Brief Report

Germination stimulatory activity of bacterial butenolide hormones from *Streptomyces albus* J1074 on seeds of the root parasitic weed *Orobanche minor*

Atsushi Okazawa,[1,]* Hiroaki Samejima,[2,#] Shigeru Kitani,[3] Yukihiro Sugimoto[2] and Daisaku Ohta[1]

[1] *Department of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599–8531, Japan*
[2] *Department of Agrobioscience, Graduate School of Agricultural Sciences, Kobe University, Kobe, Hyogo 657–8501, Japan*
[3] *International Center for Biotechnology, Osaka University, Suita Osaka 565–0871, Japan*

Damage caused by Orobanchaceae root parasitic weeds is a substantial agricultural problem for global food security. Many studies have been conducted to establish practical methods of control, but efforts are still required for successful management. Seed germination of root parasitic weeds requires host-derived germination stimulants including strigolactones (SLs). Studies on SLs have revealed that a butenolide ring is the essential moiety for SL activity as a germination stimulant. Interestingly, recent studies have revealed that butenolide hormones regulate the biosynthesis of secondary metabolites and mediate communication in actinomycete bacteria. Because of the structural similarity between SLs and the bacterial butenolides, we evaluated the germination stimulatory activity of butenolides isolated from *Streptomyces albus* J1074 on root parasitic weeds. These butenolides were found to specifically induce seed germination of *Orobanche minor*. Our findings contribute to understanding the molecular mechanisms of germination stimulant perception and to the development of a method for their biological control.
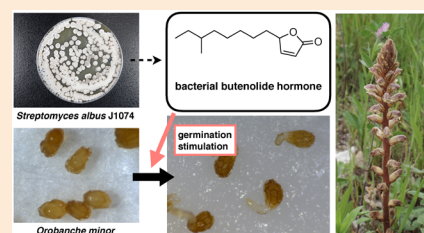
Keywords: butenolide, broomrape, *Orobanche minor*, parasitic weed, *Streptomyces albus*, strigolactone.

Introduction

Damage caused by Orobanchaceae root parasitic weeds is a substantial agricultural problem for global food security.[1] Many studies have been conducted to establish practical methods for root parasitic weed control,[2] but efforts are still required for their successful management.

Life cycles of root parasitic weeds depend on their hosts, and seed germination requires host-derived germination stimulants.[3–5] The most general germination stimulants are strigolactones (SLs),[4–6] which also act as a class of plant hormones[7,8] and
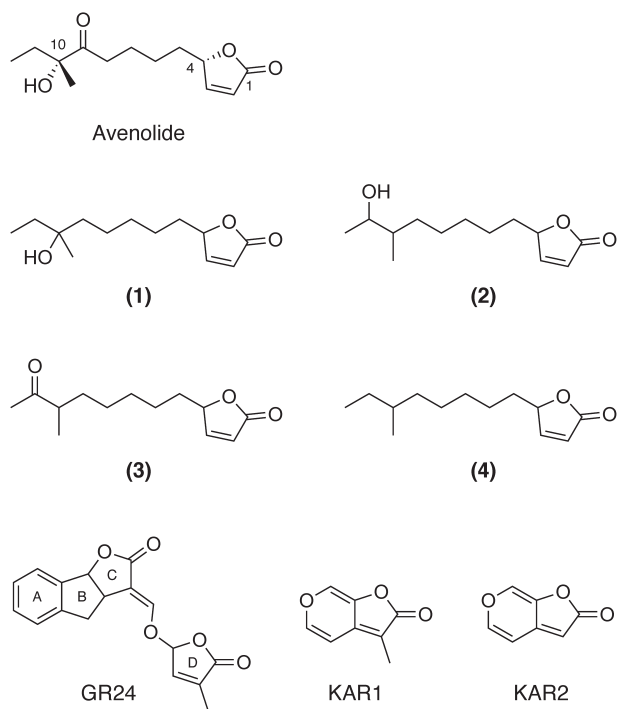
* To whom correspondence should be addressed.
  E-mail: okazawa@plant.osakafu-u.ac.jp
Present address: Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Sapporo, Hokkaido 062–8555, Japan

as signaling molecules in symbiosis with arbuscular mycorrhizal fungi.[9,10] If seeds of root parasitic weeds are forced to germinate by exogenous application of germination stimulants, they wither and die soon after because of the lack of a vitalizing host. This control strategy is called "suicidal germination", and many synthetic germination stimulants have been designed based on SL biochemisty.[11] Expanding structural information on natural SLs enables their classification into canonical SLs composed of four ring structures (A–D rings) (Fig. 1) and non-canonical SLs, possessing diverse skeletons with butenolide rings (D rings).[5,6] All active SLs possess D rings, indicating that they are a minimum and essential requirement for SL activity.[11]

Karrikins (Fig. 1) are compounds present in smoke derived from burning plant materials and have been found to promote seed germination.[12,13] Karrikins are also butenolides, although they do not induce germination of root parasitic weeds.[14] Structural similarity between SLs and karrikins has been focused on in terms of their interaction with receptor proteins.[15–17] Karrikins are known to be perceived by KARRIKIN-INSENSITIVE2 (KAI2) receptors, the intrinsic ligands of which still remain to be elucidated.[15,16] Recently, sesquiterpene lactones possessing butenolide rings in sunflowers (*Helianthus an-*

Fig. 1. Structures of butenolides **1**–**4** isolated from *Streptomyces albus* J1074; strigolactone, GR24; and karrikins, KAR1 and KAR2. Canonical SLs like GR24 consist of four ring structures, A, B, C, and D rings.

*nuus*) were shown to be bound with HaKAI2s *in silico*.[18]

SL receptors mediating seed germination of root parasitic weeds are members of a subclade of KAI2 paralogues, KAI2d,[15,16,19] while another class of KAI2 paralogues, DWARF14 (D14) consists of SL receptors in other plants that mediate SL signaling to regulate plant architecture.[8,15,16,20–22] Genomic information reveals that KAI2ds are highly diversified in Orobanchaceae through multiple gene duplications.[15,16,19,23] Diversities of SLs in plant root exudates and KAI2ds might be a result of co-evolution during battles between parasites and hosts.[5] Characterization of specificity between each KAI2d and its ligand still remains a big challenge.

A butenolide-type autoregulator, avenolide, acts as a hormone to regulate biosynthesis of secondary metabolites in the actinomycete *Streptomyces avermitilis*.[24] A recent study indicated a wide distribution of butenolide hormones in actinomycetes,[25] which were shown to act as external signals in actinomycete communication.[26] The structural similarity of these *Streptomyces* hormones with SLs and karrikins motivated us to evaluate their germination activity in root parasitic weeds. Elucidation of a new class of germination stimulants will contribute to the understanding of molecular mechanisms of germination stimulant perception in root parasitic weeds and to the development of a root parasitic weed control method.

Materials and methods

1. Butenolide preparation
Four butenolides (Fig. 1) were isolated from *Streptomyces albus*
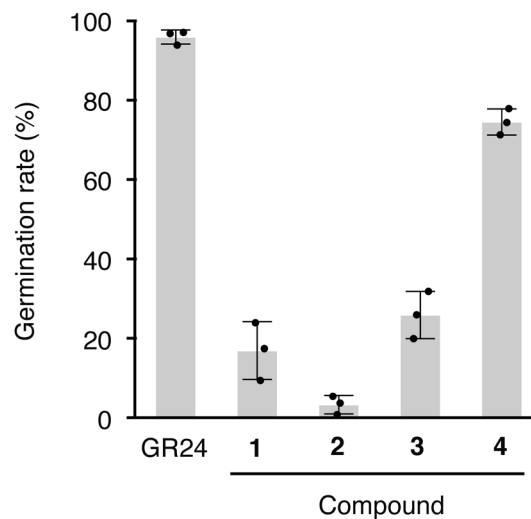
Fig. 2. Germination stimuratory activity of butenolides on *O. minor* seeds. Each butenolide was applied at 10^{-5} M, and germination rates were calculated 7 days after treatment. *rac*-GR24 at 1 ppm was applied as a positive control. Data are presented as means of three replicates. Error bars indicate standard deviations.
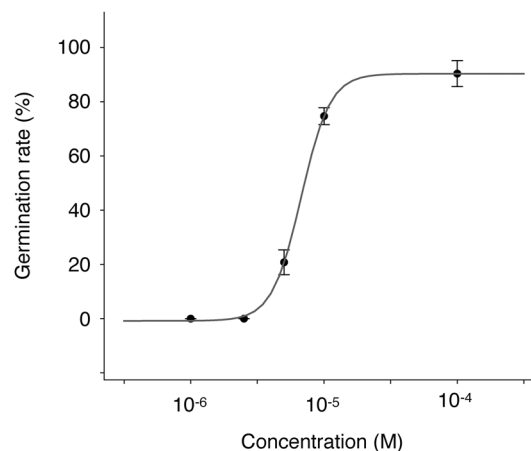

Fig. 3. Dose–dependent germination stimuratory activity of compound **4** on *O. minor* seeds. Germination rate was calculated 7 days after treatment. Data are presented as means of three replicates. Error bars indicate standard deviations.

J1074 as reported previously.[26] The culture broth of *S. albus* J1074 was extracted with ethyl acetate, and successively purified through silica gel column chromatography and preparative reversed-phase HPLC, yielding pure butenolide compounds **1**–**4**.

2. Plant material and germination treatment
Orobanche minor seeds were collected in Yokohama, Japan, in June 2013 and stored at 4°C. Seeds of *Orobanche crenata*, *Phelipanche aegyptiaca*, and *Striga hermonthica* were supplied by Dr. Walid El-Rodeny (Agricultural Research Center, Egypt), Dr. Yaakov Goldwasser (The Hebrew University of Jerusalem, Israel) and Prof. Abdel Gabar Babiker (National Center for Research, Sudan), respectively. The seed germination assay was conduct-
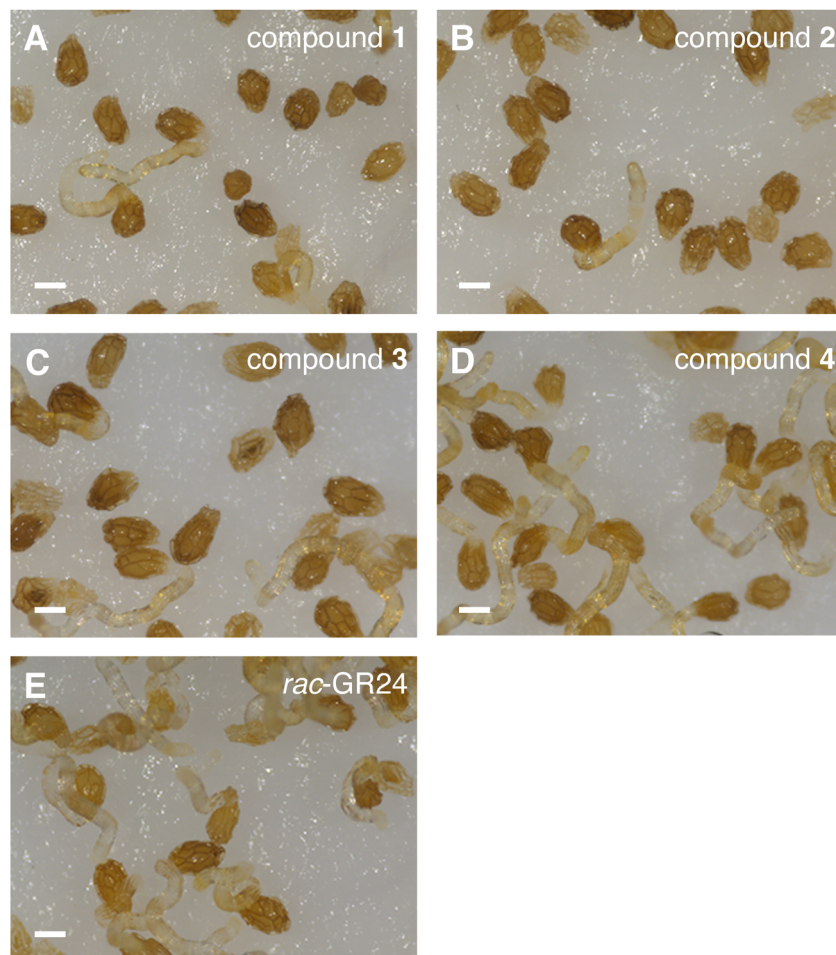
Fig. 4.   Stimulation of *O. minor* seed germination by butenolide compounds **1** (A), **2** (B), **3** (C), and **4** (D) at 10^{-5} M. *rac*-GR24 (1 ppm) was applied as a positive control (E). Microscopic images were taken 7 days after treatment of each compond. Scale bar, 0.2 mm.

ed as reported previously.[27,28] Surface-sterilized seeds were conditioned on two layers of glass filters (Whatman GF/D; GE Healthcare, Chicago, IL, USA) moistened with distilled water in a Petri dish in the dark for 7 days at 23°C for *O. minor*, for 5 days at 21°C for *O. crenata*, for 5 days at 23°C for *P. aegyptiaca*, and for 9 days at 21°C for *S. hermonthica*. After conditioning, the upper layer of the glass filter with the seeds was transferred to a new Petri dish containing a fresh glass filter (Whatman GF/D). Germination was induced by the application of a solution of each compound, and the seeds were incubated at the same temperature for conditioning. Synthetic strigolactone, *rac*-GR24 (Chiralix, Nijmegen, Netherlands), at 1 ppm was used as a positive control. The number of germinated seeds was counted under a microscope, and the germination stimulatory activity of each compound was evaluated as the percentage of germinated seeds per total treated seeds.

Results

1. Germination stimulatory activity of butenolides toward O. minor seed

All butenolides isolated from *S. albus* J1074 induced the ger-

mination of *O. minor* seeds. Compound **4** was the most potent germination stimulant among the tested butenolides (Fig. 2), inducing germination of more than 70% of the treated seeds, while other compounds stimulated less than 30% at 10^{-5} M. Higher concentrations of compounds **1**, **2**, and **3** did not improve the efficiency (data not shown). On the other hand, compound **4** induced the germination of *O. minor* in a dose–dependent manner (Fig. 3). The maximum germination rate (90.4%) was achieved at 10^{-4} M, and the no activity was confirmed at 10^{-6} M. EC_{50} value for germination stimulatory activity of compound **4** was calculated to be 6.3×10^{-6} M. Although germination stimulatory activity of butenolides other than compound **4** was low, radicles induced by compounds **1**–**3** exhibited normal growth (Fig. 4).

2. Germination stimulatory activity of compound 4 toward root parasitic weeds

Since compound **4** exhibited dose–dependent germination stimulatory activity on *O. minor* seeds, its activity was also evaluated for the Orobanchaceae root parasitic weeds, *O. crenata*, *P. aegyptiaca*, and *S. hermonthica*. However, compound **4** did not induce the germination of *O. crenata* or *S. hermonthica* in the concen-

tration range of 10^{-7}–10^{-3} M (data not shown). Gemination of *P. aegyptiaca* was induced occasionally, but the germination rate was very low and the result was not reproducible. From these experiments, we concluded that the germination stimulatory activity of compound **4** is specific to *O. minor*, at least among the tested root parasitic weeds.

Discussion

SLs are key germination stimulants for root parasitic weeds, and the structural diversity of canonical and non-canonical SLs have been identified.[5,6)] The diversity of SLs is proposed to be one of the molecular bases of host specificity in root parasitic weeds.[5,15,16,29)] Host-specific induction of germination of a range of root parasitic weeds has been shown using root exudates from various host and non-host plants.[30)] A synthetic SL, *rac*-GR24 is used widely as a germination stimulant for root parasitic plants.[31)] However, *rac*-GR24 does not induce germination of *Striga gesnerioides*.[32)] Precise evaluation of germination induction using optical isomers of GR24 derivatives revealed that *S. gesnerioides* perceives specific optical isomers as germination stimulants and some isomers as SL antagonists, while *O. minor* and *S. hermonthica* perceive all isomers as germination stimulants.[33)] Those results clearly indicate species-dependent structural requirements of SLs as germination stimulants. Species-specific germination stimulants other than SLs have also been reported. For example, isothiocyanates released as glucosinolate-breakdown products from *Brassica napus* roots strongly and specifically induce germination of *Phelipanche ramosa*.[14,34)] Since glucosinolates are secondary metabolites characteristic to Brassicaceae, response to isothiocyanates might have evolved in *P. ramosa* under host-driven selection pressure.[34)]

As expected considering the diversity of germination strategies in Orobanchaceae, transcriptomic and genomic information reveals highly diversified SL receptors.[15,16,19,23)] Most plants perceive SLs by α/β hydrolase D14s,[8,15,16,20–22)] while SL receptors in Orobanchaceae are encoded by paralogues, *KAI2ds*, in a divergent subclade of *KAI2*.[15,16,19,23)] Since *KAI2* is paralogous to *D14*, KAI2ds in Orobanchaceae and D14s in other flowering plants may have undergone convergent evolution to perceive SLs.[15,16,23)] Four to six copies of *KAI2d* were confirmed in *Orobanche* and *Phelipanche* spp.,[15)] and more than ten *KAI2d* copies exist in *Striga* genome.[23)] Here we found that butenolides from *S. albus* J1074 specifically induced *O. minor* gemination. It is highly possible that these butenolides were perceived by one or more KAI2ds in *O. minor*, resulting in the induction of seed germination.

Structure–activity relationship studies of SL and its mimics as germination stimulants of root parasitic weeds have been widely conducted, because synthetic germination stimulants can be utilized in suicidal gemination for root parasitic weed control.[11,35)] Numerous studies concluded that a terminal butenolide (D-ring, Fig. 1) is essential for the germination stimulatory activity.[11)] A methyl substituent on the D-ring is important for high activity but not essential.[36)] It is possible that the introduction

of a methyl substituent on the butenolide rings of compounds **1–4** will improve their germination stimulatory activities. Naturally, enol-ether bridges between C and D rings are hydrolyzed by D14 upon their reception to reduce active SLs.[22)] This structure can be changed to others, such as carbamate, in germination stimulants for root parasitic weeds.[36,37)] Hydrolysis-resistant analogs of potent carbamate-type germination stimulants still possess germination stimulatory activity on *S. hermonthica*, indicating that hydrolysis of the bridge between the C and D rings is not essential for the activity.[36)] Germination stimulatory activities of compounds **1–4**, substances with octyl residues directly attached to the butenolide rings, indicated that hydrolytic release of the butenolide ring is not necessary for stimulation to occur.

These butenolide hormones trigger avermectin production in *S. avermitilis*, and the C-10 hydroxy group is important for the activity, suggesting the importance of the C-10 hydroxy group in the interaction with the receptor, AvaR1 (Fig. 1).[24,26)] The minimum effective concentration of compound **1** for the induction of avermectin production is 6×10^{-9} M, whereas that of compound **4** is 3×10^{-6} M.[26)] On the contrary, this study demonstrated that compound **4** is the most potent germination stimulants toward *O. minor*, implying that hydrophobic interaction may be a major factor in the binding to receptor(s). Elucidation of the receptor(s) will reveal the precise binding-mechanism and structural requirements for their germination stimulatory activity.

Biological control of weeds is becoming increasingly important in terms of sustainable agriculture, especially in developing countries. Screening of herbivorous insects and pathogenic fungi specific to root parasitic weeds has been conducted, and some *Fusarium* species were found to reduce the emergence of root parasitic weeds.[38)] *Striga* germination is induced by ethylene, and ethylene application succeeded in eradicating *Striga asiatica* in the United States.[39,40)] Ethylene-producing bacterium *Pseudomonas syringae* induced germination of *Striga* spp. efficiently, and is expected to be utilized as a biological inducer of suicidal germination.[41,42)] Since ethylene is a volatile plant hormone, the effect of applied *P. synringae* on crops and surrounding ecosystems should be carefully assessed. In this study, we demonstrated that bacterial butenolide hormones specifically induce *O. minor* germination. Thao *et al.* reported that the concentration of active hormones towards actinomycetes is 1,000 units/mL in the culture broth, corresponding to *ca.* 8.0×10^{-6} M in total.[25)] However the concentration of each butenolide hormone in the culture broth remains to be determined. Here, we determined that EC_{50} of compound **4** is 6.3×10^{-6} M, which might be comparable to the concentration in the culture broth. Further evaluation of germination stimulatory activity of the culture broth will help us to consider the feasibility of its utilization as a germination stimulant for root parasitic weed control. Bioinformatics revealed the diversity of secondary metabolism in *Streptomyces* even at a strain level.[43)] There is a possibility that other metabolites in actinomycetes also exhibit germination stimulatory activity toward root parasitic weeds.

Previously, we found that nojirimycin, an antibiotic produced by *Streptomyces ficellus*, inhibits the germination of root parasitic weeds.[28,44,45] Based on these findings, the application of *Streptomyces* spp. as a novel approach to biological control of root parasitic weeds is expected.

Acknowledgements

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