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ARTICLE

Tumescenamide C, a cyclic lipodepsipeptide from *Streptomyces* sp. KUSC_F05, exerts antimicrobial activity against the scab-forming actinomycete *Streptomyces scabiei*

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Abstract

The antimicrobial activity of tumescenamide C against the scab-forming *S. scabiei* NBRC13768 was confirmed with a potent IC_{50} value (1.5 μg/mL). Three tumescenamide C-resistant *S. scabiei* strains were generated to compare their gene variants. All three resistant strains contained nonsynonymous variants in genes related to cellobiose/cellotriose transport system components; *cebF1*, *cebF2*, and *cebG2*, which are responsible for the production of the phytotoxin thaxtomin A. Decrease in thaxtomin A production and the virulence of the three resistant strains were revealed by the LC/MS analysis and necrosis assay, respectively. Although the nonsynonymous variants were insufficient for identifying the molecular target of tumescenamide C, the cell wall component wall teichoic acid (WTA) was observed to bind significantly to tumescenamide C. Moreover, changes in the WTA contents were detected in the tumescenamide C-resistant strains. These results imply that tumescenamide C targets the cell wall system to exert antimicrobial effects on *S. scabiei*.

Introduction

Streptomyces scabiei (synonym: *Streptomyces scabies*) is a well-known pathogenic actinomycetes that is distributed in worldwide. This phytopathogen causes common scab disease, which results in scab-like lesions on the surface of potato tubers [1]. These lesions decrease the market value of potato crop, leading to significant economic losses [2]. Common scab lesions have also been detected on various agriculturally important root vegetables, including radish, carrot, beet, and turnip [3]. Thus, there is a critical need for effective methods for controlling *S. scabiei*.

Conventional methods for controlling *S. scabiei*, such as soil acidification, fumigation, pre-sowing seed tuber treatments, and crop rotations, are insufficient for preventing scab disease and environmentally burdensome [4]. A biocontrol-based strategy was recently revealed as a sustainable alternative to traditional disease control measure [5–7]. This strategy involves the use of plant-beneficial bacteria that produce various types of secondary metabolites. For example, *Bacillus* spp. producing cyclic lipopeptides (e.g., fengycin, iturin A, and surfactin) [6] and *Streptomyces melanosporofaciens* EF-76 producing the polyketide geldanamycin have inhibitory effects on scab-causing *Streptomyces* spp [8, 9].

Tumescenamide C (Fig. 1b) is a cyclic lipodepsipeptide produced by *Streptomyces* sp. KUSC_F05 strain. Its isolation, structure elucidation, and total synthesis have been accomplished in our laboratory [10, 11]. Interestingly, the antimicrobial activity of tumescenamide C is considered to be specific to certain *Streptomyces* spp. including the producer *Streptomyces* sp. KUSC_F05. According to a paper disc diffusion assay, the minimal amounts of tumescenamide C required to inhibit growth were 3 μg/disc for *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* TK23 and 30 μg/disc for *Streptomyces* sp. KUSC_F05. In contrast, >100 μg/disc was required to inhibit the growth of the gram-positive

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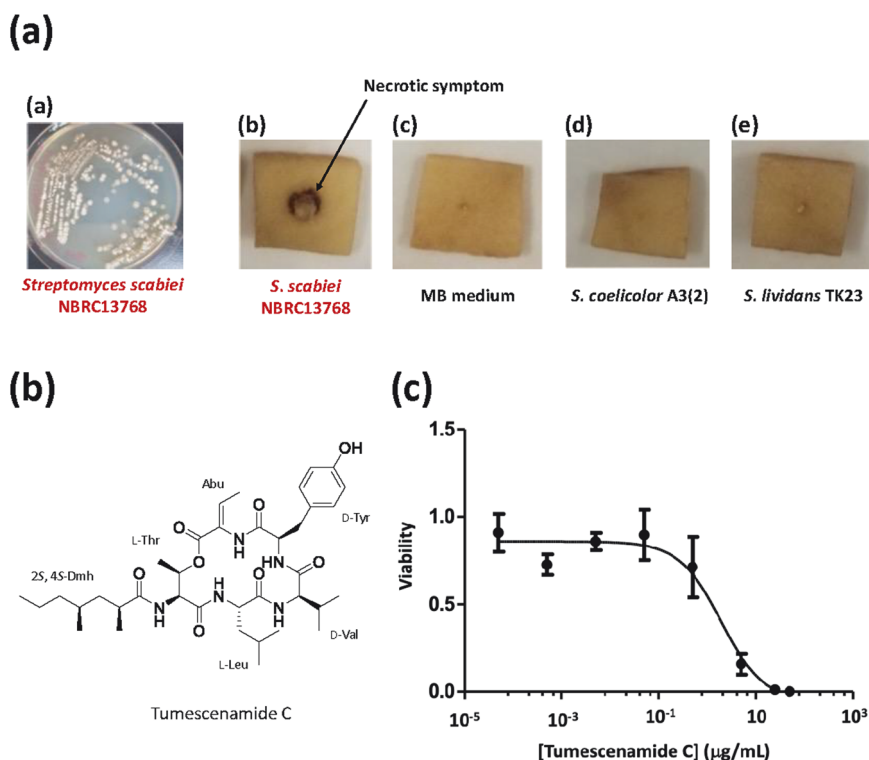
Fig. 1 Virulence assay of *S. scabiei* NBRC13768 and antimicrobial activity of tumescenamide C. **a** Assay of the virulence for *S. scabiei* NBRC13768. (a) *S. scabiei* NBRC13768 on MB medium containing 2% agar. (**b**)−(**e**) Results of the virulence bioassay involving sliced potato cubes (approximately $2 \times 2 \times 0.5$ cm). Necrosis was detected around the site inoculated with *S. scabiei* NBRC13768 (**b**), but not around the site incubated with MB medium (**c**) or the non-phytopathogenic *S. coelicolor* A3(2) (**d**) and *S. lividans* TK23 (**e**). **b** Chemical structure of tumescenamide C. **c** Antimicrobial activity of tumescenamide C against *S. scabiei* NBRC13768. Viability was calculated as the absorbance at 595 nm for each well containing tumescenamide C divided by the absorbance at 595 nm of the well lacking tumescenamide C. The estimated IC_{50} value was 1.5 µg/mL

bacteria *Staphylococcus aureus* NBRC13276 and *Bacillus subtilis* NBRC3134 as well as the gram-negative bacteria *Escherichia coli* NBRC3972 and *Pseudomonas aeruginosa* NBRC13275 [10]. The synthetic derivatives of tumescenamide C revealed the importance of the α,β-unsaturated moiety of Abu and the branched lipophilic side chain (Dmh) for the antimicrobial activity [11]. Because of the selective antimicrobial activity against *Streptomyces* spp., tumescenamide C and its producer *Streptomyces* sp. KUSC_F05 may respectively be effective chemical and biological agents for controlling scab-forming *Streptomyces* spp., with no adverse effects on plant-beneficial microbes in the rhizosphere.

In this study, the antimicrobial activity of tumescenamide C against *S. scabiei* was evaluated. Moreover, we clarified the mode of action of tumescenamide C via comparative genetic and phenotypic analyses of the wild-type (WT) and tumescenamide C-resistant strains that we generated.

Materials and methods

General information

All commercially available reagents were purchased from Nacalai (Kyoto, Japan), Fujifilm-Wako (Osaka, Japan), Kanto Chemical (Tokyo, Japan), and Tokyo Kasei (Tokyo, Japan) unless described. Tumescenamide C was purified from *Streptomyces* sp. KUSC_F05 strain according to our previous report [10]. *Streptomyces scabiei* NBRC13768 strain was distributed from National Institute of Technology and Evaluation (Tokyo, Japan). The strain was restored with Maltose-Bennett's (MB) medium (0.1% yeast extract, 0.1% beef extract, 0.2% N-Z-Amine A, 1% Maltose, pH 7.3) according to the distributer's instruction. For each experiment, a laboratory glycerol stock of the strain was spread on MB 2% agar medium and incubated at 27 °C for 3−7 days. Then, further experiments or inoculation in liquid MB medium were conducted appropriately under the conditions of 27 °C for 3−7 days with rotary agitation at 95 rpm/min. The laboratory

glycerol stocks were prepared as 4:1 ratio of the strain mycelia suspension with MB liquid medium and 80% glycerol in sterile water and stored at −80 °C until use. *S. coelicolor* A3(2) and *S. lividans* TK23 were distributed from Prof. H. Ikeda (Kitasato University) and maintained on ISP-2 medium (4% yest extract, 10% malt extract, 4% glucose, pH7.0).

Virulence assay for *Streptomyces* spp

To determine the phytopathogenic virulence of *S. scabiei* NBRC13768, potato tuber disc assay was conducted [12, 13]. Potato (Irish Cobbler potato; "Danshaku potato" in Japanese) purchased at supermarket was cut approximately 2 × 2 × 0.5 cm cubes after peeling. The cubes were put onto the petri dishes laid on moistened filter paper and wipes. Mycelia of *S. scabiei* on MB 2% agar broth maintained at 27 °C was picked up with sterilized 200 μL pipet tip and inoculated gentry onto potato cube described above. As control groups, MB medium and non-pathogenic *Streptomyces* spp.; *S. coelicolor* A3(2) and *S. lividans* TK23 were treated as the same procedure. Petri dishes were covered and placed at 20 °C in the dark for 5 days. After 5 days-incubation, the inoculated potato was pictured.

Antimicrobial activity of tumescenamide C

A dilution series of tumescenamide C in methanol (final conc. 0.001, 0.01, 0.1, 1, 10, 100, 500, and 1000 μg/mL) were dispensed in a 96 well plate (10 μL/well). The wells were fill up to 50 μL/well with flesh MB liquid medium. *S. scabiei* NBRC13768 maintained in MB liquid medium was inoculated 150 μL into the wells after unifying 0.04 in an absorbance at 595 nm. The plates were incubated at 27 °C for 72 h without shaking. After 72 h, absorbance at 595 nm was measured using iMark (Bio-Rad Laboratories, Hercules, CA) ($n = 3$). The IC_{50} values were calculated by GraphPad Prism (GraphPad Software, San Diego, CA).

DiSC$_3$(5) fluorescence assay

The membrane permeability of tumescenamide C was tested using the voltage-sensitive dye DiSC$_3$(5) (3,3'-dipropylthiadicarbocyanine iodide) [14]. *S. scabiei* NBRC13768 maintained in 5 mL MB liquid medium was centrifuged at 1000 × g for 10 min to recover the cell pellet. The pellet was washed with 5 mL PBS in twice and centrifuged again. After decantation, the pellet was suspended into 10 mL PBS containing 25 mM glucose to be 0.05 in an absorbance at 595 nm and incubated at 28 °C for 15 min without shaking. After 15 min, the cell suspension was dispended into a 96 well shading plate at 180 μL/well. Then, DiSC$_3$(5) in DMSO solution (10 μL/well) was added to be a final concentration of 400 nM. The fluorescence intensity was recorded every 1 min for 5 min to stabilize the fluorescence baseline. When the fluorescence baseline was stabilized, tumescenamide C in methanol (10 μL) was quickly added to each well to be a final concentration of 2.5 μg/mL and measured fluorescence every 30 s for 30 min. As controls, methanol, water, gramicidin, and penicillin (final conc. 10 μg/mL in each compound) were tested. All fluorescence assay was in triplicate. The fluorescence plate reader utilized was Envision 2103-0020 (PerkinElmer, Waltham, MA) with excitation/emission 620/685 nm.

Peptidoglycan (PG) binding assay

Peptidoglycan (PG) in *S. scabiei* NBRC13768 and its derivative strains conferring tumescenamide C-resistance described below was isolated by the methods subjected to other gram-positive bacteria including *S. coelicolor* [15–17]. After random digestion of PG with mutanolysin [16], the mass spectra of PG fragments were recorded using a LC-ESI-IT-TOF-MS (Shimadzu, Kyoto, Japan) on the ESI-positive mode. YMC Pack Pro C18 (2.0 × 150 mm) (YMC, Kyoto, Japan) was chosen as the analytical column. The elution program was set to an isocratic of 2% B in A (0−5 min), a linear gradient of 5 to 15% B in A (5−30 min), a linear gradient of 15 to 100% B in A (30−40 min), and an isocratic of 100% B (40−55 min) at a flow rate of 0.2 mL/min. The solutions A and B are water and MeCN, respectively. Both A and B contain 0.1% HCOOH.

After verifying PG fragments with LC/MS analysis, binding ability of tumescenamide C to peptidoglycans (PG) was surveyed according to the previous procedure [14]. Tumescenamide C (15 μL of 0.2 mg/mL in methanol) and PG without mutanolysis (75 μL of 0.4 or 20 mg/mL in water) were filled up to 300 μL with 20 mM phosphate buffer (pH 7.1) in a 1.5 mL tube and co-incubated at 27 °C for 3 h with gentle shaking. The concentrations of PG in 0.4 and 20 mg/mL were set to make ratio of tumescenamide C: PG as 1:10 and 1:500 (w/w), respectively. The co-incubated solution was centrifuged at 11,000 × g for 10 min. For quantification of non-binding free tumescenamide C in the incubation mixture, the supernatant was analyzed by LC/MS/MS as selected reaction monitoring (SRM) mode using a LC-ESI-TQ-MS of LCMS8030plus (Shimadzu). YMC Pack Pro C18 (2.0 × 150 mm) was used as an analytical column. The elution program was set to an isocratic of 5% B in A (0−5 min), a linear gradient of 5 to 100% B in A (5−20 min), and an isocratic of 100% B (20−32 min) at a flow rate of 0.2 mL/min. The solution A and B are as described above. Three MS/MS fragment ions *m/z* 86.15 (CE − 44 V), 337.15 (CE − 29 V), and 136.00 (CE − 40 V), which originated from the precursor ion of the [M + H]$^+$ of tumescenamide C (*m/z* 700.43), were selected to verify the

probability of SRM monitoring. The SRM peak areas were calculated using LabSolution software (Shimadzu). As a positive control, binding ability of vancomycin was tested. The fragment ions were selected as m/z 144.05 (CE − 19 V), 100.15 (CE − 43 V), and 83.10 (CE − 46 V) from $[M + 2H]^{2+}$ of vancomycin (m/z 725.40).

Binding assay to wall teichoic acid (WTA)

The wall teichoic acid (WTA) in *S. scabiei* NBRC13768 and its derivative strains conferring tumescenamide C-resistance described below was prepared by the previous procedure and analyzed by polyacrylamide gel electrophoresis (PAGE) [18].

As the lyophilized WTA extracts showed unfavorable solubility to adjust the concentrations for subsequent binding assay using LC/MS/MS analysis and none of the remarkable UV-Vis absorbance, contents of the extracts were alternatively measured at an absorbance at 260 nm. After checking the preparation of WTA by PAGE, WTA was diluted with 1 M Tris-HCl (pH 7.8) to be 0.5, 1.0, and 2.0 in an absorbance at 260 nm. Each diluted WTA (190 μL) was mixed with 10 μL of 0.2 mg/mL tumescenamide C. The mixture was incubated for 3 h at 27 °C with gentle shaking. After centrifugation at $11,000 \times g$ for 10 min, supernatant was recovered and subjected LC/MS/MS SRM mode for quantification of liberating tumescenamide C. Analytical conditions were the same described in PG binding assay above.

Generation of tumescenamide C-resistant strains

The resistant strains for tumescenamide C were generated from *S. scabiei* NBRC13768 by successive inoculation with the crescent of tumescenamide C exposure concentration by referencing the previous report [19]. *S. scabiei* NBRC13768 maintained in MB liquid medium was diluted to be 0.02 in an absorbance at 595 nm. The diluted sample was inoculated to the flat-bottom 24 well plate (10 μL/well) filled with 980 μL/well of MB liquid medium containing the different concentrations of tumescenamide C (final conc. 0.1, 0.2, 0.4, and 0.8 μg/mL). The plate was incubated at 27 °C for 7 days with a rotary agitation (95 rpm/min). After 7 days, the strain in each well was inoculated into the new flat-bottom 24 well plate containing two-fold higher concentrations of tumescenamide C in MB medium at the same incubating conditions. By repeating this inoculation procedure three times, five resistant strains (R1, R2, R3, R4, and R5) were obtained. No contamination of the other bacteria was confirmed by inoculating them on MB agar medium for the direct colony PCR to read 16S rRNA partial sequence [20]. The laboratory glycerol stocks of the resistant strains R1-R5 were prepared and stored at −80 °C for further

experiments. The IC_{50} values of tumescenamide C for R1-R5 were evaluated as described above.

Genome mapping analysis

To compare the genetic backgrounds between wild type (WT; *S. scabiei* NBRC13768) and tumescenamide C-resistant strains, three resistant strains of R1, R3, and R4 exhibiting weak or strong resistance to tumescenamide C were selected and subjected to NGS-aided whole genome resequencing. The IC_{50} values of tumescenamide C for R1, R3, and R4 were 9.6, 19.3, and 24.1 μg/mL, corresponding to 6.4, 12.9 and 16.1 times higher IC_{50} values compared to that for the wild type (1.5 μg/mL), respectively. The genomic DNAs were prepared from the wild type and three resistant strains (R1, R3, and R4) by the conventional phenol-chloroform extraction for sequencing analysis. The sequencing analysis was outsourced to Macrogen (Seoul, South Korea) with a paired ends of 151 read length of Illumina platform (Illumina, San Diego, CA). Reads were mapped to the reference complete genome sequence of *S. scabiei* 87-22 as a gene bank ID FN554889.1 [15]. The discovery of SNPs and indels was utilized SAMTools (http://www.htslib.orgwww.htslib.org). Reads were assembled by Bio-Linux package (http://environmentalomics.org/bio-linux/) and genes containing SNP/indel were analyzed with IGV (https://software.broadinstitute.org/software/igv/). Gene annotations were searched by KEGG (https://www.genome.jp/kegg/kegg_ja.html) and NCBI (https://www.ncbi.nlm.nih.gov/). Alignment figure was depicted using BioEdit [21].

Evaluation of thaxtomin A production

Thaxtomin A production was analyzed in each strain. The ISP-4 2% agar medium containing 5% cellobiose was selected as the production medium of thaxtomin A [22]. The agar plate inoculated each strain was placed at 27 °C for 30 days to cover up aerial mycelia on gel surface sufficiently. Then, gels were crushed into 0.5−0.7 cm cubes and moved to 50 mL conical tubes for extraction with methanol. Extraction was conducted at 27 °C for 3 h with rotary shaking (95 rpm/min). After centrifugation at $3600 \times g$ for 20 min, supernatant was filtered through gauze and concentrated *in vacuo*. After desalting by the SPE cartridge Strata C18-E (Phenomenex, Torrance, CA), organic component absorbed in the cartridge was eluted with methanol and dried up *in vacuo*. The dried eluent was resolved in methanol to be a concentration of 1 mg/mL for the LC/MS/MS analysis. Mass spectra were obtained using LC-ESI-TQ-MS of LCMS8030plus (Shimadzu) on the ESI positive and negative modes. Unison UK-C18 (2.0 × 150 mm) (Imtakt, Kyoto, Japan) was used as the analytical column. The elution program was set to an isocratic of 5% B in A

Fig. 2 Membrane permeability of tumescenamide C. Membrane permeability was indicated by increases in the fluorescence intensity following the reaction involving the voltage-sensitive fluorescent dye DiSC$_3$(5). Gramicidin, which has membrane disrupting activity, was selected as the positive control, penicillin, which targets cell wall components, was selected as the negative control
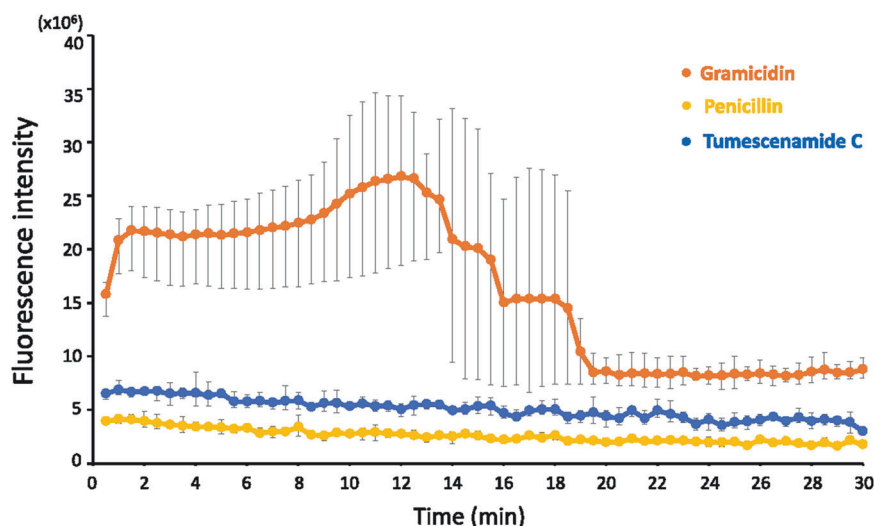
(0−5 min), a linear gradient of 5−100% B in A (5−20 min), an isocratic of 100% B (20−32 min) at a flow rate of 0.2 mL/min. The solution A and B are as described above. The ^{1}H NMR spectrum of the extract was recorded on a JEOL spectrometer at 500 MHz (Tokyo, Japan). The ^{1}H chemical shifts (δ) are shown relative to the residual solvent in CD$_3$OD: δ_H 3.30. Chemical shifts (δ) are shown in parts per million (ppm).

Cross-resistance assay

Cross-resistance for the other antibiotics were tested in three tumescenamide C-resistant strains (R1, R3, and R4) by a paper disc diffusion assay. An optional series of antibiotics (novobiocin, puromycin, tetracycline, and penicillin) were separately loaded onto the paper discs (ϕ 8.0 mm, ADVANTEC Toyo Roshi, Tokyo, Japan) to be the concentrations at 0.5−100 μg/disc. The discs containing each antibiotic was placed MB 2% agar medium after inoculation of each resistant strain. Incubation was conducted at 27 °C for 9 days. The compound amounts on disc were tested by preliminary experiments; novobiocin (0.5, 1, 10, and 100 μg/disc), puromycin (1, 10, and 100 μg/disc), tetracycline (1, 10, and 100 μg/disc), and penicillin (0.5, 1, 2.5, and 10 μg/disc). The inhibitory zones were measured every day for 9 days.

Results

Antimicrobial activity of tumescenamide C against *Streptomyces scabiei*

We found that tumescenamide C is a cyclic lipodepsipeptide with selective antimicrobial activity against *Streptomyces* spp [10, 11]. This *Streptomyces*-selective feature of tumescenamide C could be a promising controlling agent against scab-forming *Streptomyces* spp. First, the virulence of the scab-forming *Streptomyces scabiei* NBRC13768 was assessed. The development of a necrotic lesion on a sliced potato cube verified the virulence of the strain that causes common scab (Fig. 1a). Then, the analysis of the antimicrobial activity of tumescenamide C against *S. scabiei* NBRC13768 indicated the IC$_{50}$ value was 1.5 μg/mL (Fig. 1c), which was consistent with the minimal amount required to inhibit the growth of other *Streptomyces* spp [10, 11].

Evaluation of the membrane permeability and cell wall binding ability of tumescenamide C

To clarify the mechanism underlying the antimicrobial activity of tumescenamide C, its membrane permeability was measured using the voltage-sensitive fluorescent reagent DiSC$_3$(5) [14]. The positive control (gramicidin) increased the intensity of the DiSC$_3$(5) fluorescence, reflecting its membrane permeability. In contrast, the cell wall-targeting antibiotic penicillin and tumescenamide C did not increase the fluorescence intensity, indicative of a lack of membrane permeability (Fig. 2). We subsequently examined the ability of tumescenamide C to bind to cell wall components. First, peptidoglycan (PG), which is a major bacterial cell wall component, was isolated from *S. scabiei* NBRC13768 and compared with the structurally characterized PG of *S. coelicolor* [15]. The LC/MS analysis detected various ions with *m/z* values corresponding to *S. coelicolor* PG fragments [13768 (WT) in Table S1]. Notably, the detection of the monomers Tetra and Penta as well as the dimer TetraTetra suggests that the PG of *S. scabiei* NBRC13768 is structurally similar to that of *S. coelicolor*.
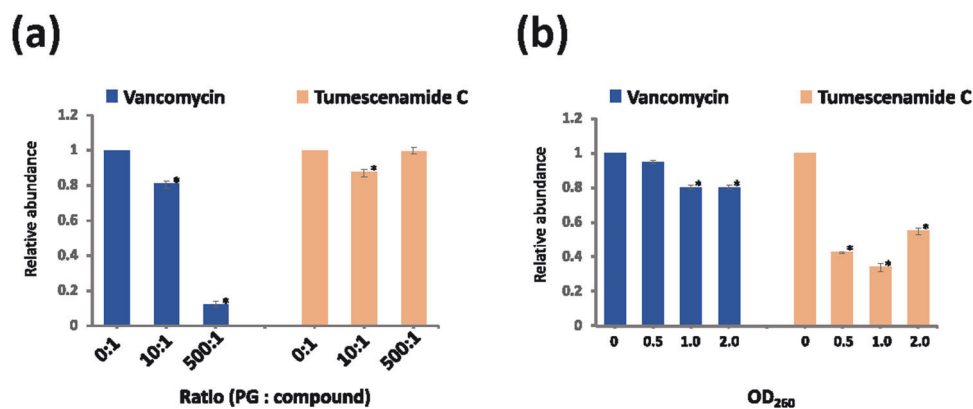
Fig. 3 Binding of tumescenamide C to peptidoglycan and wall teichoic acid. **a** Assay of the binding to peptidoglycan (PG). The *x*-axis presents the PG:compound ratio (w/w). The *y*-axis presents the relative abundance, which was calculated using the following formula: (SRM peak area of the compound in PG:compound = 10:1 or 500:1)/(SRM peak area of PG:compound = 0:1). **b** Assay of the binding to wall teichoic acid (WTA). The extract content was determined according to the absorbance at 260 nm (*x*-axis). The *y*-axis presents the relative abundance as in (**a**). Vancomycin was selected as the positive control. The asterisk indicates a significant difference ($P < 0.01$) when compared with the relative abundance for 0:1 in (**a**) or 0 in (**b**)

Second, the potential binding between *S. scabiei* NBRC13768 PG and tumescenamide C was analyzed on the basis of the decreasing peak area of the tumescenamide C SRM ions. The peak area for positive control (vancomycin) decreased in a dose-dependent manner, which was in accordance with the fact this antibiotic targets PG. However, the lack of a dose-dependent decrease in the peak area implied tumescenamide C does not bind to PG (Fig. 3a).

We also examined the potential binding between tumescenamide C and wall teichoic acid (WTA), the characteristic cell wall component of gram-positive bacteria. After analyzing the WTA obtained from the strain by PAGE (Fig. S1) [18], the binding between tumescenamide C and WTA was evaluated. The vancomycin peak area decreased slightly in accordance with the change in the WTA content. In contrast, the tumescenamide C peak area decreased to 0.43 (relative abundance) during the incubation with the low absorbance WTA extracts (absorbance at 260 nm of 0.5) (Fig. 3b). Although a distinct dose-dependent decrease in the peak area was not observed for WTA, tumescenamide C may target this cell wall component and/or affect its biosynthesis.

Generation of tumescenamide C-resistant strains and comparison of their genetic backgrounds

S. scabiei NBRC13768 strains resistant to tumescenamide C were generated, after which their genetic backgrounds were compared with that of the tumescenamide C-susceptible WT strain. Inoculations with increasing tumescenamide C concentration produced five resistant strains (R1, R2, R3, R4, and R5). The IC$_{50}$ values were >6.4-times higher for the
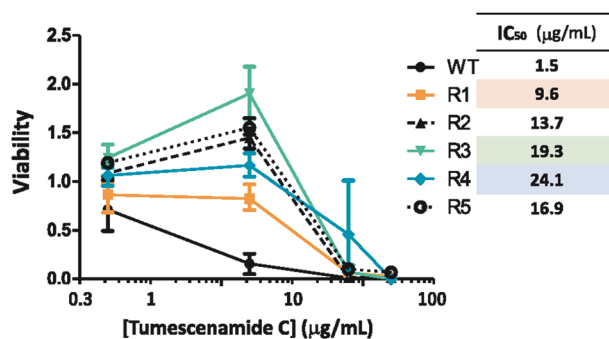

Fig. 4 Antimicrobial activity of tumescenamide C against tumescenamide C-resistant strains. The IC$_{50}$ values were >6.4 times higher for the tumescenamide C-resistant strains than for the wild-type (WT) strain. The resistant strains that underwent the whole genome resequencing analysis are highlighted in orange (R1), green (R3), and blue (R4)

R1-R5 strains than the WT *S. scabiei* NBRC13768 (Fig. 4). More specifically, the IC$_{50}$ values of tumescenamide C for the R1, R3, and R4 strains were 9.6, 19.3, and 24.1 µg/mL, which were 6.4-, 12.9-, and 16.1-times higher than that of the WT control (1.5 µg/mL), respectively. To compare the genetic backgrounds, the WT control and the three resistant strains (R1, R3, and R4) exhibiting weak or strong resistance to tumescenamide C were subjected to NGS-aided whole genome resequencing. The assembled NGS sequences revealed the high GC contents, which were consisted with that of the complete *S. scabiei* 87-22 strain sequence genome (71.5%) [23]. The reads for WT, R1, R3, and R4 were mapped to the reference genome sequence (Table S2). Genes containing variants, such as SNPs and Indels, were extracted from the mapping data (Table S3). The annotation

Fig. 5 The annotation process to extract the mutation genes in the tumescenamide C-resistant strains; R1, R3, and R4.
a Variants (SNPs and indels) in WT, R1, R3, and R4 were analyzed by mapping sequences on the complete *S. scabiei* 87-22 reference genome (FN554889.1, see Tables S2 and S3). By comparing each resistant strain vs WT, variants specific in the three resistant strains were picked up. In them, 162 genes with 409 variants were commonly observed in the three resistant strains, but not in WT. By BLAST searching of the 162 genes, a total of 19 genes were identified and listed in Table 1 as the variant genes common in the three resistant strains. Other genes were uncharacterized by the BLAST searching. **b** The variants specific genes in each resistant strain are listed in the supplementary Excel files entitled R1 vs WT, R3 vs WT, and R4 vs WT
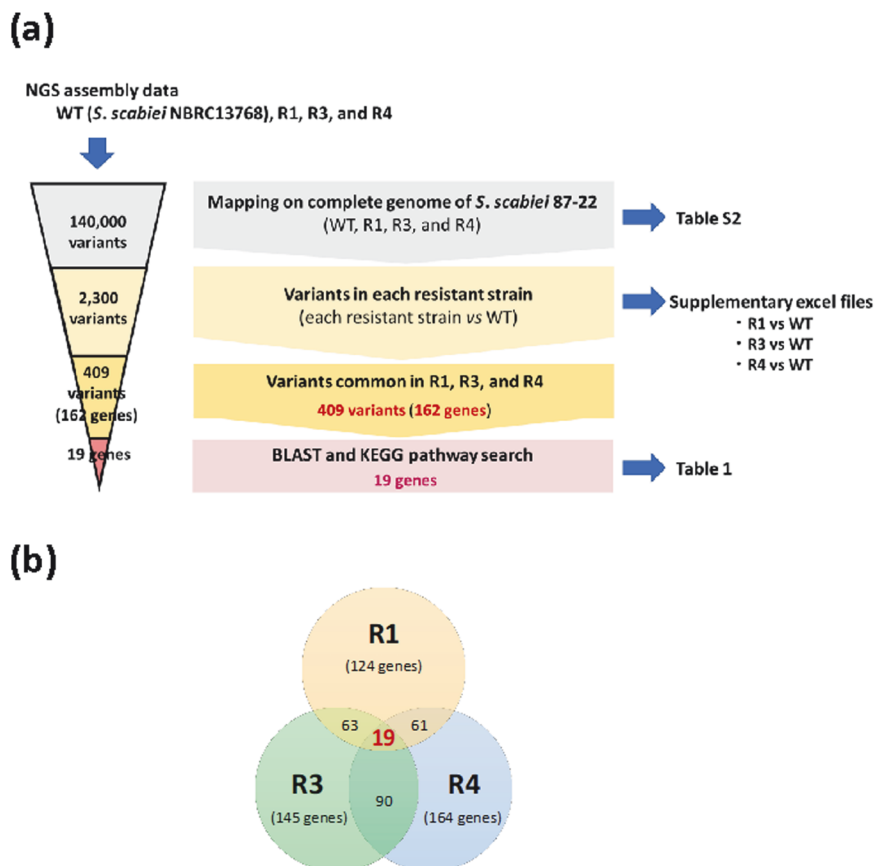
process is depicted in Fig. 5. A total of 19 genes with variants affecting amino acid sequences in the three resistant strains (R1, R3, and R4), but not in WT, were identified (Table 1).

Eight of the 19 genes were predicted to encode enzymes that catalyze the degradation of alcohols, sugars, and proteins. The structure/motif associated with their variant positions was unidentified. Additionally, the motif of one gene encoding a stress response protein was uncharacterized. The structure/motif associated with the valiant positions in the other 10 genes were predicted. Interestingly, of these 10 genes, the following four were related to sugar transport systems: *cebF* (SCAB_2411), *cebG* (SCAB_2401), *cellobiose transport system permease* (SCAB_57741), and *sugar transport system permease* (SCAB_28151). Moreover, SCAB_2411, SCAB_2401, and SCAB_57741 encode components of the cebEFG cellobiose/cellotriose transport system [22, 24]. This system triggers the production of the phytotoxic compound thaxtomin A, which contributes to the necrosis observed in root vegetables infected with the common scab disease [7, 12, 24–26].

To date, two cebEFG systems have been reported [24]. The main cellobiose/cellotriose transporter genes (*cebEFG1*) in *S. scabiei* 87-22 are located in SCAB_57721-

57761. As orthologous sequences, *cebEFG2* (SCAB_2391-2431) encode proteins that are reportedly similar to cebE1, cebF1, and cebG1 (46%, 58%, and 54% amino acid sequence identities, respectively). Experiments in which *cebE1* and *cebE2* are deleted revealed both genes encode proteins responsible for thaxtomin production. Among the phytopathogenic *Streptomyces* spp., only *S. scabiei* contains cebEFG2, which probably serves as a safeguard against the loss of the main transporter cebEFG1 [24]. The genes *cebF* (SCAB_2411), *cebG* (SCAB_2401), and *cellobiose transport system permease* (SCAB_57741) in Table 1 likely correspond to *cebF2*, *cebG2*, and *cebF1*, respectively (Table 1).

Thaxtomin A production by the three tumescenamide C-resistant strains

Because the variants in the tumescenamide C-resistant strains were in genes encoding components of the cellobiose/cellotriose transport system, we analyzed the production of thaxtomin A by the three resistant strains. Thaxtomin A was undetectable in the cellobiose-containing liquid media (e.g., ISP-4 medium) inoculated with *S. scabiei* NBRC13768. Thus, extracts were prepared from solid ISP-4 medium containing 5% cellobiose and 2% agar [27].

Table 1 Gene variants common to all three tumescenamide C-resistant strains

			Position in variant					
Gene No.	Gene name	Function	87-22	WT	R1	R3	R4	Variant position
SCAB_2401 carbohydrate ABC transporter permease	carbohydrate transport		A119	A119	S119	S119	S119	Adjacent of F118, a residue constructing dimer interface
SCAB_2411 sugar ABC transporter permease	carbohydrate transport		I184	I184	V184	V184	V184	Three residues away of Q181, a residue constructing putative periplasmic-BP binding loops
SCAB_3831 GabD (aldehyde dehydrogenase family protein)	GABA shunt pathway		D245	D245	G245	G245	G245	D245, G247 ... catalytic residues with NAD(P) binding site
			G247	G247	D247	D247	D247	
			D259	D259	A259	A259	A259	D259, W273 ... within catalytic region
			W273	W273	R273	R273	R273	
SCAB_3851 α-L-fucosidase	N-glycan degradation		C90	C90	W90	W90	W90	
SCAB_4031 HAMP domain-containing sensor histidine kinase	stress response, two component system		R170	R170	H170	H170	H170	Adjacent of L169, a residue constructing dimer interface
SCAB_4241 universal stress protein	stress response		H202	D202	N202	N202	N202	
SCAB_4611 LuxR family transcriptional regulator	transcriptional regulator		N878	N878	Y878	Y878	Y878	A residue for DNA binding site
SCAB_6541 alcohol dehydrogenase catalytic domain-containing protein	alchol metabolism ?		V30	V30	L30	L30	L30	
			F34	F34	Y34	Y34	Y34	
			Q54	Q54	A54	A54	A54	
			N92	N92	D92	D92	D92	
			Q98	Q98	R98	R98	R98	
			R101	N101	H101	H101	H101	
			Q102	Q102	R102	R102	R102	
			I104	I104	V104	V104	V104	
			H106	H106	Q106	Q106	Q106	
SCAB_6551 SDR family oxidoreductase	NAD- or NADP-dependent redox		T78	N78	A78	A78	A78	T78, H104 ... within NAD(P) binding site
			H104	Q104	D104	D104	D104	T119, T123, I128 ... within active site
			T119	T119	S119	S119	S119	
			T123	T123	A123	A123	A123	
			I128	I128	V128	V128	V128	
SCAB_6581 aldo/keto reductase	carbohydrate degradation		E45	E45	K45	K45	K45	
			F106	F106	S106	S106	S106	
			T140	T140	S140	S140	S140	
			I171	I171	M171	M171	M171	
			G172	S172	R172	R172	R172	
			E219	E219	A219	A219	A219	
			V225	A225	G225	G225	G225	
SCAB_8501 aldehyde dehydrogenase family protein	primery metabolism ?		D42	D42	V42	V42	V42	
			K145	M145	R145	R145	R145	
SCAB_12341 dioxygenase (catechol 1,2-dioxygenase)	benzoate degradation etc		E110	E110	K110	K110	K110	
SCAB_16371 DUF4357 domain-containing protein	restriction endonuclease		H435	H435	Y435	Y435	Y435	Adjacent of F436, a residue constructing walker B motif
SCAB_19651 putative quinone binding protein	unknown		L90	G90	A90	A90	A90	
			A91	deletion 91	G91	G91	G91	
SCAB_28151 carbohydrate ABC transporter permease	multiple carbohydrate transport		K162	K162	E162	E162	E162	Adjacent of successive 13 residues (E163-M175) constructing ABC-ATPase subunit interface
SCAB_36291 chaperonin GroEL	protein folding		Q154	K154	H154	H154	H154	Five residues away of F149, a residue constructing ATP/Mg binding site
SCAB_53281 putative peptidase	protein degradation		S237-D238	Insertion S237-P-D238	Insertion S237-WWP-D238	Insertion S237-WWP-D238	Insertion S237-WWP-D238	
SCAB_57741 putative cellobiose binding-protein-dependent transport system	cellobiose transport		Y99	deletion Y99	deletion Y99	deletion Y99	deletion Y99	Y99, Q179 ... residues constructing dimer interface
			Q179	deletion	deletion	deletion	deletion	
			S203	Q179	Q179	Q179	Q179	S203 ... a residue for ABC-ATPase subunit interface
			I152	deletion S203	deletion S203	deletion S203	deletion S203	I152 ... adjacent of K151, putative periplasmic BP binding loops
				I152	F152	F152	F152	
SCAB_69911 transaldolase	pentose phosphate pathway etc		N234	Y234	H234	H234	H234	

The 19 listed genes contain variants that resulted in amino acid substitutions. Amino acid substitutions in important motifs (e.g., active site, NAD-binding site, and dimer-forming region) are indicated in red

The genes in bold and highlighted in blue encode components of the cellobiose/cellotriose transport system *cebEFG*


Fig. 6 Extracted ion currents of thaxtomin A. Chromatograms were drawn as an extracted ion current (EIC) with m/z 439.15 corresponding to the $[M + H]^+$ of thaxtomin A. The differences in the thaxtomin A peak were confirmed via MS/MS fragmentation, ^{1}H NMR analysis, and necrosis assay (see Fig. S2)
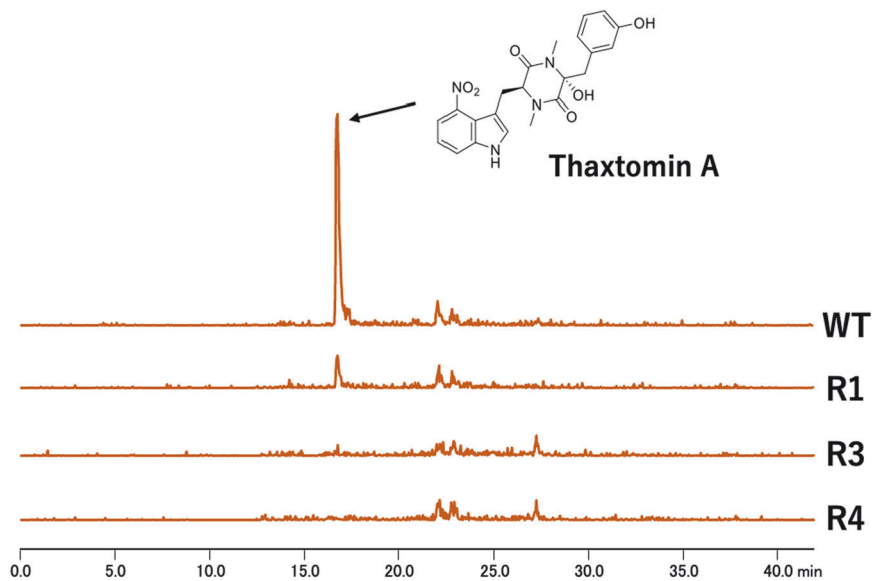
Hence, ensuring the turbidity of the samples with consistent was difficult. The weight of the gel extracts varied among the samples (1.3–1.6 mg for WT, 1.8–2.5 mg for R1, 1.4–1.6 mg for R3, and 1.0–1.3 mg for R4). All gel extracts (1 mg/mL in methanol) were included in the LC/MS analysis. Surprisingly, the thaxtomin A peaks were substantially lower for R1, R3, and R4 than for WT (Fig. 6, Fig. S2). For R3 and R4, which were highly tolerant to tumescenamide C (IC$_{50}$ of 19.3 and 24.1 μg/mL for R3 and R4, respectively; Fig. 4), the EIC peaks of thaxtomin A were nearly disappeared. Consistent with this observation, the virulence of R3 and R4 were also decreased (Fig. 7).
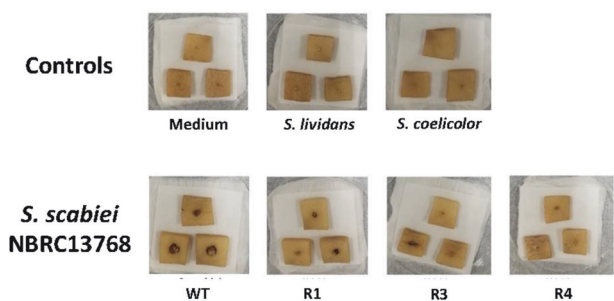
Fig. 7 Necrosis assay for the tumescenamide C-resistant strains. Medium in control is the MB medium alone. *S. lividans* and *S. coelicolor* were placed as the paradigms of non-phytopathogenic *Streptomyces* spp. WT denotes *S. scabiei* NBRC13768. The R1, R3, and R4 are tumescenamide C-resistant strains derived from WT. The phytopathogenesis in the resistant strains was compared to WT

Table 2 Cross-resistance of three tumescenamide C-resistant strains to selected antibiotics

Strain	Novobiocin (0.5 µg/disc)	Puromycin (100 µg/disc)	Tetracycline (10 µg/disc)	Penicillin (2.5 µg/disc)
WT	21.7	12.7	27.1	13.2
R1	28.3	14.1	27.7	25.2
R3	24.6	12.4	26.1	14.6
R4	25.2	8.7	29.4	9.5

Novobiocin, puromycin, tetracycline, and penicillin affect DNA synthesis (DNA gyrase), protein synthesis (ribosome), protein synthesis (30S ribosomal subunit), and cell wall synthesis (penicillin-binding protein), respectively. Values refer to the average diameters of the growth inhibition zones (mm)

Cross-resistance assay and phenotypic evaluation of the three tumescenamide C-resistant strains

The WT, R1, R3, and R4 strains were compared in terms of their cross-resistance to the following antibiotics affecting different cellular processes: novobiocin (DNA synthesis), puromycin and tetracycline (protein synthesis), and penicillin (cell wall synthesis). For the novobiocin, puromycin, and tetracycline treatments, the growth inhibition zones were similar between the resistant strains and the WT strain, implying these antibiotics and tumescenamide C have different targets. However, the inhibitory effect of the penicillin treatment clearly differed between the WT and resistant strains. Furthermore, the growth inhibition zone diameters varied among the three resistant strains (Table 2). The R1 and R4 strains were respectively more and less susceptible to penicillin than the WT strain. In contrast, the R3 and WT strains were similarly susceptible to penicillin (Fig. S3). The changes in the susceptibility to penicillin among R1, R3, and R4 were suggestive of cell wall modifications. Accordingly, the strains were compared regarding their tolerance to temperature and osmotic stress. As expected, these three strains were differentially tolerant to the two tested abiotic stress (Fig. S4). More specifically, R1 (highly susceptible to penicillin) was highly tolerant to the temperature and osmotic stresses, whereas R3 and R4 were relatively susceptible to these stresses.

Wall teichoic acid (WTA) contents and cell wall-related gene variants in the three tumescenamide C-resistant strains

In the gram-positive methicillin-resistant *Staphylococcus aureus* (low GC content), changes in the susceptibility to penicillin and the tolerance to temperature and osmotic stresses are reportedly related to modifications to the cell wall component WTA [28]. In the current study, WTA and PG in the three tumescenamide C-resistant strains were analyzed. The detected PG-related ions were abundant in three resistant strains compared to those of WT strain (Table S1). In WTA, some remarkable differences were found in the migration of WTA in the PAGE gel (Fig. S5). The WTA bands for R3 and R4, which were respectively similarly susceptible and less susceptible to penicillin (compared with WT; Fig. S3), were less intensely stained and shifted upward compared with the WT WTA band, suggesting a high WTA content may enhance the susceptibility to tumescenamide C. The WTA band of R1 (highly susceptible to penicillin) migrated to a similar position as the WT WTA band, but it was more intensely stained (Fig. S5).

The differences in the susceptibility to the cell wall-targeting antibiotic penicillin, the susceptibility to temperature and osmotic stress, and the WTA band patterns among R1, R3, and R4 implied the resistance of these three strains to tumescenamide C was due to diverse modifications to the cell wall system. We re-analyzed the whole genome resequencing data to examine the gene variants in each resistant strain (WT vs R1, WT vs R3, and WT vs R4). The main gene variants in R1, R3, and R4 were detected in sequences encoding a response regulator transcription factor (18 nonsynonymous mutations), an ABC transporter substrate-binding protein (seven nonsynonymous mutations), and formyltetrahydrofolate deformylase (eight nonsynonymous mutations), respectively, indicating the tumescenamide C resistance of the three strains was not due to changes in the same functional protein or genetic locus (supplementary Excel files entitled R1 vs WT, R3 vs WT, and R4 vs WT). Next, genes encoding proteins contributing to cell wall synthesis were identified in the three tumescenamide C-resistant strains. After excluding the gene encoding unknown proteins, 12 of 70 in R1, 15 of 69 in R3, and 7 of 80 genes in R4 were related to cell wall synthesis (Fig. S6). Gene variants affecting PG synthesis were detected in all three strains, but variants were not detected in genes associated with WTA (Table S4).

Discussion

The results of the present study indicate tumescenamide C has potent antimicrobial effects on *S. scabiei*, the major phytopathogen causing common scab disease (Fig. 1c). To identify the gene variants associated with the resistance to tumescenamide C, three resistant strains R1, R3, and R4 were selected the whole genome resequencing because they had relatively low and high IC_{50} values (i.e., 9.6, 19.3, and 24.1 µg/mL for R1, R3, and R4, respectively) (Fig. 4). Intriguingly, 4 of 19 genes with variants in the three resistant strains encoded components of the carbohydrate transport system located in the periplasmic region (Table 1). Notably, SCAB_2411, SCAB_2401, and SCAB_57741 encode cellobiose/cellotriose transport system components; *cebF2*, *cebG2*, and *cebF1*, respectively, which are responsible for the production of phytotoxic compound thaxtomin [3, 22, 24].

In accordance with the mutations in the genes encoding *ceb*s, a substantial decrease in the thaxtomin A peak was observed in the R1, R3, and R4 (Fig. 6). Moreover, the phytopathogenic effects of these three resistant strains decreased (Fig. 7). These results suggested that resistance of tumescenamide C might affect phytopathogenicity because of the associated decrease in the production of the major phytotoxin thaxtomin A. Although the nonsynonymous variants in *cebF2*, *cebG2*, and *cebF1* were located near or in the sequence encoding amino acid residues in important motifs, such as dimer interfaces and ATP-binding motifs (Table 1), most of the substituted amino acids were replaced by structurally similar amino acids (Table 1). The effects of these substitutions on the conformations of cebF1, cebF2, and cebG2 remain unknown because of a lack of information regarding their crystallographic structures. These findings are quite interesting, but decreases in thaxtomin A production and phytopathogenicity are not directly correlated with the antibacterial activity of tumescenamide C.

The other genes listed on Table 1 were encoding the transcriptional regulator LuxR family (SCAB_4611), two-component systems (SCAB_4031), and chaperon GroEL (SCAB_36291), respectively. These proteins are known as attractive targets of antibiotics with a wide range of antibacterial spectra because of their conserved amino acid residual sequences and functional redundancy [29, 30]. However, these proteins might not be an important for the tumescenamide C mode of action because of its characteristic antibacterial spectrum feature (e.g., 3−30 µg/disc in *Streptomyces* spp. and >100 µg/disc in *S. aureus*) [10]. Thus, further refinement for the molecular target of tumescenamide C could not work well. To obtain some clues regarding the molecular target, cross-resistance assay involving known antibiotics was performed.

In the cross-resistance assay, the antibacterial effects of penicillin differed between the WT and resistant strains. Notably, the susceptibility of the R1 strain to penicillin increased (Table 2, Fig. S3). Earlier research on methicillin-resistant *S. aureus* indicated that an increase in the susceptibility to penicillin is correlated with defective WTA biosynthesis [28, 31–33]. According to the in vitro binding assay, tumescenamide C can bind to WTA, but not to PG (Fig. 3b). These observations suggest that the cell wall components could be a target of tumescenamide C. The cell wall biosynthesis-related systems in the tumescenamide C-resistant strains may have been altered, thereby limiting the adverse effects of tumescenamide C. PG, which is the main cell wall component, was also analyzed for each strain. For PG, in addition to the variable fragment ions due to the random digestion with mutanolysin during the preparation of PG fragments for the mass spectroscopy, fragments of the same PG dimer and monomer structures were obtained for the WT and three resistant strains (Table S1). For WTA, the R1 strain (increased susceptibility to penicillin) had the highest WTA content among the examined strains (Fig. S5). This result was unexpected because the high susceptibility of methicillin-resistant *S. aureus* to penicillin is reportedly correlated with a decrease in the WTA content [28, 31–33]. The relationship between a high WTA content and enhanced susceptibility is currently unknown. However, high WTA contents might affect the overall cell wall structure or cell wall system, ultimately leading to increased susceptibility to penicillin. The other strains R3 and R4 slightly altered migration patterns on WTA-PAGE (Fig. S5), suggesting some changes of the cell wall system both in quality and quantity. The relatively high frequency in PG-fragment ions detected in three resistant strains compared to WT might be due to the diverse extraction efficiencies caused by the changes in cell wall intensity or system in each strain (Table S1). Taking these data and the result that tumescenamide C showed the binding ability to WTA but not PG (Fig. 3), WTA is a crucial factor of antimicrobial activity by tumescenamide C. The cell wall system (including WTA) is a vital, but structurally diverse, part of gram-positive bacteria [34, 35]. The structure of the outer part of the cell wall is recognized as an allergen by the mammalian immune-system [35]. It is reasonable to speculate that the *Streptomyces*-selective antibiotic activity of tumescenamide C is related to the fact this compound targets the peripheral cell wall system. We previously determined that the α,β-unsaturated moiety of Abu and branched lipophilic side chain (Dmh) are crucial for the antimicrobial activity of tumescenamide C [11]. The lipophilic side chain might anchor the compound in the cell wall, whereas the α,β-unsaturated moiety may act as the Michael acceptor [36] of cell wall component, including WTA.

Because we should consider the uncharacterized or overlooked genes as well as the mapping gaps, future studies will need to identify the molecular target of

tumescenamide C. A recent transcriptomic analysis clarified the virulome response to cello-oligosaccharide elicitor in *S. scabiei* 87-22, suggesting the similar approach may be useful for further characterizing tumescenamide C [25]. Moreover, molecular probe-based examinations of tumescenamide C may be an alternative strategy.

In conclusion, the findings of this study elucidated the antimicrobial activity of tumescenamide C against the scab-causing actinobacteria *S. scabiei*. Additionally, three tumescenamide C-resistant strains generated in this study contained nonsynonymous mutations in the genes related to the cellobiose/cellotriose transport system (i.e., *cebEFG1* and *cebEFG2*). This transport system is crucial for the production of thaxtomin A, which promotes necrosis in root crops infected by the scab-causing phytopathogen *S. scabiei*. Comparing with the WT strain, the three tumescenamide C-resistant strains with nonsynonymous variants in *cebEFG* genes produced less thaxtomin A and were less phytopathogenic. Although the molecular target of tumescenamide C was not identified, our results suggest that cell wall components, such as WTA, may be target. More specifically, changes in the WTA contents were detected in the tumescenamide C-resistant strains, while the binding between WTA and tumescenamide C was confirmed. Research focused on identifying the molecular target and detailed mode of action of tumescenamide C is currently underway in our laboratory.

Data availability

Data sets generated for this study are included in this article or supporting information; further information can be directed to the corresponding author (H.K.).

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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