce. The cichofactin-deficient mutant of *P. cichorii* SF1-54 was also included in the pathogenicity assay. Tap water was used as the un-inoculated control. Virulence is represented as mean number of rotten midribs. Bars indicated with the same letter are not statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$). **B,** Population sizes of *P. cichorii* strains in lettuce. Values represent the mean standard deviation of three independent experiments. No significant difference was observed among population sizes of three *P. cichorii* strains in lettuce.

**Cichopeptin** $R_1$-dhAbu-Pro-Ala-Ala-Ala-**Ala**-Val-dhAbu-**Gly**-Val-Ile-**Gly**-Ala-**Val**-Ala-Val-dhAbu-*α*Thr-Ala-Dab-Ser-Leu/Ile/Val

**Corpeptin** $R_2$-dhAbu-Pro-Ala-Ala-Ala-**Val**-Val-dhAbu-**Hse**-Val-Ile-**dhAbu**-Ala-**Ala**-Ala-Val-dhAbu-*α*Thr-Ala-Dab-Ser-Ile

**Fuscopeptin** $R_3$-dhAbu-Pro-Leu-Ala-Ala-Ala-Ala-Val-Gly-Ala-Val-Ala-Val-dhAbu-Dab-*α*Thr-Ala-Dab-Dab-Phe

**Syringopeptin** $R_4$-dhAbu-Pro-Val-Val-Ala-Ala-Val-Val-dhAbu-Ala-Val-Ala-Ala-dhAbu-*α*Thr-Ser-Ala-dhAbu-Ala-Dab-Dab-Tyr

$R_1$: $COOH-CH_2-CHOH-(CH_2)_n-CH=CH-(CH_2)_m-CH_3$ (cichopeptin A and B)
$R_2$: 3-hydroxydecanoate (corpeptin A); cis-3-hydroxy-5-dodecenoate (corpeptin B)
$R_3$: 3-hydroxyoctanoate (fuscopeptin A); 3-hydroxydecanoate (fuscopeptin B)
$R_4$: 2-hydroxydecanoate (syringopeptin 22A); 3-hydroxydodecanoate (syringopeptin 22B)

**Figure 7.** Structures of cichopeptin, corpeptin, fuscopeptin and syringopeptin SP22. The fatty acid chain of each cyclic lipopeptide is indicated. For cichopeptins, the general formula of the fatty acid chain is presented because the exact location of the double bond was not elucidated. Abbreviation of nonstandard amino acids: Dab, 2,4-diaminobutyric acid; dhAbu, 2,3-dehydroaminobutyric acid; Hse, homoserine; *α*Thr, allothreonine. The four amino acids that differ between cichopeptin and corpeptin are indicated in bold.

**Table S1.** Bioactivities of the seven biosurfactant compounds produced by *Pseudomonas cichorii* SF1-54[a]

| Compound | Antimicrobial activity | | | Phytotoxicity |
|---|---|---|---|---|
| | ***B. megaterium*** | ***G. candidum*** | ***R. mucilaginosa*** | |
| A | + | - | - | - |
| B | - | + | + | - |
| C | - | - | + | - |
| D (cichofactin A) | + | - | + | - |
| E (cichofactin B) | + | - | + | - |
| F | + | - | - | + |
| G | + | - | - | + |

[a] The bioactive properties of *P. cichorii* SF1-54-produced biosurfactant compounds presented in this table were obtained and summarized from the results of Pauwelyn et al. (2013).

**Table S2.**

Masses and assignment of b- and y”-ions yielded upon MS/MS fragmentation of doubly charged forms of compounds F and G[a] . The corresponding spectrum for compound G (cichopeptin A) is shown in Fig. S1.

| Fatty acid | Peptide | Compound F | | Compound G | |
|---|---|---|---|---|---|
| | | b-ion | y”-ion | b-ion | y”-ion |
| C12:1-OH | dhAbu | | | | |
| | dhAbu-Pro | | | | |
| | dhAbu-Pro-Ala | 448 | | 448 | |
| | dhAbu-Pro-Ala-Ala | 519 | 1533 | 519 | 1547 |
| | dhAbu-Pro-Ala-Ala-Ala | 590 | 1462 | 590 | 1476 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala | 661 | 1391 | 661 | 1405 |
| | dhABu-Pro-Ala-Ala-Ala-Ala-Val | 760 | 1292 | 760 | 1306 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu | 843 | 1209 | 843 | 1223 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly | 900 | 1152 | 900 | 1166 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val | 999 | 1053 | 999 | 1067 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile | 1112 | 939 | 1112 | 953 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile-Gly | 1169 | (882) | 1169 | 896 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile-Gly-Ala | 1240 | 811 | 1240 | 825 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile-Gly-Ala-Val | 1339 | 712 | 1339 | 726 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile-Gly-Ala-Val-Ala | (1411) | (641) | 1411 | 655 |
| | dhABu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile-Gly-Ala-Val-Ala-Val | 1510 | 542 | 1510 | 556 |
| | dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile-Gly-Ala-Val-Ala-Val-dhAbu | (1594) | 459 | (1594) | 473 |

[a]The ion masses between brackets were not observed but are expected upon calculation

**Table S3.** Analysis of amino acid specificity codes of A domains of cichopeptin synthetases

| NRPS module of cichopeptin synthetase | Specificity code[b] | Highest homology with[c] | Predicted amino acid |
|---|---|---|---|
| M1 | DFWNIGMVHK<br>DFWNIGMVHK<br>DFWNIGMVHK | Q83VS0_m5<br>Q9FDB3_m1 | Thr<br>dhAbu |
| M2 | DVQYVAHVTK<br>DVQYIAHVVK | Q9FDB3_m2 | Pro |
| M3 | DLYNNALTYK<br>DLYNNALTYK | Q83VS1_m1 | Ala |
| M4 | DLYNNALTYK<br>DLYNNALTYK | Q83VS1_m1 | Ala |
| M5 | DLYNNALTYK<br>DLYNNALTYK | Q83VS1_m1 | Ala |
| M6 | DLYNNALTYK<br>DLYNNALTYK | Q83VS1_m1 | Ala |
| M7 | DALWIGGTFK<br>DALWIGGTFK | Q9FDB3_m4 | Val |
| M8 | DFWNIGMVHK<br>DFWNIGMVHK<br>DFWNIGMVHK | Q83VS0_m5<br>Q9FDB3_m1 | Thr<br>dhAbu |
| M9 | DILQLGVIWK<br>DILQLGLIWK | Q9RAH2_m2 | Gly |
| M10 | DALWIGGTFK<br>DALWIGGTFK | Q9FDB3_m4 | Val |
| M11 | DALFMGCTYK<br>DAMFLGCTYK | Q84BQ4_m4 | Ile |
| M12 | DILQLGVIWK<br>DILQLGLIWK | Q9RAH2_m2 | Gly |
| M13 | DLYNNALTYK<br>DLYNNALTYK | Q83VS1_m1 | Ala |
| M14 | DALWIGGTFK<br>DALWIGGTFK | Q9FDB3_m4 | Val |
| M15 | DLYNNALTYK<br>DLYNNALTYK | Q83VS1_m1 | Ala |
| M16 | DALWIGGTFK<br>DALWIGGTFK | Q9FDB3_m4 | Val |
| M17 | DFWNIGMVHK<br>DFWNIGMVHK<br>DFWNIGMVHK | Q83VS0_m5<br>Q9FDB3_m1 | Thr<br>dhAbu |
| M18 | DFWNIGMVHK<br>DFWNIGMVHK<br>DFWNIGMVHK | Q83VS0_m5<br>Q9FDB3_m1 | Thr<br>dhAbu |
| M19 | DLYNNALTYK<br>DLYNNALTYK | Q83VS1_m1 | Ala |

| | | | |
|---|---|---|---|
| M20 | DLEHNTTVSK | | |
| | DLEHNTTVSK | Q83VS0_m10 | Dab |
| M21 | DVWHMSLVDK | | |
| | DVWHMSLVDK | Q84BQ5_m4 | Ser |
| M22 | DALFMGATFK | | |
| | DAFFLGVTFK | Q9K5L9_m1 | Ile |
| | DAFFLGVTFK | Q9RAH4_m1 | Ile/Val/Leu |
| | DALWIGGTFK | Q9FDB3_m4 | Val |

[a] Module number of cichopeptin NRPS of *P. cichorii* SF1-54

[b] The specificity code of the adenylation domain of the NRPS modules was identified using the web-based software NRPSpredictor2 (Rausch et al., 2005, 2007). The most homologues specificity codes are grouped together.

[c] UniprotKB query_module number

**Table S4.** Genes involved in choline uptake and metabolic pathway in *Pseudomonas cichorii* SF1-54

| Gene | Gene product | Size (bp) | Homolog | Nucleotide identity (%) |
|---|---|---|---|---|
| *betT* | High affinity choline uptake protein | 1998 | BetT of Pst DC3000[a] | 98 |
| *cbcX* | Betaine, choline, and carnitine transporter | 948 | CbcX of Pst DC3000 | 86 |
| *cbcW* | Betaine, choline, and carnitine transporter | 846 | CbcW of Pst DC3000 | 88 |
| *cbcV* | Betaine, choline, and carnitine transporter | 1179 | CbcV of Pst DC3000 | 86 |
| *opuCA* | Osmoprotectant transporter | 1170 | OpuCA of Pst DC3000 | 88 |
| *betA* | Choline dehydrogenase | 1707 | BetA of Pst DC3000 | 94 |
| *betB* | Betaine aldehyde dehydrogenase | 1743 | BetB of Pst DC3000 | 93 |
| *gbcA* | Glycine betaine demethylase subunit A | 1299 | GbcA of Pss B728a[b] | 96 |
| *gbcB* | Glycine betaine demethylase subunit B | 1101 | GbcB of Pss B728a | 93 |
| *dgcA* | Dimethylglycine demethylase subunit A | 2061 | Putative DgcA of Pss B728a | 97 |
| *dgcB* | Dimethylglycine demethylase subunit B | 1962 | Putative DgcB of Pss B728a | 92 |
| *soxB1* | Sarcosine oxidase beta subunit | 1251 | SoxB of Pst DC3000 | 99 |
| *soxD1* | Sarcosine oxidase delta subunit | 297 | SoxD of Pst DC3000 | 91 |
| *soxA1* | Sarcosine oxidase alpha subunit | 3021 | SoxA of Pst DC3000 | 95 |
| *soxG1* | Sarcosine oxidase gamma subunit | 633 | SoxG of Pst DC3000 | 88 |
| *soxB2* | Sarcosine oxidase beta subunit | 1242 | SoxB of Pst DC3000 | 97 |
| *soxD2* | Sarcosine oxidase delta subunit | 306 | SoxD of Pst DC3000 | 90 |
| *soxA2* | Sarcosine oxidase alpha subunit | 2907 | SoxA of Pst DC3000 | 89 |
| *soxG2* | Sarcosine oxidase gamma subunit | 552 | SoxG of Pst DC3000 | 74 |
| *glyA1* | Serine hydroxymethyltransferase | 1254 | Putative GlyA2 of Pst DC3000 | 87 |
| *glyA1* | Serine hydroxymethyltransferase | 1254 | Putative GlyA1 of Pst DC3000 | 91 |
| *sda* | L-serine dehydratase | 1377 | Putative Sda of Pst DC3000 | 85 |

[a] *Pst* DC3000, *Pseudomonas syringae* pv. *tomato* DC3000.
[b] *Pss* B728a, *Pseudomonas syringae* pv. *syringae* B728a.

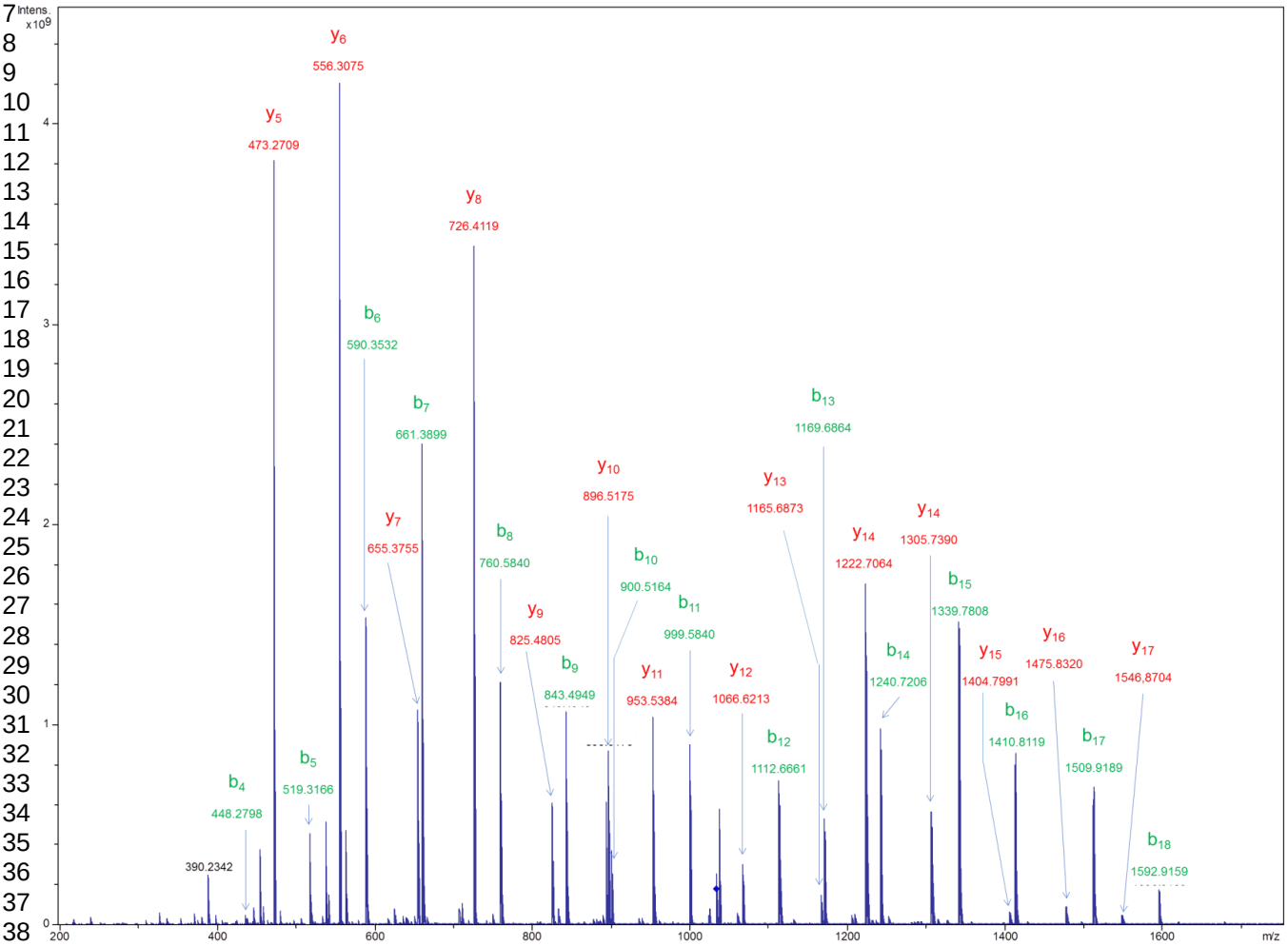



**Figure S1**. MS/MS Spectrum of doubly charged ions of cichopeptin (compound G). Tandem mass spectrum recorded with m/z 1033.10 as precursor ion. Red-labelled ions correspond to y-type ions (containing the C terminus) whereas green-labelled ions correspond to b-type ions (containing the N-terminus).

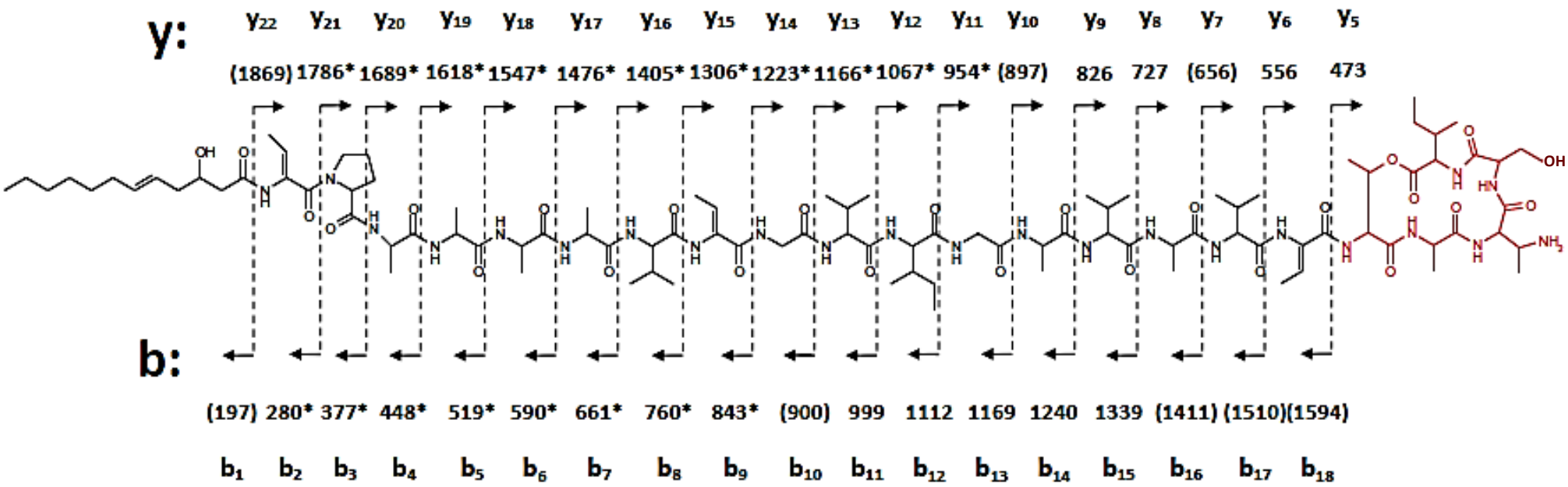



**Figure S2.** Proposed structure for a part of compound G, which could be deduced from the LC-ESI-MS the fragmentation spectrum of the $[M+H]^+$ ion of compound G. The sites of cleavage, yielding b- and y-ions are represented and the observed masses of b- and y-ion fragments are indicated. The ion masses between brackets are expected upon calculation. All fragments labeled with * have also been detected in HR-ESR-MS-MS. The chemical nature of the fatty acid moiety is expected to be a $C_{12:1}$-OH residue upon calculation. The unsaturated C12-fatty acid found in corpeptin B (Emanuele *et al.*, 1998) has been drawn as an example.

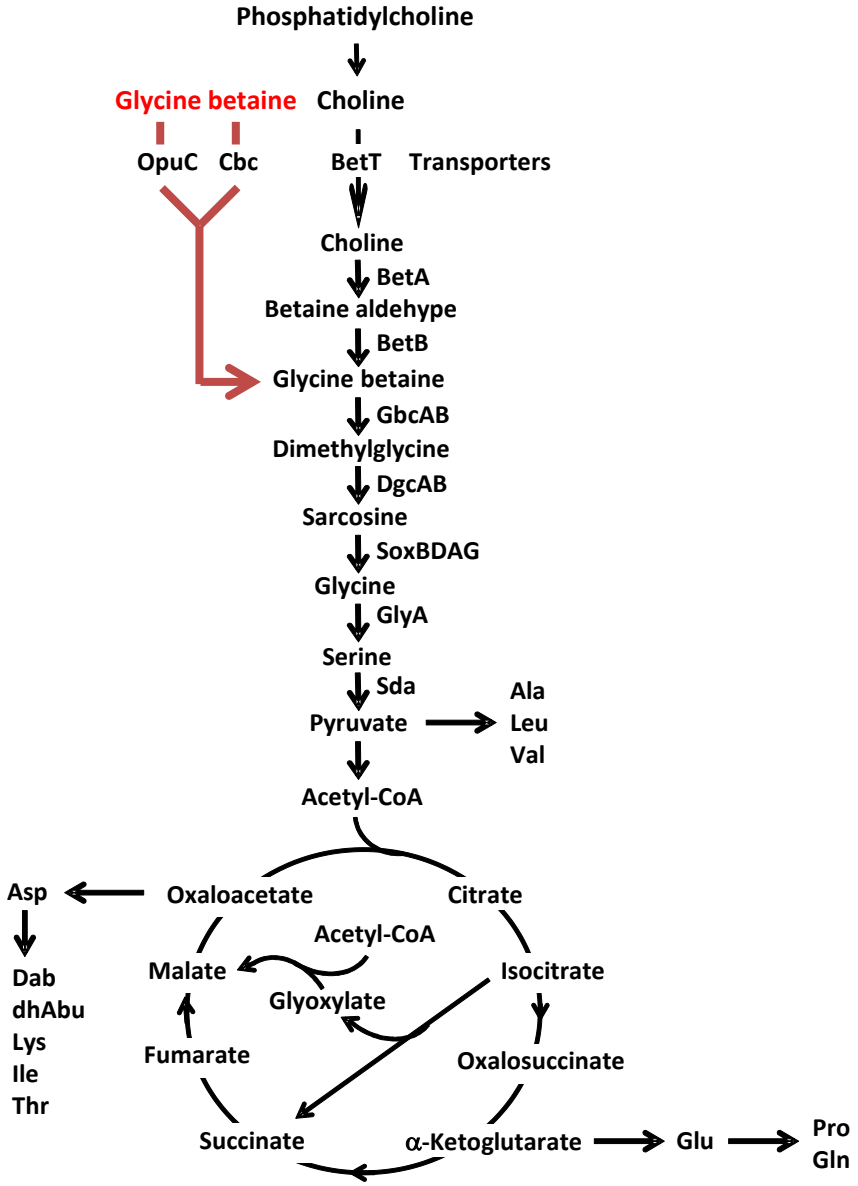



**Figure S3.** Proposed metabolic pathways in *P. cichorii* when growing on choline or glycine betaine to produce the amino acid precursors of phytotoxic cichopeptin. Abbreviation of nonstandard amino acids: Dab, 2,4-diaminobutyric acid; dhAbu, 2,3-dehydroaminobutyric acid. The transporters and enzymes responsible for uptake and catalysis of choline are as follows: BetT, high affinity choline uptake protein; Cbc, betaine, choline and carnitine transporter; OpuC, osmoprotectant transporter; BetA, choline dehydrogenase; BetB, betaine aldehyde dehydrogenase; GbcAB, glycine betaine demethylase; DgcAB, dimethylglycine demethylase; SoxBDAG, sarcosine oxidase; GlyA, serine hydroxymethyltransferase; Sda, L-serine dehydratase.

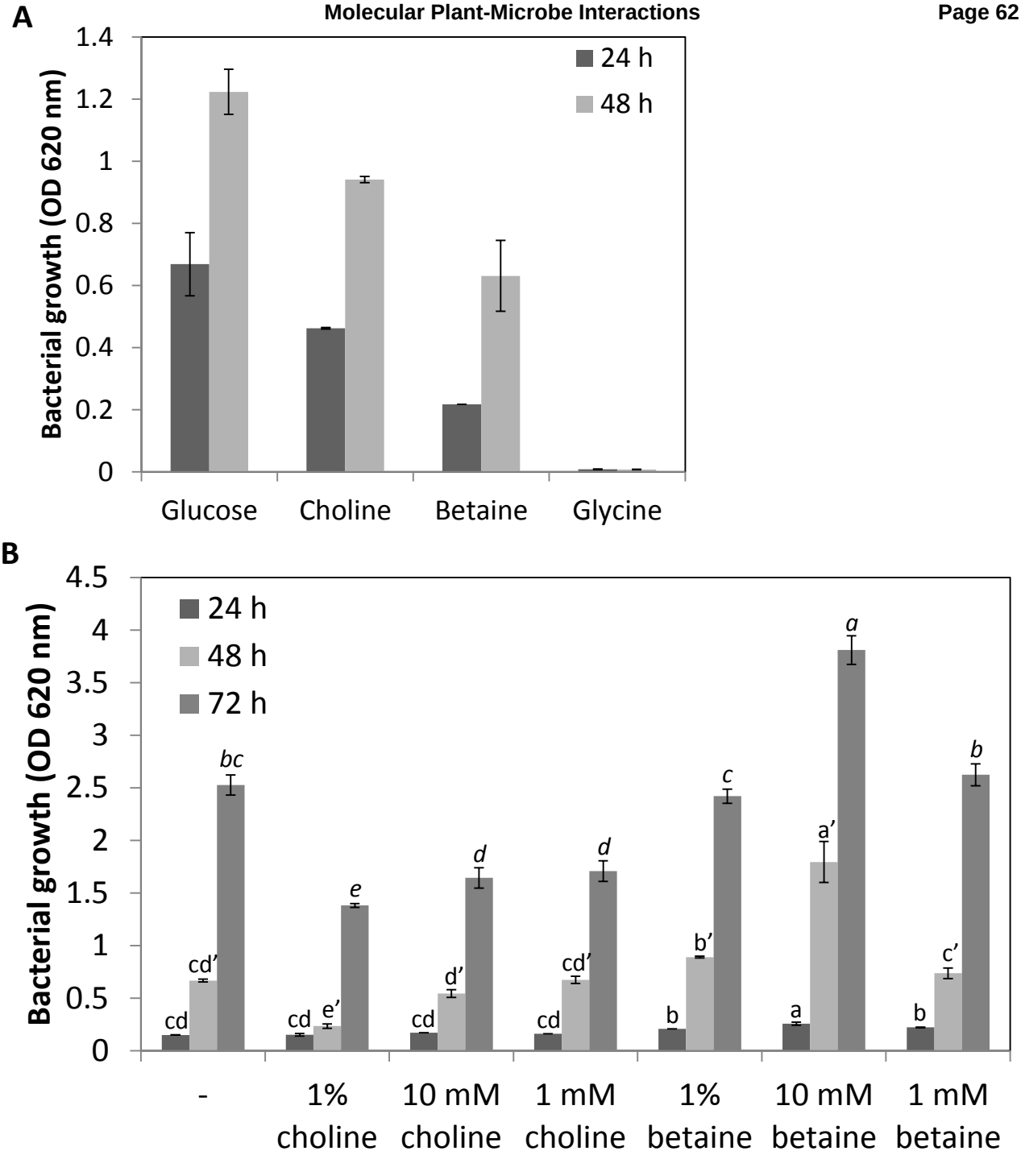



**Figure S4.** Effect of choline, glycine, and glycine betaine (betaine) on growth of *Pseudomonas cichorii*. *P. cichorii* SF1-54Nal$^R$ was cultured in standard minimal medium containing 1% choline chloride (71 mM), glycine (133 mM) or glycine betaine (85 mM) as the sole carbon source **(A)**. Glucose (1% or 55 mM) was used in the positive control. Bacterial growth was determined by measuring optical density at 620 nm after 24 and 48 h cultivation. Each treatment had three repetition and the experiment was repeated once. Data represent the mean ± standard deviation of two experiments. **B,** Effect of choline and betaine on *P. cichorii* SF1-54Nal$^R$ growth under static culture conditions for lipopeptide production. Choline or betaine was added into $SRM_{AF}$ medium at indicated concentrations. Bacterial growth was determined by measuring optical density at 620 nm after 24, 48, and 72 h cultivation. Each treatment had three repetitions. The experiment was repeated once and showed similar results. Data represent the mean ± standard deviation of three repetitions in one experiment. Bars indicated with the same letter are not statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

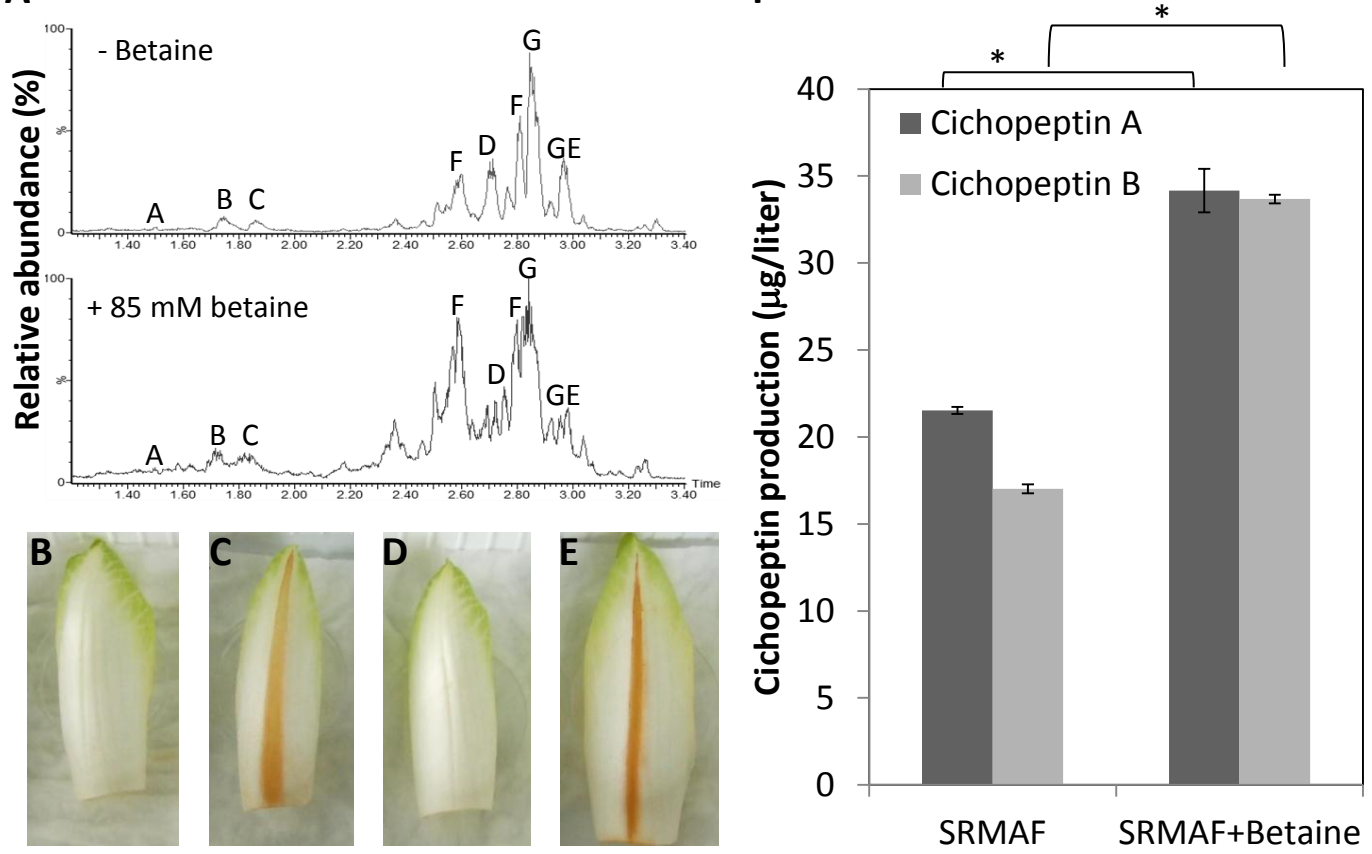



**Figure S5.** Effect of glycine betaine (betaine) on cichopeptin production by *Pseudomonas cichorii*. *P. cichorii* SF1-54Nal$^R$ was cultured in SRM$_{AF}$ medium with or without 85 mM betaine and the culture filtrates were analyzed by reverse phase ultra-performance liquid chromatography coupled with electrospray ionization mass spectrometry (UPLC-ESI-MS) and by phytotoxicity assay. **A,** Chromatogram of semipurified extracts of SF1-54Nal$^R$. Compounds A – F are indicated. D and E, cichofactins. F and G, cichopeptins. **B-E,** Assays of phytotoxicity of SF 1-54Nal$^R$ culture filtrates on witloof chicory leaves. Chicory leaves were inoculated with **B,** SRM$_{AF}$ medium; **C,** crude culture filtrate of SF1-54Nal$^R$ grown in SRM$_{AF}$ medium; **D,** SRM$_{AF}$ medium+betaine; **E,** crude culture filtrate of SF1-54Nal$^R$ grown in SRM$_{AF}$ medium supplemented with betaine. Photographs were taken one day post inoculation. **F,** Quantification of production of cichopeptins by SF1-54Nal$^R$. Cichopeptin production of each sample was measured by high-performance liquid chromatography electrospray ionization mass spectrometry by using single-ion recording integration of peaks corresponding to the exact mass of A and B homologues. Values represent the mean ± standard deviation of three independent cultures.

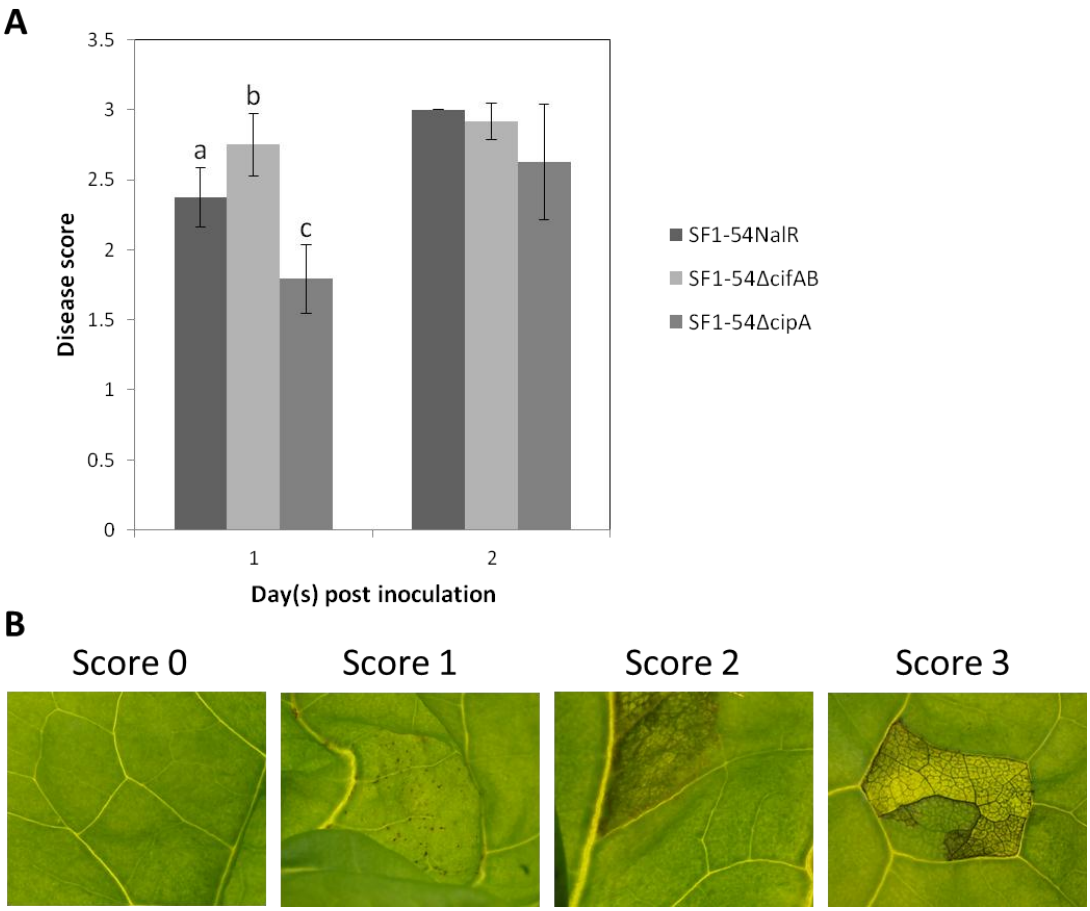



**Figure S6.** Virulence of *Pseudomonas cichorii* strains on detached leaves of butterhead lettuce. **A,** Detached leaves were infiltrated with *P. cichorii* strains and the symptom was scored at one and two days post inoculation. *P. cichorii* strains are indicated. Values represent the mean ± SD of three repetitions. **B,** Illustration of the scores of disease symptoms. 0, no disease symptoms; 1, discoloration or small spot of the infiltrated tissue; 2, brown necrosis of the infiltrated tissue; and 3, collapse of the infiltrated tissue.