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Research

Solanoeclepin C, a root-secreted molecule converted by rhizosphere microbes to hatching factors for potato cyst nematodes

Ryota Akiyama[1,2], Yui Kawano[1], Kosuke Shimizu[1], Soichiro Makino[1], Karen Akanuma[1], Haru Nagatomo[1], Masami Yokota Hirai[2], Yukihiro Sugimoto[1], Atsuhiko Kushida[3], Keiji Tanino[4] and Masaharu Mizutani[1]

[1]Graduate School of Agricultural Science, Kobe University, Rokkodai 1-1, Nada, Kobe, Hyogo, 657-8501, Japan; [2]RIKEN Center for Sustainable Resource Science, Suehiro-cho 1-7-22, Tsurumi-ku, Yokohama, Kanagawa, 230-0045, Japan; [3]Technology Application Research Team, Department of Research Promotion, Hokkaido Agricultural Research Center, NARO, 1 Hitsujigaoka, Toyohira, Sapporo, Hokkaido, 062-8555, Japan; [4]Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo, Hokkaido, 060-0810, Japan

Authors for correspondence:
Ryota Akiyama
Email: ryota.akiyama@riken.jp

Masaharu Mizutani
Email: mizutani@gold.kobe-u.ac.jp

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Summary

- Eclepins are root-secreted compounds that induce the hatching of cyst nematodes. Solanoeclepin A (SEA) and B (SEB) have been isolated as potent hatching factors for potato cyst nematodes (PCNs). SEB is biosynthesized in roots, released into the rhizosphere, and converted into SEA by soil microorganisms. However, given that SEB and SEA exhibit comparable hatching-inducing activity toward PCNs, the ecological significance of microbial solanoeclepin metabolism in eclepin-mediated communication remains unclear.
- In this study, we identified solanoeclepin C (SEC), a previously unrecognized solanoeclepin secreted by tomato and potato roots. Structural analysis revealed that SEC is an acetylated derivative of SEB. Soil incubation experiments demonstrated that SEC is converted into SEB and subsequently into SEA. SEC exhibits 10 000-fold lower hatching-inducing activity than SEA.
- Gene expression analysis in hydroponically grown tomatoes showed that solanoeclepin biosynthesis is upregulated under nitrogen and phosphorus deficiencies, with nitrogen starvation having the strongest effect.
- Our results demonstrate that although SEC itself exhibits low hatching-inducing activity, it is converted by soil microorganisms into SEB and SEA, which are then exploited by PCNs to trigger their hatching. These findings reveal a previously unrecognized three-way interaction among plants, soil microbes, and nematodes mediated by solanoeclepins.

Introduction

Plants have evolved the ability to produce various specialized metabolites with diverse biological activities. Plants use these compounds to regulate their surroundings through interactions with other plants, insects, animals, and microbes. These metabolites are produced in response to certain environmental or developmental conditions and act as signaling molecules, attractants, repellents, or inhibitors of other organisms (Peters *et al.*, 1986; Akiyama *et al.*, 2005; McCormick *et al.*, 2012). For instance, strigolactones (SLs) induce hyphal branching of arbuscular mycorrhizal (AM) fungi, which provide water and nutrients, particularly phosphorus, to the host plants (Akiyama *et al.*, 2005). However, SLs were originally discovered as host plant-derived cues that stimulate the germination of Striga seeds, a notorious group of root-parasitic weeds (Cook *et al.*, 1966). Plant metabolite-mediated communication systems are multidimensional and highly complex.

Eclepins, a group of specialized metabolites in plants, were isolated as hatching factors (HFs) for cyst nematodes. Cyst nematodes are root-parasitic nematodes, with the genera *Heterodera* and *Globodera* being particularly damaging to agricultural production world-wide (Williamson & Gleason, 2003; Nicol *et al.*, 2011; Jones *et al.*, 2013). At the end of a life cycle, cyst nematodes form a cyst, a hardened female body enclosing hundreds of eggs forming the next generation (Masler & Perry, 2018). The cyst remains in the soil even after the host plant dies, and the eggs in the cyst can survive in a dormant state; in the case of potato cyst nematodes (PCNs), they are capable of surviving for up to 20 yr (Wright & Perry, 2006; Devine, 2010). When suitable host plants grow nearby, the eggs hatch in response to HFs secreted by the host roots. In the 1980s, three eclepins – glycinoeclepins A, B, and C (GEA, GEB, and GEC) – were isolated from kidney bean roots as HFs for soybean cyst nematode (SCN, *Heterodera glycines*) (Masamune *et al.*, 1982, 1987; Fukuzawa *et al.*, 1985). Similarly, solanoeclepin A (SEA)

was isolated from potato root exudates as an HF for PCNs (*Globodera rostochiensis* and *Globodera pallida*) (Mulder *et al.*, 1996; Schenk *et al.*, 1999). More recently, solanoeclepin B (SEB) was identified from potato and tomato as an HF for PCNs (Shimizu *et al.*, 2023). SEA and SEB can induce the hatching of PCNs at extremely low concentrations (10^{-8} to 10^{-10} g ml^{-1}). To date, five solanoeclepin biosynthetic genes have been identified in tomato, and knocking out these genes has been shown to reduce the risk of PCN hatching (Shimizu *et al.*, 2023). In this way, eclepins function as signaling molecules in the soil, inadvertently exposing the plant to enemy threats. On the other hand, the production of eclepins in evolutionarily distant Legume and Solanaceae plants suggests a potential role in plant survival strategies beyond nematode hatching, although their specific functions in this context remain uncharacterized. An example indicating the involvement of organisms other than plants and cyst nematodes in eclepin-mediated interspecies communication is the microbial conversion of solanoeclepins in the soil. Recent research has demonstrated that plants do not produce SEA directly; rather, SEA is generated through the microbial conversion of SEB (Shimizu *et al.*, 2023). However, given that SEB and SEA exhibit comparable hatching-inducing activity toward PCNs, the impact of microbial solanoeclepin metabolism on eclepin-mediated communication systems remains unclear.

In this study, we discovered a previously unknown eclepin, solanoeclepin C (SEC), which is converted into SEB and SEA by soil microorganisms. Notably, SEC itself exhibits *c.* 10 000-fold lower hatching-inducing activity than SEB and SEA but is efficiently converted into these hatch-inducing active forms by soil microorganisms. These findings highlight the critical role of soil microorganisms in mediating plant–cyst nematode interactions and provide insights into the complex and multidimensional interactions mediated by plant metabolites in the soil ecosystem. Furthermore, we found that solanoeclepin production is induced under nitrogen- or phosphate-deficient conditions, with nitrogen deficiency having a particularly strong effect. This study highlights the critical role of soil microorganisms in mediating plant–cyst nematode interactions. Moreover, our findings lay the foundation for uncovering additional, yet unexplored functions of eclepins beyond cyst nematode hatching, particularly under nutrient-limiting conditions.

Materials and Methods

Chemicals

Solanoeclepin A used in this study was synthesized in a previous study (Tanino *et al.*, 2011). SEB used in this study was isolated in a previous study (Shimizu *et al.*, 2023).

UPLC-MS/MS analysis of solanoeclepins

The LC-MS/MS analyses other than solanoeclepin profiling shown in Figs 1(a), 3(b), and 5(b) (will be discussed later) were performed using the same method as the solanoeclepin analysis described in our previous study (Shimizu *et al.*, 2023). Specifically, the procedure was as follows. The LC-MS system (Waters, Milford, MA, USA) comprised an ACQUITY UPLC H-Class System coupled with an ACQUITY quadruple tandem mass spectrometer (TQ detector). Data acquisition and analysis were conducted using the MassLynx 4.1 software (Waters). Each sample was injected into an ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm; Waters) protected by an ACQUITY UPLC HSS T3 VanGuard Pre-column (10 mm × 2.1 mm, 1.8 μm; Waters) with a column at 40°C and a flow rate of 0.2 ml min^{-1}. The mobile phase comprised solvent A (0.1% formic acid in water) and solvent B (acetonitrile), and the following gradient was used: 10% B at 0 min, 60% B at 20 min, 100% B at 20 min, held for 25 min, reduced to 10% B after 25 min, and held for 30 min. The eluent was introduced into the electrospray ionization (ESI) ion source of the mass spectrometer, operating at optimized settings: source temperature 120°C, desolvation temperature 350°C, cone N_2 gas flow 50 l h^{-1}, and desolvation N_2 gas flow 600 l h^{-1}. The multiple reaction monitering (MRM) mode was employed in the positive ESI mode. The MRM transitions were *m/z* 499 > 399 with a cone voltage of 35 V and a collision energy of 30 eV for SEA, *m/z* 501 > 429 with a cone voltage of 30 V and a collision energy of 10 eV for SEB, and *m/z* 543 > 483 with a sample cone voltage of 30 V and a collision energy of 10 eV for SEC.

The concentration of solanoeclepins in the hydroponic tomato culture medium analyzed in Fig. 5(b) (will be discussed later) was approximately several tens of times lower than that typically found in tomato hairy root culture medium used in our previous studies. In addition, Figs 1(a) and 3(b) (will be discussed later) show time-course analyses of solanoeclepin levels in soil. Therefore, these experiments required a more sensitive and accurate LC-MS/MS method than the one previously employed. To meet this need, the analyses corresponding to Figs 1(a), 3(b), and 5(b) (will be discussed later) were conducted using a newly developed method with a Xevo TQ-S tandem quadrupole mass spectrometer (MS/MS). The detailed procedure will be described later. The ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) analysis for nutrient deficiency responses in hydroponically grown tomatoes and conversion experiments in soil was conducted under the following conditions. The UPLC I-Class System (Waters) was equipped with a binary solvent and sample manager and coupled to a Xevo TQ-S tandem quadrupole mass spectrometer (MS/MS) with an ESI interface. Each sample was injected into an ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm; Waters) protected by an ACQUITY UPLC HSS T3 VanGuard Pre-column (10 × 2.1 mm, 1.8 μm; Waters) with a column at 40°C and a flow rate of 0.3 ml min^{-1}. The mobile phase included solvent A (0.1% formic acid in water) and solvent B (acetonitrile), and the following gradient was used: 10% B at 0 min, increased to 60% B at 12 min, 100% B at 12.01 min, held for 14 min, reduced to 10% B after 14.01 min, and held for 18 min. The eluent was introduced into the ESI ion source of the mass spectrometer, operating at optimized settings: source temperature 150°C, desolvation temperature 550°C, cone N_2 gas flow 150 l h^{-1}, and desolvation N_2 gas flow 650 l h^{-1}. The MRM mode was employed in the positive ESI mode. The MRM

transitions were *m/z* 499 > 399 with a cone voltage of 30 V and a collision energy of 25 eV for SEA, *m/z* 501 > 429 with a cone voltage of 25 V and a collision energy of 20 eV for SEB, and *m/z* 543 > 483 with a sample cone voltage of 10 V and a collision energy of 15 eV for SEC.

Hydroponic cultivation of potatoes for SEC isolation

Initially, potato culture shoots, or microtubers, were sown on vermiculite spread in pots, and the seedlings were grown for 3 wk with bottom feeding of the hydroponic solution under a 16 h : 8 h, light : dark photoperiod. The plants were planted in a hydroponic facility and grown, while the roots were sprayed with a hydroponic solution. The hydroponic solution was prepared by the OTA nos. 1, 2, and 5 fertilizers (OTA house fertilizer series; OTA Agrio, Tokyo, Japan).

SEC purification from the potato hydroponic culture solution

Approximately 5000 potato plants (*Solanum tuberosum* L.) of various varieties were grown in the hydroponic facility as described previously. Then, 3 l of Sepabeads SP207 (Mitsubishi Chemical Corp., Tokyo, Japan) was placed in a plastic column and positioned where the effluent from the medium sprayed on the roots was circulated. Sepabeads SP207 was replaced every month from July 2021 to October 2021; hence, 9 l of Sepabeads SP207 adsorbed potato root exudate was collected. The total volume of hydroponic effluent that passed through SP207 during this period was estimated to be *c.* 20 000 l. Approximately 5 l of resin was packed into a column, washed with three volumes of water, and eluted with three volumes of methanol (MeOH). The MeOH eluate from the SP207 was concentrated by drying *in vacuo* and redissolved in 200 ml of MeOH. The solution was filtered through a filter paper to remove the MeOH-insoluble fraction. The MeOH-soluble fraction was then dried *in vacuo* again. The residue was dissolved in 150 ml of water, and the resulting aqueous solution was extracted with 150 ml of *n*-hexane three times. Hydrochloric acid was then added to the remaining aqueous phase to adjust the pH to < 2, followed by liquid–liquid extraction with 150 ml of ethyl acetate. The extraction with ethyl acetate was also performed three times. The acidic ethyl acetate phase was dried *in vacuo* and redissolved in 100 ml of 5% (v/v) aqueous methanol. After adding formic acid to the obtained solution to achieve a final concentration of 0.1% (v/v), the solution was loaded onto Oasis HLB Vac Cartridges (35 cc/6 g; Waters) that were equilibrated with five column volumes of 5% (v/v) aqueous MeOH containing 0.1% (v/v) formic acid. After washing the cartridge with 50 ml of 5% (v/v) aqueous MeOH, a stepwise elution was performed from 50 ml of 10% MeOH to 100% MeOH, with each fraction analyzed by LC-MS/MS. The 60% MeOH fraction, in which SEC was detected at the highest intensity, was then subjected to silica gel column chromatography (5 g) with a stepwise elution (30 ml each step) of chloroform : MeOH (100 : 0 to 50 : 50, 5% step). SEC was eluted in the chloroform : MeOH (95 : 5) and chloroform : MeOH (90 : 10) fractions. These fractions were dried and dissolved in 20 ml of 5% (v/v) aqueous MeOH, with the pH adjusted to 6.5–7.0. The samples were loaded onto an Oasis MAX Vac cartridge (6 cc/500 mg; Waters) washed with 5% (v/v) aqueous MeOH, followed by activation with 5 ml of water containing 5% (v/v) ammonia. The cartridge was washed with 5 ml of MeOH and subsequently eluted with 5 ml of MeOH containing 0.2 M formic acid. The acidic MeOH fraction was dried and redissolved in 100 µl of 35% (v/v) aqueous MeOH. This solution was subjected to reverse-phase high-performance liquid chromatography (HPLC) using an Atlantis T3 column (10 × 150 mm, 5 µm; Waters). Isocratic elution was performed with a mixture of water and MeOH (65 : 35, v/v) containing 0.1% formic acid at 5.0 ml min^{-1}. The eluent collected at a retention time of 30–31 min was combined and dried. The resulting residue was then dissolved in 100 µl of chloroform : MeOH (95 : 5) and subjected to normal-phase HPLC using a YMC (Kyoto, Japan)-Pack Diol-120-NP column (4.6 × 250 mm, 5 µm; YMC). The mobile phase included solvent A (chloroform) and solvent B (MeOH), with a flow rate of 1.0 ml min^{-1}, employing the following linear gradient system: 0% B at 0 min, held for 10 min; ramped to 10% B at 50 min; increased to 100% B at 50 min, and held until 70 min. The eluents were monitored at 270 nm, and the SEC was collected from the eluents at a retention time of 30.5 min.

The molecular formula of SEC was determined as $C_{29}H_{34}O_{10}$ based on high-resolution mass spectrometry (HRMS; ESI) analysis by Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), which revealed *m/z* 543.2228 $[M + H]^+$ (calculated for $C_{29}H_{35}O_{10}$, *m/z* 543.2230 $[M + H]^+$). The nuclear magnetic resonance (NMR), Tokyo, Japan spectra were recorded in deuterium oxide (Sigma-Aldrich) on a JNM-ECZ400S/L1 spectrometer (JEOL, Tokyo, Japan) at 400 MHz for ^1H.

Soil incubation experiment

The tomato hairy root culture medium was incubated with soil in 14-ml round tubes. After 3 wk of cultivation, 1 ml of the culture medium was collected and added to 2 ml of soil. Five hundred microliters of water was added to ensure an even distribution of the medium throughout the soil. The mixture was incubated at 25°C for 0, 1, 3, 5, and 7 d. After each incubation period, 10 ml of water was added to the tubes, and the suspension was filtered. The filtrate was then centrifuged to remove any residual soil. The resulting supernatant was subjected to solanoeclepin extraction using Oasis HLB Vac Cartridges (3 cc/60 mg; Waters) as follows. Formic acid was added to the prepared supernatant to a final concentration of 0.1% (v/v), and the solution was passed through an Oasis HLB cartridge pre-equilibrated with water. The cartridge was then washed with 0.1% (v/v) formic acid in water, and the solanoeclepins were eluted with 100% MeOH. The eluted fractions were dried under an N atmosphere, and the resulting residue was dissolved in 200 µl of 100% MeOH. An aliquot (1 µl) was injected into the UPLC–MS/MS

system for analysis. The pure SEC incubation was conducted in the same manner as described previously with minor modifications. Ten microliters of SEC dissolved in water was added to 2 ml of soil, followed by the addition of 1.5 ml of water. The mixture was then incubated at 25°C.

Tomato plant hydroponic culture and solanoeclepin extraction from culture media

Tomato (*Solanum lycopersicum* L. cv Micro-Tom) seeds were sown on agar plates and grown at 23°C in the dark for 7 d. The seedlings were then transferred into conical tubes and cultivated hydroponically in 50 ml of half-strength Hoagland nutrient solution with a 16 h : 8 h, 23°C, light : dark photoperiod for 2 and 4 wk, respectively. During this period, the nutrient solution was replaced every 7 d. Plants were subsequently subjected to different nutrient conditions, with control plants maintained in a half-strength Hoagland solution. Nitrogen- and phosphorus-deficient solutions were prepared by omitting the respective elements from the half-strength Hoagland solution. After 7 d of acclimatization to the test media, plants were harvested and divided into shoots and roots. The roots were briefly rinsed with tap water and stored at −80°C until further analysis. Each hydroponic solution was subjected to solanoeclepin extraction using Oasis HLB Vac Cartridges (3 cc/60 mg; Waters) as described previously. Each residue was redissolved in 200 µl of 100% MeOH, and an aliquot (1 µl) was injected into the UPLC–MS/MS system.

qRT-PCR analysis

Total RNA from tomato hairy roots treated with each phytohormone was extracted using the RNeasy Plant Mini Kit (Qiagen) and the RNase-Free DNase Set (Qiagen). The extracted total RNA was used for the synthesis of first-strand cDNAs by the ReverTra Ace qPCR RT Master Mix with genomic DNA remover. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with a LightCyclerNano (Roche) using GeneAce SYBR qPCR Mix α No ROX (Nippon Gene, Tokyo, Japan) with the primers shown in Supporting Information Table S2. Cycling was performed at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s for amplification, followed by holding at 95°C for 30 s, and then ramping up from 60°C to 95°C at 0.1°C s^{-1} to perform a melting curve analysis. Eight biological replicates were analyzed. The values obtained for the Ubi gene were used as internal references in tomato, and the gene expression levels were normalized to these values. Data acquisition and analysis were performed using the LightCyclerNano software (Roche).

Hatching assay

The hatching assay was conducted as previously described (Sakata *et al.*, 2021) with slight modification. Briefly, 1 ml of a suspension of *G. rostochiensis* pathotype Ro1 eggs (containing *c.* 300 eggs ml^{-1}) was placed in a glass vial. Then, 1 ml of each test solution containing SEA or SEC at various concentrations (100 ng ml^{-1}, 10 ng ml^{-1}, 1 ng ml^{-1}, 100 pg ml^{-1}, 10 pg ml^{-1}, 1 pg ml^{-1}, and 100 fg ml^{-1}) was added to the vial. The vials were incubated at 18°C for *c.* 2 wk. After incubation, 1 ml from each vial was transferred to a Syracuse dish, and the number of hatched second-stage juveniles (J2s) and unhatched eggs was counted under a binocular microscope. Distilled water and synthetic SEA were used as negative and positive controls, respectively.

Results

Identification of the SEB precursor converted into SEB in the soil

We previously demonstrated that soil microorganisms convert SEB into SEA (Shimizu *et al.*, 2023). In this study, we investigated the time course of this conversion in the soil. Tomato root exudates containing SEB, but not SEA, were added to the soil and incubated for 1–7 d. After the incubation period, the water extracts from the soil were analyzed using UPLC-MS/MS. SEA formation was observed after 3 d of incubation. By the seventh day, SEB was almost completely converted into SEA (Fig. 1a). Interestingly, increased SEB concentrations were observed during the first day of incubation (Fig. 1a). Based on these results, we hypothesized that tomato hairy root exudates contain an SEB precursor that is converted into SEB in the soil. We named this putative precursor SEC. We conducted a series of fractionation experiments on the tomato hairy root exudates to confirm the presence of the SEC (Fig. 1b). Each fraction was analyzed by LC-MS/MS before and after soil incubation. After removing the salts using the synthetic adsorbent SP207, the exudates were fractionated using an Oasis® MAX (Waters) solid-phase extraction cartridge, which combines the reversed-phase and anion exchange retention mechanisms. SEB was not detected in the pass-through fraction, which contained compounds that did not adsorb on the MAX cartridge (Supporting Information Fig. S1). Similarly, no SEB was found in the methanol (MeOH) fraction, which contained low-polar compounds. By contrast, SEB was detected in the 0.2 M formic acid in the MeOH fraction, which contained acidic compounds (Fig. S1). When each fraction was added to the soil and incubated for 7 d, SEB and SEA were detected only in the soil extract derived from the 0.2 M formic acid in the MeOH fraction (Fig. S1). We then further fractionated the 0.2 M formic acid MeOH fraction using a reversed-phase SPE cartridge (Oasis® HLB; Waters) with stepwise elution using water and MeOH (Fig. 1b). SEA was not detected in any of the fractions before soil incubation. SEB was detected in the 40% and 50% MeOH fractions (Fig. 1c). The 60% MeOH-derived fraction did not contain SEA and SEB before soil incubation; however, SEA was detected after incubating this fraction in the soil (Fig. 1a). These results indicate the presence of a SEC in the 60% MeOH fraction.

Isolation and structural determination of the SEC

Given its behavior during solid-phase extraction, SEC is likely an acidic compound with lower polarity than SEB. Previous research

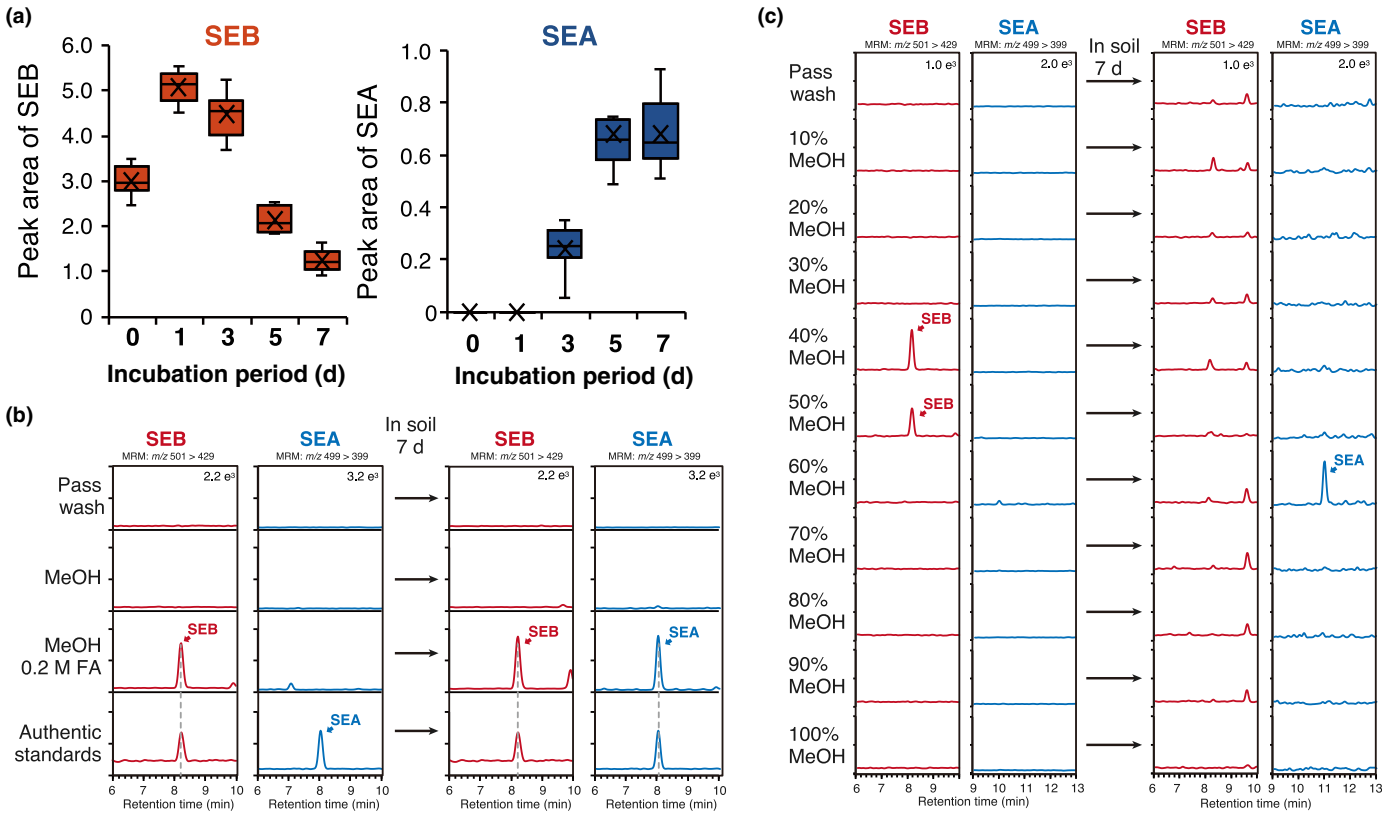

Fig. 1 Identification of the solanoeclepin C in the tomato hairy root culture medium. (a) Time course of changes in solanoeclepin B (SEB) and solanoeclepin A (SEA) amounts during incubation of the tomato (*Solanum lycopersicum*) hairy root culture medium in the soil. Samples were analyzed in eight replicates. The median (line within the box), first and third quartiles (box), and nonoutlier range (whiskers) are displayed. (b) Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis of the tomato hairy root culture medium fractions fractionated using Oasis MAX before and after soil incubation. (c) UPLC-MS/MS analysis of the tomato hairy root culture medium fractions fractionated using Oasis HLB presented in (b) before and after soil incubation. Multiple reaction monitoring (MRM) transitions of *m/z* 501 > 429 (red) and *m/z* 499 > 399 (blue) were used to detect SEB and SEA, respectively.

on triterpenoids in *Arabidopsis thaliana* reported that acylated forms of thalianol or its derivatives – including acetate, laurate, myristate, and palmitate – are released from the roots into the soil, in which their acyl groups are cleaved by soil bacteria (Huang *et al.*, 2019). Therefore, we hypothesized that SEC may be a decorated form of SEB acylated on the C2, C15, or C19 hydroxy group (Fig. 2a). Based on this hypothesis, we set up MRM channels for detecting acety-, lauroyl-, myristoyl-, and palmitoyl-SEB using LC-MS/MS and analyzed the tomato hairy root medium (Fig. 2b). As a result, a clear peak was observed only in the MRM channel for detecting acetyl-SEB at a retention time of 12.7 min (Fig. 2b). Next, we analyzed the fractionated tomato hairy root exudates used in the analysis described previously (Fig. 2c). Consequently, the peak at 12.7 min was clearly detected in the 60% MeOH fraction, which was estimated to contain SEC (Fig. 2c). After soil incubation, the peak's intensity in the 60% MeOH fraction decreased significantly (Fig. 2c). These findings indicate that the compound eluting at 12.7 min is a SEC. Therefore, we proceeded with the isolation and purification of the compound corresponding to this peak to determine its structure.

We used the hydroponic solution from the large-scale hydroponic cultivation of potatoes used for SEB isolation to obtain a sufficient quantity of SEC for structural determination (Shimizu *et al.*, 2023). The hydroponic solution was adsorbed onto the synthetic resin SP207, and the MeOH eluate was analyzed by LC-MS/MS (Fig. S2), and SEA, SEB, and SEC were detected (Fig. S2). SEC was then purified from the MeOH eluate through a series of purification and fractionation steps guided by LC-MS/MS analysis (Figs S3–S9). Using LC-Orbitrap-MS, the molecular formula of the purified SEC was determined to be $C_{29}H_{34}O_{10}$ based on the proton adduct ion $[M + H]^+$ at *m/z* 543.2228, which is consistent with that of the acetylated SEB (calculated for $[C_{29}H_{34}O_{10} + H]^+$, 543.2230). In the ^{1}H NMR analysis, the acetyl methyl protons at 1.89 ppm were observed (Figs S10, S11; Table S1). The other signals in the ^{1}H NMR spectrum of SEC were very similar to those of SEB except for the downfield shift of 0.37 ppm in the chemical shift of H2, clearly indicating that the hydroxy group at the C2 of SEB is acetylated (Figs S10, S11; Table S1).

Next, we examined the stability of SEC under natural conditions. The purified SEC was dissolved in water, and the solution was incubated at 25°C for 10 d in water and analyzed using LC-MS/MS (Fig. S12). The SEC peak decreased, and two new peaks with retention times of 14.1 and 14.6 min were observed

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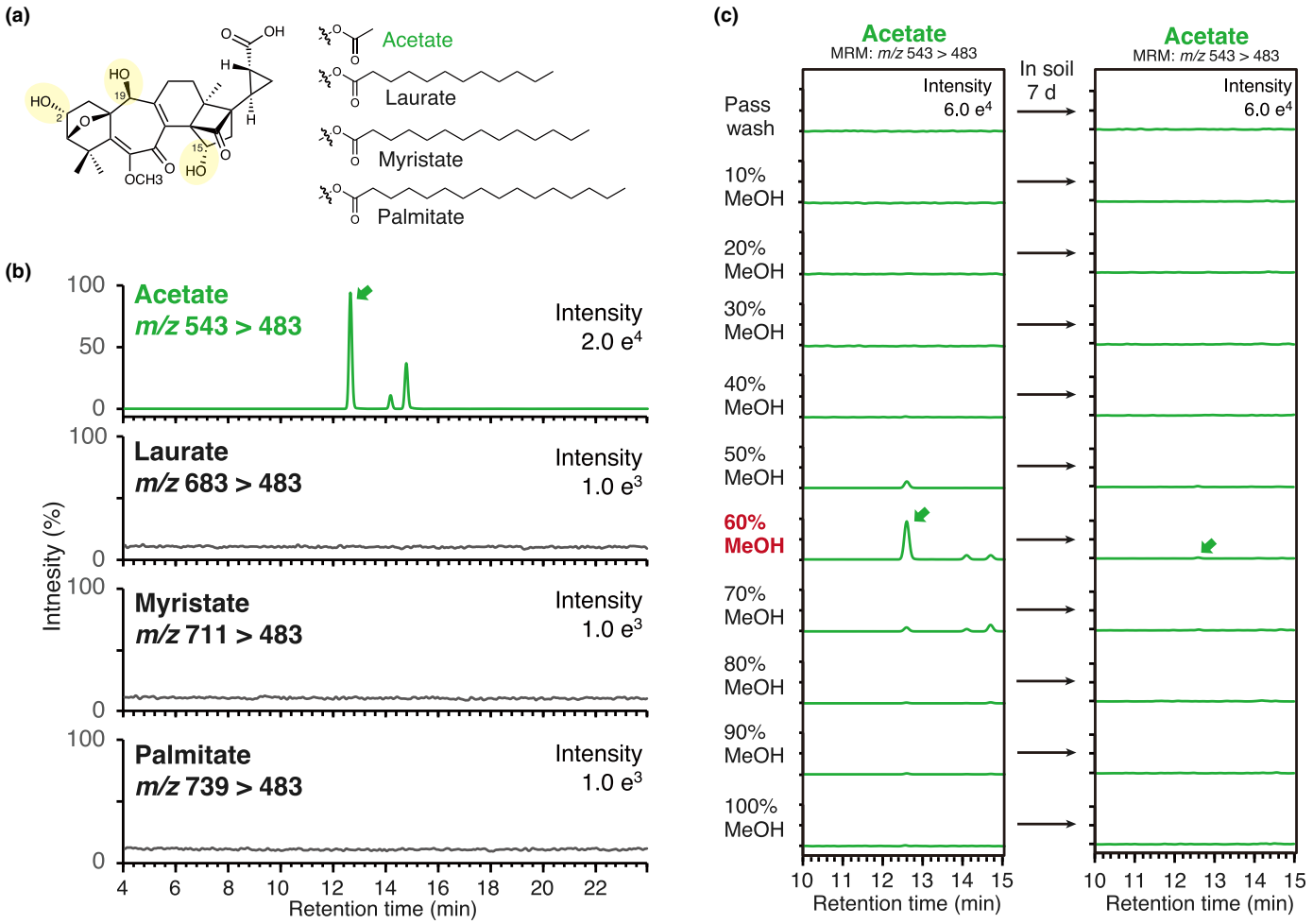

Fig. 2 Prediction of the solanoeclepin C (SEC) structure. (a) Structures of the candidate SEC. (b) Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis of the tomato (*Solanum lycopersicum*) hairy root culture medium by UPLC-MS/MS. Multiple reaction monitoring (MRM) channel targeting for solanoeclepin B (SEB)-acetate (*m/z* 543 > 483), SEB-laurate (*m/z* 683 > 483), SEB-myristate (*m/z* 711 > 483), and SEB-palmitate (*m/z* 739 > 483) was used. (c) UPLC-MS/MS analysis of the tomato hairy root culture medium fractions fractionated using Oasis HLB presented in Fig. 1, before and after soil incubation. Multiple reaction monitoring transitions of *m/z* 543 > 483 were used.

(marked as * and ** in Fig. S12), which likely correspond to the SEC decomposition products. These peaks are identical to the peaks eluting after SEC in the chromatograms shown in Figs 2(c) and S2. In the MRM chromatograms targeting SEA and SEB in Fig. S2, two additional peaks were also detected following the elution of SEA and SEB. These peaks are similarly presumed to represent the degradation products of SEA and SEB.

SEC is a less active HF and is activated by soil microorganisms

The purified SEC, verified to be highly pure by UPLC-UV and NMR analyses, was added to the soil, which was then incubated for 0–3 d. After the incubation periods, we analyzed the soil water extracts. During soil incubation, SEC levels decreased over time (Fig. 3b). Although SEB was not detected before the incubation, it began to be detected on the first day, and its levels decreased by the third day (Fig. 3b). SEA formation started on the second day and increased further by the third day (Fig. 3b). These results confirm that SEC is converted into SEB and subsequently into SEA in the soil. Further experiments using gamma-ray-sterilized soil demonstrated a decrease in SEC levels (Fig. 3b); however, the decrease rate was slower than that in non-sterilized soil. SEB production was also observed, but the levels were much lower than those in nonsterilized soil, and no SEA was detected (Fig. 3b). These results suggest that the conversion of SEC into SEB might occur abiotically but is significantly enhanced by soil microorganisms.

We next assessed the hatch-inducing activity of highly purified SEC against PCN (*G. rostochiensis*) (Fig. 3c). SEA, known as a highly active HF, exhibited a *c.* 78% hatching rate at 10^{-10} g ml^{-1}, whereas SEC at the same concentration displayed a hatching rate of 7.2%, which is equivalent to the value observed for the negative control, distilled water (Fig. 3c). Measuring the

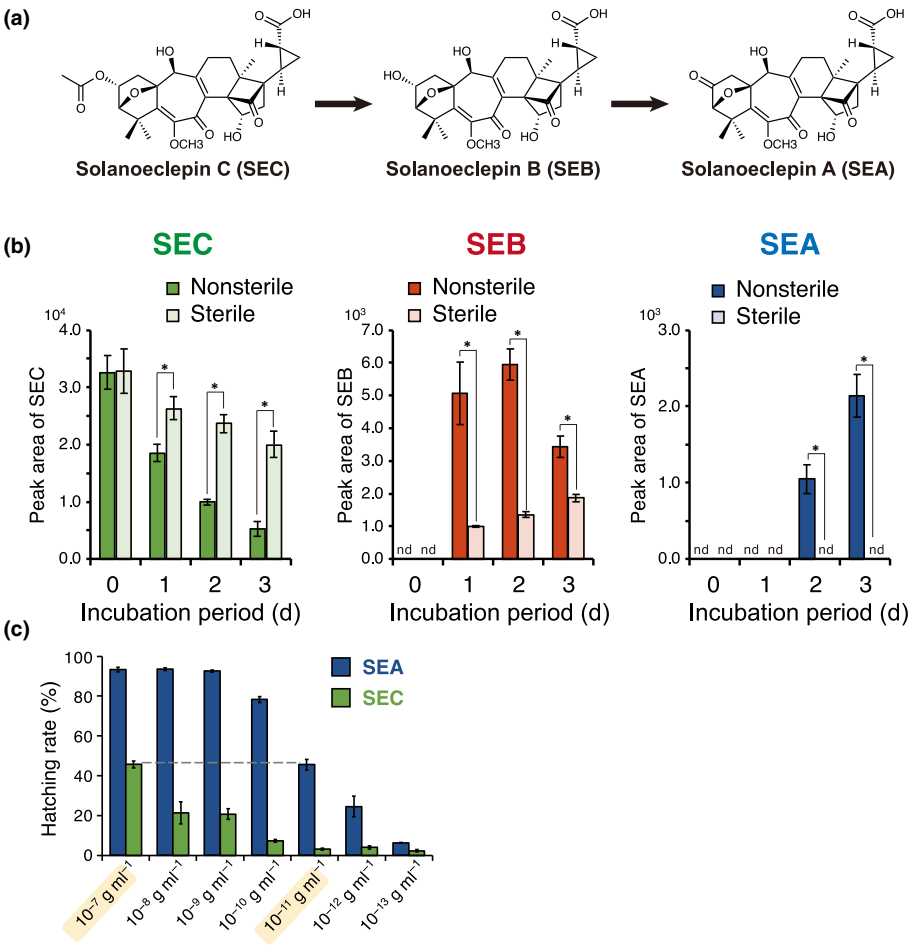

Fig. 3 Solanoeclepin C (SEC) characterization. (a) Structures of SEC, solanoeclepin B (SEB), and SEC (b) Conversion time course of pure SEC in nonsterile or sterile soil. The values are presented as the mean ± SE (n = 3) of three biological replicates. nd, not detected. (c) Dosage response to synthetic solanoeclepin A (SEA) and isolated SEC of *in vitro* % hatch of *Grobodera rostochiensis* after 21 d at 22°C. SEA and SEC were diluted to produce a series of concentrations from 10^{-7} to 10^{-13} g ml^{-1} in water. % hatch = (Nj_{21} − Nj_0)/Ne_0; Nj_{21}, number of hatched juveniles at 21 d; Nj_0, number of hatched juveniles at 0 d; Ne_0, number of eggs at 0 d, 250 < Ne_0 < 350 eggs/each in 10 ml of Petri dish. Results are presented as mean ± SE of the means (n = 3). Significant differences are indicated by asterisks (P < 0.01; Student's t-test).

hatch-inducing activity of SEC at higher concentrations revealed a *c.* 45% hatching rate at 10^{-7} g ml^{-1}, which is comparable to the rate observed for SEA at 10–11 g ml^{-1} (Fig. 3c). These data indicate that SEC is *c.* 10 000-fold less active than SEA in inducing PCN hatching.

Solanoeclepin C quantification in the hairy root extracts and exudates revealed that it was present in *c.* 20 times greater quantities in the exudates (Fig. S13). Furthermore, SEB was not detected in the root extracts (Fig. S13). Although this comparison is based on peak area rather than on absolute quantification, SEC appears to be secreted in *c.* 50 times greater amounts than SEB (Fig. S13). These findings indicate that SEC is actively released from the roots and subsequently converted into active HFs, SEB, and SEA by soil microorganisms.

SEC is biosynthesized from SEB

Our previous study identified five SEB biosynthetic genes (*SOLA1–SOLA5*) and generated knockout tomato hairy roots for these genes (Shimizu *et al.*, 2023). Here, we investigated SEC production in these mutant hairy roots (Fig. 4a). The LC-MS/MS analysis of the hairy root line culture media revealed that SEB was not produced in the knockout lines, consistent with our previous findings (Fig. 4a). Similarly, SEC was not detected in the media from these knockout hairy roots (Fig. 4a). This result indicates that *SOLA1–SOLA5* are involved in SEB and SEC biosynthesis, which are biosynthesized through the same pathway. Since SEC has an additional acetyl group attached to the C2 hydroxy group of SEB, SEC is likely biosynthesized from SEB. We conducted SEB feeding experiments to test this hypothesis (Fig. 4b). Purified SEB was added to the sola1 mutant hairy root culture media. This mutant could not produce SEB and SEC due to the loss of function of a dioxygenase encoded by SOLA1. The LC-MS/MS analysis of the media collected after a 10-d incubation revealed the presence of SEB and SEC in the media. These results indicate that SEB is taken up by sola1 mutant hairy roots, converted into SEC, and secreted into the culture media (Fig. 4b). These observations suggest that SEC is biosynthesized from SEB as an intermediate, possibly through the action of an unknown acetyltransferase.

Solanoeclepin biosynthesis is induced under nitrogen and phosphate deficiency

In response to varying nutrient conditions, plants secrete specialized metabolites from their roots, influencing interactions with

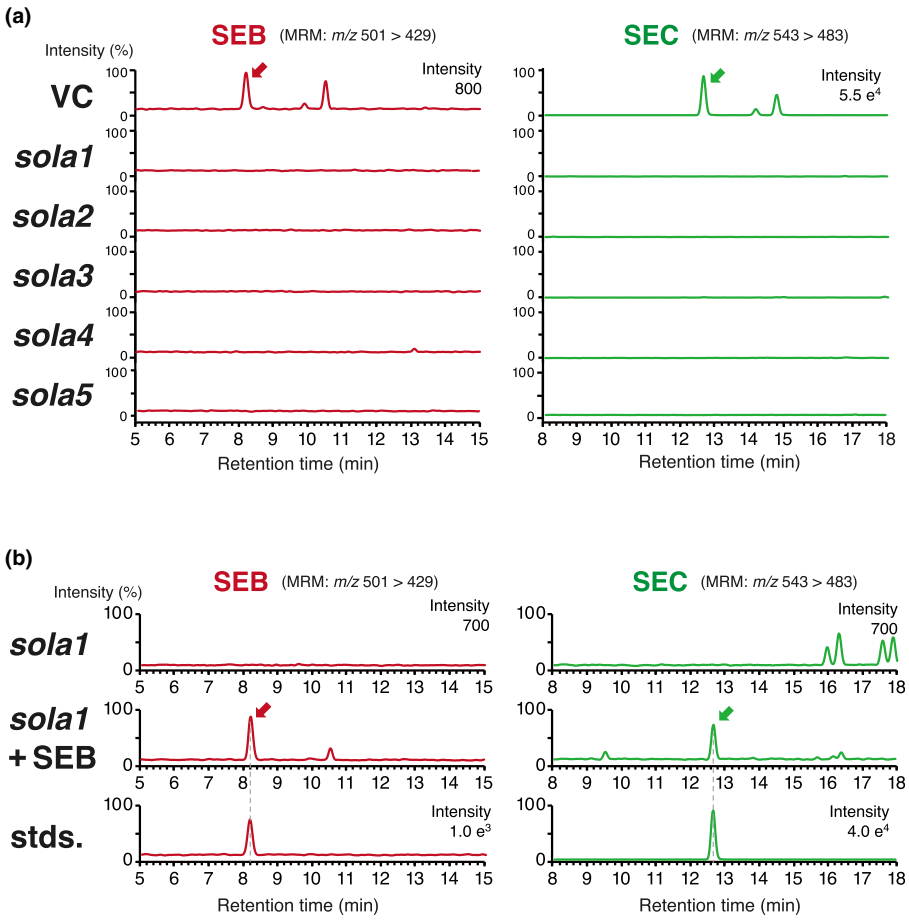

Fig. 4 Analysis of the solanoeclepin C (SEC) biosynthetic pathway using tomato hairy roots. (a) Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis of solanoeclepin B (SEB) and SEC in the culture medium of vector control and *solanoeclepin biosynthesis 1* (*sola1*)–*sola5* mutant tomato (*Solanum lycopersicum*) hairy roots lines. (b) UPLC-MS/MS detection of the conversion from administered SEB to SEC in *sola1* mutant tomato hairy roots. sds, authentic standards of SEB and SEC. Multiple reaction monitoring (MRM) transitions of *m/z* 501 > 429 (red) and *m/z* 543 > 483 (green) were used to detect SEB and SEC, respectively.

soil organisms, including symbiosis, attraction, and repulsion. Here, we assessed how different combinations of phosphate and nitrogen levels affect solanoeclepin biosynthesis in tomato (*S. lycopersicum* cv Micro-Tom). Three-week-old tomato plants were hydroponically cultivated for 1 wk in standard, phosphate-deficient, nitrogen-deficient, or phosphate- and nitrogen-deficient media. Total RNA was extracted from the roots, and solanoeclepin levels in the medium were quantified by LC-MS/MS over the cultivation period (Fig. 5). The expression levels of five solanoeclepin biosynthetic genes (SOLA1–SOLA5) were analyzed using qRT-PCR (Fig. 5a). Phosphate deficiency alone, under sufficient nitrogen, significantly increased gene expression compared with the control; nitrogen deficiency elicited an even stronger response. The highest gene induction was observed under simultaneous phosphate and nitrogen deficiency (Fig. 5b). A similar pattern was observed in the semiquantitative analysis of SEC and SEB (Fig. 5b). SEC production increased *c.* 3.4-fold under phosphate deficiency, 5.5-fold under nitrogen deficiency, and 8.4-fold under phosphate and nitrogen deficiency compared to the control condition (Fig. 5b). SEB levels were below the detection limit in the control condition but exhibited a similar pattern to that of SEC (Fig. 5b). Additionally, SEA was not detected under any of the conditions tested. These findings highlight that solanoeclepin biosynthesis is significantly enhanced under phosphorus and nitrogen deficiencies, with nitrogen limitation having a particularly strong effect, resulting in increased solanoeclepin secretion from the roots.

Discussion

Eclepins have been studied extensively as signal molecules that connect plants and cyst nematodes and as crucial targets for controlling cyst nematodes (Masamune *et al.*, 1982, 1987; Fukuzawa *et al.*, 1985; Mulder *et al.*, 1996; Schenk *et al.*, 1999; Tanino *et al.*, 2011; Shimizu *et al.*, 2023). In the 1980s, three glycinoeclepins – GEA, GEB, and GEC – were isolated and structurally characterized. SEA was identified in the 1990s. However, likely due to their trace natural abundance, no reports were made on eclepin detection for 20 yr following their initial discovery. In the 2020s, SEA detection was reported, and a novel solanoeclepin, SEB, was subsequently isolated and identified (Guerrieri *et al.*, 2021; Vlaar *et al.*, 2022; Shimizu *et al.*, 2023). This study identified and structurally characterized SEC, a third solanoeclepin (Fig. 3a). We demonstrated that SEC has an *c.* 10 000-fold lower hatching activity for PCNs than SEA and SEB. Previous studies have demonstrated that GEB and GEC are 1000-fold less active than GEA in inducing SCN hatching (Masamune *et al.*, 1982; Fukuzawa *et al.*, 1985), indicating that cyst nematodes can precisely recognize HF structures for hatching. A 'suicide hatching' strategy has been proposed to control cyst

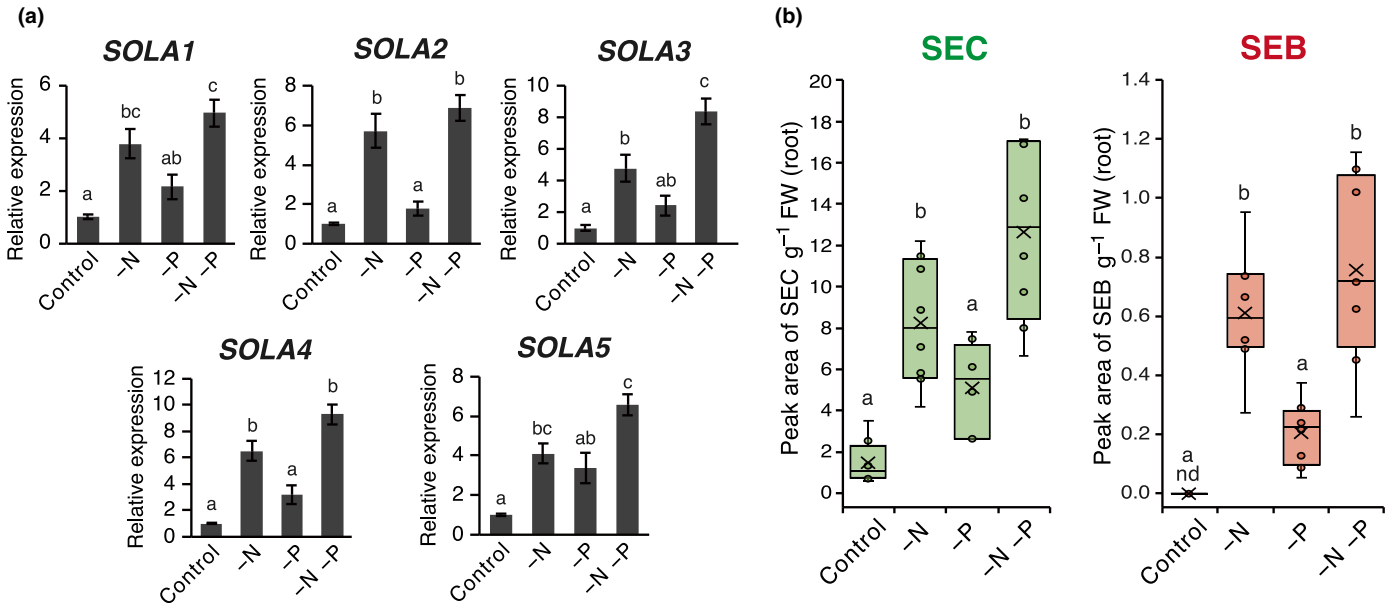

Fig. 5 Effect of nitrogen or phosphate deficiency on solanoeclepin production in hydroponically grown tomato plants. (a) Analysis of the transcription levels of five solanoeclepin biosynthetic genes by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in hydroponically grown tomato (*Solanum lycopersicum*) under different nutrient conditions. The values are presented as the mean ± SE (n = 8) of three biological replicates. (b) Semiquantification of solanoeclepin C (SEC) and solanoeclepin B (SEB) in tomato root exudates. The green and red boxes indicate the quantities of SEC and SEB (peak area g^{-1} root fresh weight (FW)), respectively. Samples were analyzed in eight replicates. The median (line within the box), first and third quartiles (box), and nonoutlier range (whiskers) are presented. Control, optimal nitrogen (N) and phosphate (P) conditions: −N, without N and optimal P conditions; −P, optimal N and without P conditions; −N − P, without N and P conditions; nd, not detected. Different letters indicate statistically significant differences between samples using a Tukey's multiple comparison test ($P < 0.05$).

nematode spread by applying HFs directly to the soil. Although synthetic methods for GEA and SEA have been reported (Murai *et al.*, 1988; Tanino *et al.*, 2011), their highly complex structures require multistep reactions, making their synthetic production currently impractical for nematode control. Additional structural analogs of eclepins are thought to exist in nature. Isolating and characterizing these analogs and identifying their hatching activities may allow us to design simpler yet highly active HFs for effective cyst nematode control.

Steroidal glycoalkaloids (SGAs), such as α-solanine and α-tomatine, are representative specialized metabolites in *Solanum* plants that exhibit weak hatching-inducing activity toward PCNs (Devine *et al.*, 1996; Byrne *et al.*, 2001; Shimizu *et al.*, 2020). Although these SGAs are secreted from tomato and potato roots, their contribution to the hatching activity in root exudates appears to be minimal (Shimizu *et al.*, 2020; Nakayasu *et al.*, 2021). The hatching-inducing activity of SECs observed in this study is comparable to, or even weaker than, those of SGAs (Fig. 3c; Shimizu *et al.*, 2020). Since solanoeclepin secretion levels are *c.* 1000 times lower than those of SGAs (Guerrieri *et al.*, 2021; Nakayasu *et al.*, 2021), SEC likely plays a minimal role in promoting cyst nematode hatching. SEC only effectively induces hatching after being converted into SEB or SEA, highlighting this conversion as a key step in cyst nematode–plant interactions. Interestingly, the conversion of SEC to SEB was also observed in sterilized soil, suggesting that it can occur even in the absence of microbial activity. Although the reaction proceeded more rapidly in unsterilized soil, indicating that microorganisms facilitate the process, potential abiotic mechanisms should also be considered. Acetyl groups are relatively labile under aqueous conditions and may undergo hydrolysis, particularly at elevated temperatures or acidic pH. Thus, soil pH and other physicochemical factors may contribute to the spontaneous conversion of SEC. In addition, residual hydrolytic enzymes such as lipases could remain active poststerilization and participate in this transformation. Further studies using various soil types and controlled abiotic conditions will help clarify these mechanisms. These findings indicate that soil microorganisms contribute to the conversion of SEC, thereby activating the hatching stimulant. To our knowledge, this is the first study to demonstrate microbial involvement in interspecies interactions mediated by solanoeclepins.

In recent years, accumulating evidence has demonstrated that the metabolism of specialized metabolites secreted from plant roots by soil microorganisms affects the microbial community in the rhizosphere. For example, α-tomatine secreted from tomato roots is degraded by certain *Sphingobium* spp. strains (Nakayasu *et al.*, 2021, 2023). Although α-tomatine has anti- and antibacterial activities, these *Sphingobium* strains can detoxify α-tomatine, allowing them to preferentially colonize the tomato rhizosphere. Since several species in the family *Sphingobium* promote plant growth, tomato roots are expected to secrete α-tomatine to facilitate the colonization of the *Sphingobium* microbes in the rhizosphere for their benefit (Nakayasu *et al.*, 2021, 2023). Similar examples have also been

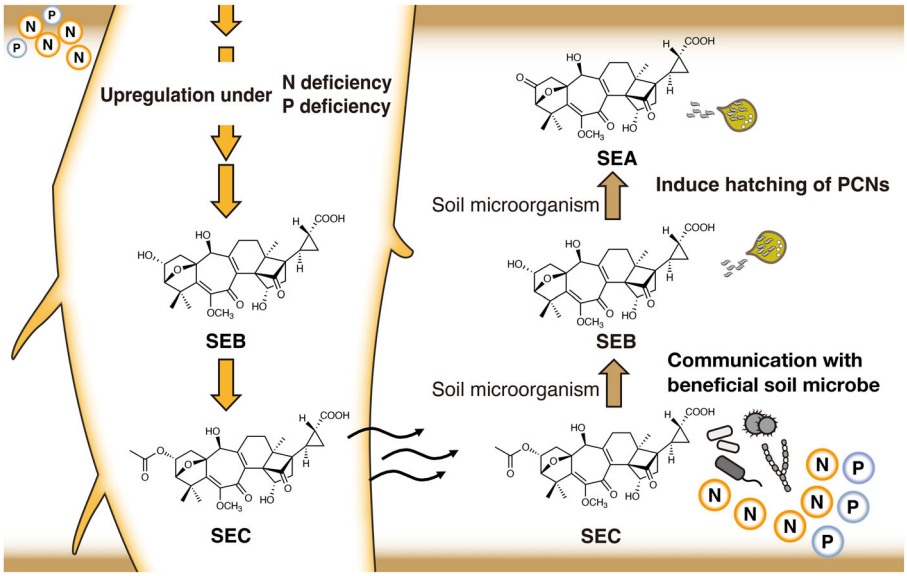

Fig. 6 Summary of the solanoeclepin-mediated communication system.

demonstrated with thalianol derivatives in *A. thaliana* and nicotine in *Nicotiana tabacum* (Huang *et al.*, 2019; Shimasaki *et al.*, 2021). The production of these toxic metabolites is typically induced by the plant hormone methyl jasmonate (MeJA) as part of a defense response (Shoji *et al.*, 2008; Thagun *et al.*, 2016; Hu *et al.*, 2018). By contrast, solanoeclepin production decreased significantly under MeJA treatment (Shimizu *et al.*, 2023). Therefore, we expect eclepins to influence the rhizosphere microbiome in a manner distinct from the toxic compounds mentioned previously.

This study demonstrated that solanoeclepin production is induced in tomato plants under nitrogen or phosphorus deficiency, with a particularly pronounced increase under nitrogen deficiency. Since both nitrogen and phosphorus are essential macronutrients for plant growth, nutrient deprivation is known to drive changes in root exudation to recruit beneficial microorganisms that assist in nutrient acquisition. For example, under nitrogen deficiency, legumes secrete cocktails of flavonoids to attract nitrogen-fixing bacteria that establish symbiosis within root nodules (Peters *et al.*, 1986; Masson-Boivin & Sachs, 2018). Phosphate starvation induces the production of SLs, which promote symbiosis with AM fungi. Although SLs were initially characterized as germination stimulants for parasitic plants (Cook *et al.*, 1966), they are now recognized as key signals for AM fungi, triggering hyphal branching and enhancing symbiotic colonization under low-phosphate conditions. By analogy, solanoeclepins may also function as rhizosphere signals that promote beneficial interactions with microbes, potentially facilitating colonization or optimizing symbiosis under nutrient stress. In this context, solanoeclepin production may reflect a plant-driven strategy to cope with nutrient limitation. However, this beneficial role may be hijacked by PCNs (Fig. 6).

Previous reports have shown that PCN hatching activity of potato root exudates changes significantly depending on the presence or absence of AM fungal colonization (Ryan *et al.*, 2000; Deliopoulos *et al.*, 2010). Although the underlying mechanism remains unclear, this observation suggests a possible link between AM symbiosis and solanoeclepin-related signaling. Whether such effects result from changes in solanoeclepin biosynthesis, other plant-derived compounds, or microbial metabolites produced by AM fungi remains to be determined. Nevertheless, these findings raise the intriguing possibility that AM fungi may be among the ecological targets of solanoeclepins. Further studies using solanoeclepin-deficient mutants of tomato and potato will be necessary to clarify the broader ecological roles of these compounds beyond nematode hatching.

While solanoeclepins are currently known to be produced only by Solanaceae species, structurally related GEA, GEB, and GEC have been identified in legumes such as kidney bean, in which they act as HFs for SCN. Notably, glycinoeclepins do not induce hatching in PCN, and even among the glycinoeclepin variants, there are substantial differences in SCN hatching activity. These observations highlight the strong specificity in the interactions mediated by different eclepins and suggest that structural variation among these molecules underpins distinct ecological functions. Given that solanoeclepins and glycinoeclepins share partial structural similarity, it is likely that the upstream steps of their biosynthetic pathways are conserved. The possibility that eclepin-like compounds exist in plant lineages beyond Solanaceae and Fabaceae also deserves consideration. Taken together, these findings support the idea that eclepins represent a previously unrecognized class of plant-derived signaling molecules, mediating diverse and context-dependent interactions in the rhizosphere.

The fact that SEC is activated to act as an HF for PCNs by soil microorganisms highlights an intriguing three-way interaction between plants, soil microbes, and nematodes, underscoring the complexity of interspecies communication mediated by specialized plant metabolites. Our work provides new insights into eclepins as potential signaling compounds in the rhizosphere, regulated by nutrient deficiencies to enhance plant–microbe

interactions. These findings open avenues for exploring eclepins' broader ecological roles and their application in sustainable agriculture.

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Competing interests

None declared.

Author contributions

RA, AK and MM were involved in conceptualization and project administration. RA, YM, AK and MM were involved in methodology. RA, YK, KS, SM, KA, HN and AK were involved in investigation. RA was involved in visualization. RA, AK, KT and MM were involved in funding acquisition. RA, YS, AK, KT, MYH and MM were involved in supervision. RA and MM were involved in writing – original draft. RA, AK, MYH and MM were involved in writing – review and editing.

ORCID

Ryota Akiyama https://orcid.org/0000-0003-2116-4226
Masami Yokota Hirai https://orcid.org/0000-0003-0802-6208
Atsuhiko Kushida https://orcid.org/0000-0002-4549-7861
Masaharu Mizutani https://orcid.org/0000-0002-4321-0644
Yukihiro Sugimoto https://orcid.org/0000-0002-8767-9671

Data availability

The data supporting the findings of this study are presented in the figures (Figs 1–6) and Supporting Information (Figs S1–S13; Tables S1, S2) of this article.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Scheme of fractionation of tomato hairy root culture medium.

Fig. S2 Ultra-performance liquid chromatography–tandem mass spectrometry analysis of solanoeclepins in the potato root exudates.

Fig. S3 Solanoeclepin C purification scheme.

Fig. S4 Ultra-performance liquid chromatography–tandem mass spectrometry analysis of the liquid phase extraction fractions.

Fig. S5 Ultra-performance liquid chromatography–tandem mass spectrometry analysis of the fractions fractionated using Oasis HLB.

Fig. S6 Ultra-performance liquid chromatography–tandem mass spectrometry analysis of the fractions fractionated by silica gel column chromatography.

Fig. S7 Ultra-performance liquid chromatography–tandem mass spectrometry analysis of the fractions fractionated by Oasis MAX.

Fig. S8 Solanoeclepin C purification by reverse-phased preparative high-performance liquid chromatography.

Fig. S9 Solanoeclepin C purification by normal phased preparative high-performance liquid chromatography.

Fig. S10 ^{1}H-NMR spectrum of solanoeclepin C.

Fig. S11 Solanoeclepin B and solanoeclepin C structures.

Fig. S12 Solanoeclepin C stability.

Fig. S13 LC-MS/MS analysis of the tomato hairy root exudates or extracts.

Table S1 Assignments of ^{1}H chemical shifts of solanoeclepin C and previously reported solanoeclepin B.

Table S2 Primers used in this study.

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