J. Pestic. Sci. **49**(1), 22–30 (2024)
DOI: 10.1584/jpestics.D23-038

JOURNAL OF PESTICIDE SCIENCE

Regular Article

Cycloheximide in the nanomolar range inhibits seed germination of *Orobanche minor*

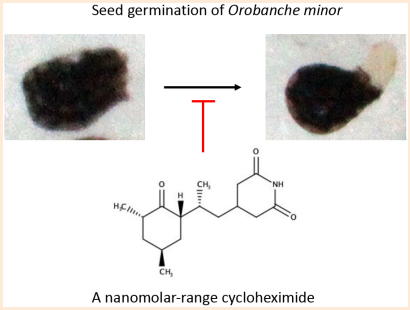
Ryosuke Nogami,[1,†] Mari Nagata,[2,†] Risa Imada,[2] Kenji Kai,[1,2] Takashi Kawaguchi[1,2] and Shuji Tani[1,2,*]

[1] *Graduate School of Agriculture, Osaka Metropolitan University, 1–1 Gakuen-cho, Sakai, Osaka 599–8531, Japan*
[2] *Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–1 Gakuen-cho, Sakai, Osaka 599–8531, Japan*

(Received July 31, 2023; Accepted October 29, 2023)

Supplementary material

From the 992 samples of culture extracts of microorganisms isolated from soil in Japan, we found that the extract of *Streptomyces* sp. no. 226 inhibited *Orobanche minor* seed germination without significantly affecting the seed germination of *Trifolium pratense* and the growth of *Aspergillus oryzae* and *Escherichia coli*. Using ESI-MS, ^{1}H-NMR, and ^{13}C-NMR, we identified the active compound as cycloheximide. Cycloheximide had half-maximum inhibitory concentrations of 2.6 ng/mL for the inhibition of seed germination of *O. minor* and 2.5 μg/mL for that of the conidial germination of *A. oryzae*. Since cycloheximide is known to inhibit translation by interacting with ribosomal protein L28 (RPL28) in yeast, we investigated whether RPL protein of *O. minor* plays a critical role in the inhibition of *O. minor* seed germination. Our data suggested that *O. minor* RPL27A was not sensitive to cycloheximide by comparing it to the strain expressing *S. cerevisiae* RPL28. These findings suggest the presence of an unidentified mechanism by which cycloheximide hinders *O. minor* seed germination.



Keywords: *Streptomyces*, chemical control, broomrape, seed germination.

Introduction

Orobanchaceae includes root parasite weeds, which are noxious parasitic weeds that represent a severe danger to numerous crops across the globe, resulting in considerable output losses and financial strain on farmers. Orobanchaceae includes the broomrape, *Orobanche*, and *Phelipanche* species, which are mostly found in the Mediterranean area and parasitize a broad variety of crops and beans.[1)] The genus *Orobanche* has over 100 species, four of which are recognized as important weeds in Europe: *Orobanche crenata* Forsk., *Orobanche cumana* Waller., *Orobanche ramosa* L., and *Orobanche aegyptiaca* Pers.[2)] Although crop loss has not yet been recorded, *Orobanche minor*, a parasite of red clovers (*Trifolium pratense*), is known to inflict economic harm. Red clovers thrive in a symbiotic relationship with nitrogen-fixing *Rhizobium* bacteria. As a result, they were employed as a form of fertilizer in numerous locations around Japan. Since *O. minor* seeds were introduced to Japan in the first part of the twentieth century together with red clover seeds (unpublished data), they have grown wild in Japan. As a result, in Japan, we initiated research on suppressing seed germination of broomrape using active chemicals from microorganisms or the microorganisms themselves, using *O. minor* as a model.

Several parasitic weed management strategies have been presented. Only after a few weeds have sprouted in a limited area may mechanical techniques (hand picking) be used to inhibit the production of weed seeds. However, because *Orobanche* and *Phelipanche* produce hundreds of thousands of small seeds that can remain dormant in the soil for more than ten years while still germinating,[3,4)] removing broomrape by hand pulling is impossible once it has spread. As a result, chemical control of broomrape has been studied since the 1970s. Glyphosate proved

* To whom correspondence should be addressed.
E-mail: shujitani@omu.ac.jp
† These authors contributed equally to this work.
Published online December 26, 2023

effective in controlling broomrape in carrots, parsley, and faba beans at low rates. Sulfosulfuron and rimsulfuron, both sulfonylurea herbicides, were also efficient in controlling broomrape in tomatoes and potatoes.[5] However, broomrape's seed dormancy makes chemical management challenging. Host plant roots lengthen and leak strigolactones in root exudates, which promote broomrape seed development at the place where the roots newly grow throughout the harvest season. Because this occurrence happened in the soil, herbicides must be continually fed into the soil, making chemical management of broomrape problematic.[5]

Broomrape may be biologically managed by destroying its seed bank or preventing it from identifying its hosts. *Phytomyza orobanchia*, an insect herbivore, has been used in the particular biological control of broomrape.[6] Filamentous fungi were utilized for biological control as well. In many situations, phenolic chemicals found in the testa of broomrape seeds inhibit seed destruction by microbes. Plant diseases, on the other hand, such as *Fusarium* sp. and *Ulocladium botrytis*, may disintegrate the endosperm cell walls, enter within, and metabolize the cytoplasm of their seeds.[7] Bacteria such as *Pseudomonas aeruginosa* and *Bacillus subtilis* may inhibit broomrape radicle formation.[8] Arbuscular mycorrhizal fungi such as *Rhizobium leguminosarum* and *Azospirillum brasilense* may lower strigolactones in mycorrhizal plant root exudates, promoting the broomrape seed bank to stay dormant.[9] Despite the fact that many weed control techniques have been explored, traditional weed management tactics have proved ineffective in regulating its expansion, emphasizing the urgent need for new and long-term solutions to tackle this deadly plant.

We tested roughly 1,000 microorganisms isolated from soil in Japan for active compounds preventing *O. minor* seed germination as the first stage in developing chemical or biological control methods for broomrape. We discovered *Streptomyces*, which generates a chemical that inhibits *O. minor* seed germination but has no influence on the seed germination of *T. pratense* and the growth of *Aspergillus oryzae* and *Escherichia coli*. Purification and identification of the substance indicated that cycloheximide is an inhibitor of *O. minor* seed germination. Interestingly, cycloheximide decreased *O. minor* seed germination at nanomolar concentrations. Cycloheximide's mode of action on seed germination in *O. minor* was investigated and addressed.

Materials and methods

1. Strains and media

Minimal medium,[10] yeast peptone dextrose broth (Takara), and Luria–Bertani (LB) broth were used to culture *A. oryzae* RIB40 (NBRC 100959), *Saccharomyces cerevisiae* Y2HGold (Takara, Shiga, Japan), and *E. coli* DH5α (Takara), respectively.

2. Isolation of actinomycetes from soil samples and preparation of culture extracts

Actinomycetes were largely isolated from soil samples obtained in Japan using the previously published modified HVG agar medium.[11] To keep isolated microorganisms alive, Maltose-Bennett's agar media[12] were utilized. Inhibitors of *O. minor* seed germination were identified using extracts generated from cultures cultured for 5 days in liquid medium A at 30°C (Supplemental Table S1) by adding an equivalent amount of acetone and mixing.

3. Screening microorganisms for compounds inhibiting seed germination of O. minor

In May, *O. minor* seeds were harvested in Hyogo, Japan, and kept at 4°C. Conditioned seeds that had been treated to aid germination were produced as previously reported.[13] The organic solvent was air-dried after 14 μL of culture extracts was applied to glass filters (Whatman GF/D; Cytiva, Tokyo, Japan), and then 27 μL of GR24 solution (0.1–1.0 mg/L (w/v)) diluted with distilled water was added to filter paper. A glass filter containing conditioned *O. minor* seeds was then put on the chemically treated filter. The seeds were examined under a microscope after one week, and the germination rate was obtained by dividing the number of germinated seeds by the total number of seeds. Professor Kuse of Kobe University in Japan generously contributed GR24.

Isolated microorganisms were identified based on 16S rRNA DNA sequence analysis. This involved polymerase chain reaction using primers 5′-AGAGTTTGATCCTGGCTCAG and 5′-AAGGAGGTGATCCAGCCGCA)[14] followed by Sanger sequencing.

4. Evaluation of the specific inhibition of the extracts and cycloheximide

T. pratense seeds were produced on glass filters (Whatman GF/D), put on another glass filter treated with chemicals, 27 μL of distilled water was added, and an average of 20 *T. pratense* seeds were checked for germination after one week. The germination rate was obtained by dividing the total number of seeds by the number of germinated seeds. 25 μL of culture extracts and 25 μL of submerged minimal medium containing 1×10^2 conidia of *A. oryzae* RIB40 were incubated at 30°C without shaking for 20 hr, and then conidium germination was seen under a microscope. The germination rate was estimated by dividing the total number of conidia by the number of germinated conidia. An overnight culture of *E. coli* DH5α was diluted 1/100 in LB broth and combined with 25 mL of actinomycete culture extract for the *E. coli* inhibition experiment. As a control, an *E. coli* dilution in 50% acetone/LB medium was employed. The mixes were incubated at 37°C for 4 hr before being tested at 600 nm for growth. The *E. coli* growth rate was estimated by dividing the OD_{600} of the extract-treated culture by the OD_{600} of the control.

To study the inhibitory impact of cycloheximide, different quantities were applied to grass filters with GR24 for the inhibition of *O. minor* seed germination or to minimal medium for the suppression of *A. oryzae* conidium germination.

5. Purification of seed germination inhibitor

Streptomyces sp. no. 226 was cultivated in medium A for 2 days at 30°C. Aliquots were transferred to 200 mL of the medium described in media A to F (Supplemental Table S1) and cultured for 3 days at 30°C. EtOAc extracts were produced and tested for their ability to suppress *O. minor* seed germination. After adjusting the culture conditions, medium B was chosen to grow *Streptomyces* sp. no. 226. To purify the chemical, this strain was grown in medium B at 30°C for 5 days. The supernatant (51.8 L) was extracted twice using an equal amount of EtOAc and then evaporated. The crude extract (7.22 g) was loaded onto a Wakogel C-200 column (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and eluted using a stepwise gradient of *n*-hexane/EtOAc (100:0 to 0:100), followed by evaporation. The dried material obtained from the fraction of *n*-hexane/EtOAc (25:75) was resuspended in MeCN before being purified using an Inertsil ODS-3 column (250×10 mm, 5 μm, GL Sciences, Tokyo, Japan). The gradient elution was as follows: 30 min of H_2O/MeCN (from 90:10 to 40/60), 10 min of H_2O/MeCN (40:60), and 10 min of H_2O/MeCN (0:100). The eluent was fractionated every 1 min, and the flow rate was adjusted to 4.0 mL/min. In the 11th fraction, the seed germination inhibitor was discovered. The bioactive component was subjected to HPLC with the Inertsil ODS-3 column (250×10 mm, 5 μm) at a flow rate of 4.0 mL/min with 30% MeCN. A significant single peak was identified at the retention time (12.6 min) and processed to obtain 4.6 mg of dry powder.

6. Spectroscopic analysis of the purified compound and cycloheximide

The structures of the purified compound and cycloheximide (Fujifilm, Tokyo, Japan) were determined using ^{1}H- and ^{13}C-NMR spectroscopy and COSY. DMSO-d_6 was used as the solvent, and the NMR measurements were conducted on a JNM-ECZ500R/S1 NMR spectrometer (JEOL Ltd., Tokyo, Japan). The chemical shifts were referenced to the solvent peak (δ_H 2.49, δ_C 39.7) as an internal standard. Additionally, the molecular mass was determined using the LCMS 2020 system (Shimadzu, Kyoto, Japan).

7. Generation of S. cerevisiae *strains that expressed the* rpl *genes*

The ribosomal 60S subunit protein L28 gene (*rpl28*; gene ID, 852775) of *S. cerevisiae* is homologous to the *rpl27A* gene of *A. oryzae* (*rpl27A*; gene ID, AO090001000413) and the *rpl27A* gene of *O. minor* (gene ID, IADW01093067). The *rpl* genes were expressed using pGADT7 that had the GAL4 activation domain deleted by PCR using primer sets, pGADT7F and pGADT7R, followed by *Eco*RI digestion and ligation to generate pGBKT7ΔBD. The *S. cerevisiae rpl28* gene was amplified by PCR using the primer pairs ScRPL28F and ScRPL28R, then digested with *Cla*I and *Bam*HI. pGBKT7ΔBD-*rpl28* was created by subcloning this fragment into their sites. *S. cerevisiae* RPL28 was amplified using primer sets ScRPL28F and ScPRL28Q38MR and ScPRL28Q38MF and ScRPL28R, and then fused by PCR with primer sets ScRPL28F and ScRPL28R. This fragment was subcloned into the *Cla*I and *Bam*HI sites, resulting in pGBKT7ΔBD-rpl28Q38M. The *O. minor rpl27A* gene was amplified using the OmRPL27AF and OmRPL27AR primer sets and ligated into the *Cla*I and *Bam*HI sites to produce pGBKT7ΔBD-rpl28Q38M. The ADH1 promoter, *rpl* genes, and *leu2* marker gene were amplified using primer sets RPL_leuF and RPL_leuR. In *S. cerevisiae*, the 5′ and 3′ franking regions of *rpl28* were amplified using primer sets ScRPLbf500F and ScRPLbf500R and ScRPLaf500F and ScRPLaf500R, respectively. These three fragments were constructed using the primer sets ScRPLbf500F and ScRPLaf500R, which include 500 bp homologous to the flanking region of *rpl28*. The amplified DNA fragments were utilized to alter the Y2HGold strain of *S. cerevisiae* (Takara Bio, Shiga, Japan). Nested PCR was used to validate transformants in which the *rpl28* locus was replaced with the inserted DNA pieces by homologous recombination. For *rpl28*-WT and *rpl28*-Q38M, primer sets F1 and R1 were used, while for *O. minor rpl27A*, primer sets F1 and R2 were used. The second PCR was amplified using primer sets F2 and R1 and F2 and R2. Supplementary Table S2 summarizes the primers utilized in this work.

8. Susceptibility test of yeast strains expressing rpl28 *orthologs cycloheximide*

Overnight cultures of *S. cerevisiae* strains, which expressed *rpl28* orthologs, were adjusted to an optical density (OD_{600}) of 1.0 using MES buffer (pH 6.0). Subsequently, these cultures were serially diluted and spotted onto SD media lacking leucine, supplemented with varying concentrations of cycloheximide as indicated. Media were kept for 3 days at 30°C. Representative images of three biological replicates are shown.

Results

1. Screening microorganisms producing inhibitors for seed germination of O. minor

Since actinomycetes are known as prolific producers of natural products with a wide range of biological activities,[15] *Actinomycetes* were primarily screened from soil samples obtained in different locations in Japan to separate microorganisms providing inhibitors for seed germination of *O. minor*. A small quantity of dirt was combined with water before being diluted with an SDS-yeast solution. The solution was warmed at 40°C for 20 min to decrease bacteria and increase actinomycetes development. A portion of the solution was applied to the humic acid medium. For 5 days, the isolated strains were grown in a submerged culture with shaking at 30°C. Acetone extracts were made by adding an equivalent quantity of acetone to the culture broth and centrifuging the mixture. The produced acetone extracts, as well as the synthetic strigolactone GR24, were employed to treat Japanese *O. minor* seeds. Seeds were kept at 25°C for 7 days, and *O. minor* seed germination was studied under a microscope.

From 992 acetone extracts, 452 samples (45%) preventing *O. minor* seed germination were found. Thus, extracts were tested

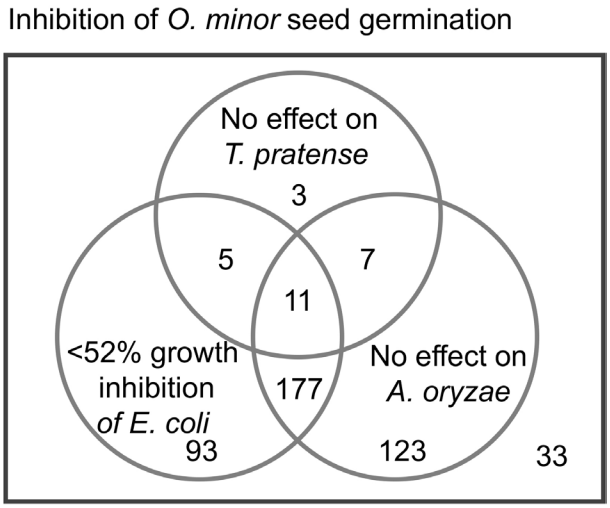


Fig. 1. Venn diagram illustrating the effects of culture extracts on the seed germination of *O. minor* and *T. pratense*, the conidial germination of *A. oryzae*, and the growth of *E. coli*.

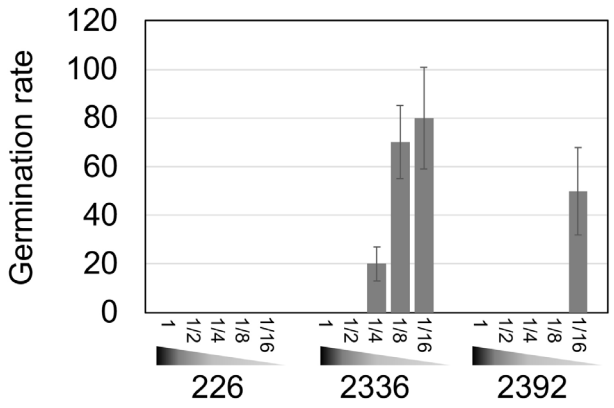


Fig. 2. Dose-dependent effect of culture extracts (sample no. 226, 2336, and 2392) on seed germination of *O. minor*. The seed germination rate under the control condition (with GR24) was set at 100%. The extract samples were diluted with sterilized water in a sequential manner and applied for the test. The X-axis represents the dilution ratio of each strain's culture extract relative to the original solution. The relative germination levels represent the means of three independent experiments, with error bars indicating the standard deviations (n=3).

for selectivity for inhibition of seed germination of *T. pratense*, a host of *O. minor*; conidium germination of *A. oryzae*, a representative of eukaryotic microorganisms; and growth of *E. coli*, a representative of prokaryotic microorganisms. Samples that preferentially inhibited *O. minor* seed germination while having little influence on each other's development were chosen. Samples with an *E. coli* growth inhibition rate of less than 52% relative to control conditions were selected, yielding 11 samples that met the criteria (Fig. 1). When 11 samples were extracted with ethyl acetate, the active chemicals from eight of them were found in the aqueous layer and three in the organic layer. The hydrophilic compound that inhibited seed germination at the lowest concentrations was derived from a strain similar to *Strep-*

Table 1. 16S Ribosomal DNA sequence of the isolated microorganism

	Top hits of BLAST analysis	Identity (%)
no. 226	*Streptomyces* sp. strain KCB16C001	99%
	Streptomyces tsukiyonensis strain: NBRC 14353	99%
	Streptomyces xanthocidicus strain: NBRC 13469	99%

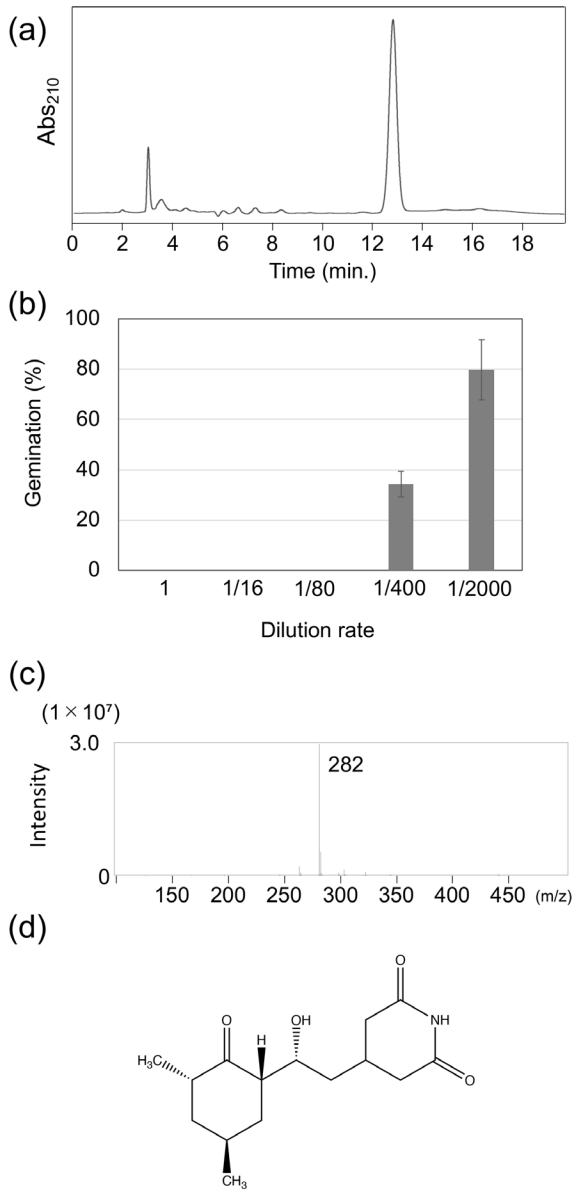


Fig. 3. LC-MS data and activity of the purified compound. (a) HPLC profile indicating the bioactive fraction at 12.6 min. (b) Dose-dependent effect of the purified compound on seed germination of *O. minor*. The seed germination rate under the control condition (with GR24) was set at 100%. The purified sample was diluted with sterilized water in a sequential manner and applied for the test. The X-axis represents the dilution ratio of each strain's culture extract relative to the original solution. The relative germination levels represent the means of three independent experiments, with error bars indicating the standard deviations (n=3). (c) ESI-MS data of the purified compound from *Streptomyces* sp. no. 226. The $[M+H]^+$ of the sample was detected at m/z 282. (d) Chemical structure of cycloheximide.

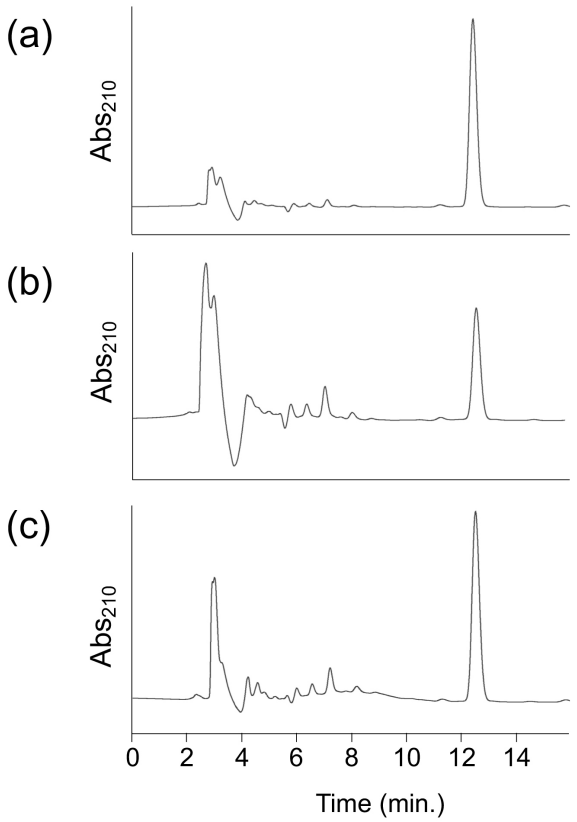


Fig. 4. HPLC analysis of the purified compound (a), cycloheximide (b), and mixture (c).

tomyces nojiriensis strain BCCO10_878 (identity, 98%) (data not shown), which is known to produce nojirimycin, which inhibited *O. minor* seed germination.[12] That information implied that hydrophilic compounds might include nojirimycin. Therefore, we decided to focus on hydrophobic compounds and made progressive dilutions of three hydrophobic samples, nos. 226, 2336, and 2392, and chose sample no. 226, which affected seed germination the least (Fig. 2). The isolated microorganism's 16S ribosomal DNA sequence is comparable to that of *Streptomyces* species (Table 1). As a result, the isolated strain was given the name *Streptomyces* sp. no. 226.

2. *Purification and identification of the inhibitor for seed germination of* O. minor

We optimized the culture conditions and proceeded with the purification of an inhibitor of *O. minor* seed germination from a 51.8 L culture of strain no. 226. The active compound was extracted using ethyl acetate, resulting in 7.2 g of dry material. We performed chromatography using Wakogel C-200 and Inertsil ODS-3 columns, which yielded 4.6 mg of the purified compound as described in the materials and methods. This compound exhibited a single peak with a retention time of 12.6 min and demonstrated inhibition of *O. minor* seed germination (Figs. 3a and b). ESI-MS analysis of the purified compound revealed a molecular ion $[M+H]^+$ at m/z 282 (Fig. 3c). Further analysis through ^{1}H-NMR, ^{13}C-NMR, COSY, and distortionless enhancement by polarization transfer experiments indicated the presence of two methyl carbons, three ketones, and five CH_2 groups in the purified compound. We searched the *Streptomyces* natural product database StreptomeDB3.0[16] and identified cycloheximide as a candidate compound that matched the criteria, with a mass of 281 (Fig. 3d). We compared ^{1}H-NMR data of the purified compound with those of cycloheximide reported previously[17] and ^{13}C-NMR data of cycloheximide, which we analyzed in this study (Supplementary Table S3). Their spectra were identical, although there were some extra signals in the spectra data in the purified compound by comparing them to those of cycloheximide probably due to contaminants. Chromatograms of the purified compound alone (Fig. 4a), cycloheximide alone (Fig. 4b), and their mixture (Fig. 4c) displayed a single peak at a retention time of 12.6 min, indicating that the purified compound contained cycloheximide. The half maximal inhibitory concentration (IC_{50}) of the purified compound for *O. minor* seed germination was determined to be 3.5 ng/mL, equivalent to that of cycloheximide, as demonstrated below.

3. *The IC_{50} of cycloheximide for inhibition of seed germination of* O. minor *and that of conidial germination of* A. oryzae

We explored the reason why cycloheximide, a well-known translational inhibitor for Eukaryote,[18,19] was identified despite preferentially selecting molecules that did not suppress fungal growth (Fig. 1). Cycloheximide had an IC_{50} of 2.6 ng/mL for inhibition of seed germination of *O. minor* and 2.5 μg/mL for that of conidial germination of *A. oryzae* (Fig. 5).

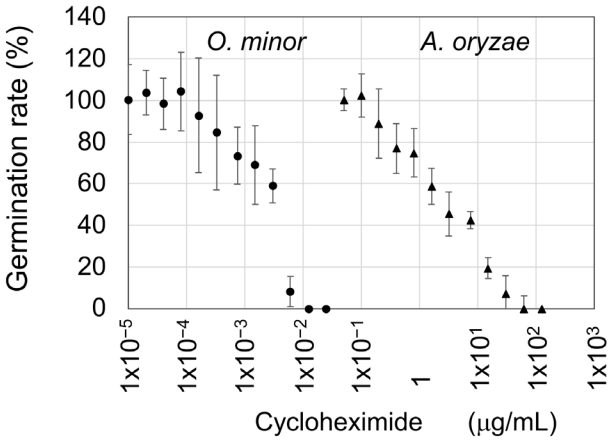


Fig. 5. Dose-dependent effect of cycloheximide on the seed germination of *O. minor* (filled circle) and the conidial germination of *A. oryzae* (filled triangle). Cycloheximide was diluted with sterilized water in a sequential manner and applied for the test. The relative germination levels represent the means of three independent experiments, with error bars indicating the standard deviations (n=3).

4. The mechanism underlying the inhibition of seed germination of O. minor *by cycloheximide differs from the translational inhibition mechanisms of* S. cerevisiae

Cycloheximide has been known to inhibit seed germination of *Triticum aestivum*, *Arabidopsis thaliana*, and *Striga hermonthica*.[20–22] Seed germination of *S. hermonthica* was inhibited to approximately 30% with 1 μM cycloheximide.[21] Interestingly, seed germination of *T. pratense* was not inhibited by 100 ng/mL cycloheximide, which completely inhibited seed germination of *O. minor*. The mechanisms by which cycloheximide inhibits the seed germination of *O. minor* at concentrations as low as one-thousandth of that required to inhibit conidium germination in *A. oryzae* remains unknown.

S. cerevisiae has an IC_{50} of 0.3 μg/mL for cycloheximide, which is approximately 100 times greater than that of *O. minor*.[23] We looked into the mechanism of action of cycloheximide in *S. cerevisiae* since genetic modification in *Saccharomyces* is easier than that in *Aspergillus*. Cycloheximide is known to inhibit translation by binding to the yeast ribosomal 60S subunit protein L28 (Rpl28) in *S. cerevisiae*.[24] The changes that gave *S. cerevisiae* cycloheximide resistance occurred in two areas: Gly37/Gln38/His39 and Gly54/Lys55/Arg59, according to a thorough mutational investigation of RPL28 in *S. cerevisiae*.[25] We found that only the 38th methionine of *O. minor* RPL27A was not conserved among the six amino acids described above by comparing them to the amino acid sequences of RPL28 of *S. cerevisiae*, RPL27A of an *A. oryzae* homolog, and RPL27A of an *O. minor* homolog (Fig. 6a). We created *Saccharomyces* strains that express the wild-type RPL28 (WT), RPL27A of *O. minor* (Om), and *S. cerevisiae* PRL28 with the 38th amino acid substitution of methionine (Q38M), allowing the expression of proteins at the endogenous *rpl28* locus (Fig. 6b), in order to determine whether this amino acid change affects the IC_{50} value of cycloheximide in *O. minor*. Through homologous recombination, each expression cassette was inserted into the *S. cerevisiae rpl28* locus (Fig. 6c). These three strains were cultivated, the cell numbers were adjusted, the medium was successively diluted, and the cells were spotted on it (Figs. 7a–e). All three strains developed uniformly in the absence of cycloheximide (Fig. 7a). The growth of WT and Q38M on cycloheximide-containing media reduced in a dose-dependent manner (Fig. 7e), while the growth of Om did not slow down even on medium containing 50 μg/mL cycloheximide (Figs. 7a–e). These findings show that *O. minor RPL27A* is not susceptible to cycloheximide and imply that cycloheximide may prevent *O. minor* seed germination *via* an unknown mechanism.

Discussion

It has been widely accepted for more than 50 years that cycloheximide has the ability to inhibit the growth of fungi,[19,26]

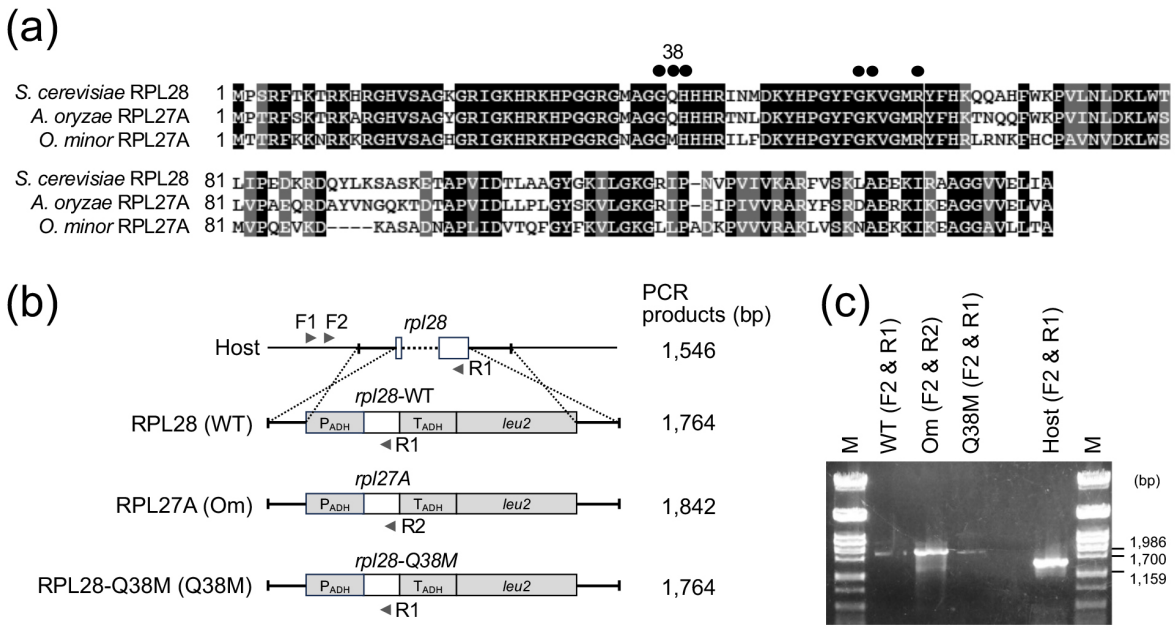


Fig. 6. Generating *S. cerevisiae* strains that produce the wild-type RPL28 of *S. cerevisiae* (WT), *O. minor* RPL27A (Om), and RPL28-Q38M (Q38M). (a) Alignment of RPL proteins. *S. cerevisiae* RPL28 (gene ID, 852775); *A. oryzae* RPL27A (gene ID, AO090001000413); *O. minor* RPL27A (gene ID, IADW01093067). Blocks of identical or similar amino acids are marked by black or gray backgrounds, respectively. Filled dots indicate critical amino acids conferring cycloheximide resistance to *S. cerevisiae*, including Gly37, Gln38, His39, Gly54, Lys55, and Arg59. (b) The illustrations depict the DNA sequence at the *rpl28* locus in *S. cerevisiae*. The dotted line represents the intron of *rpl28*. The WT strain possesses *rpl28* without its intron. P_{ADH} represents the promoter region of the alcohol dehydrogenase gene (*adh*), while T_{ADH} represents the terminator region of *adh*. The marker gene encoding β-isopropylmalate dehydrogenase is indicated as *leu2*. F1, F2, R1, and R2 denote the primers used to evaluate homologous recombination at the *rpl28* locus. The sizes of PCR products are indicated. (c) The amplified DNA bands obtained through PCR with the indicated primer sets confirm the integration of DNA cassettes into the *rpl28* locus through homologous recombination.

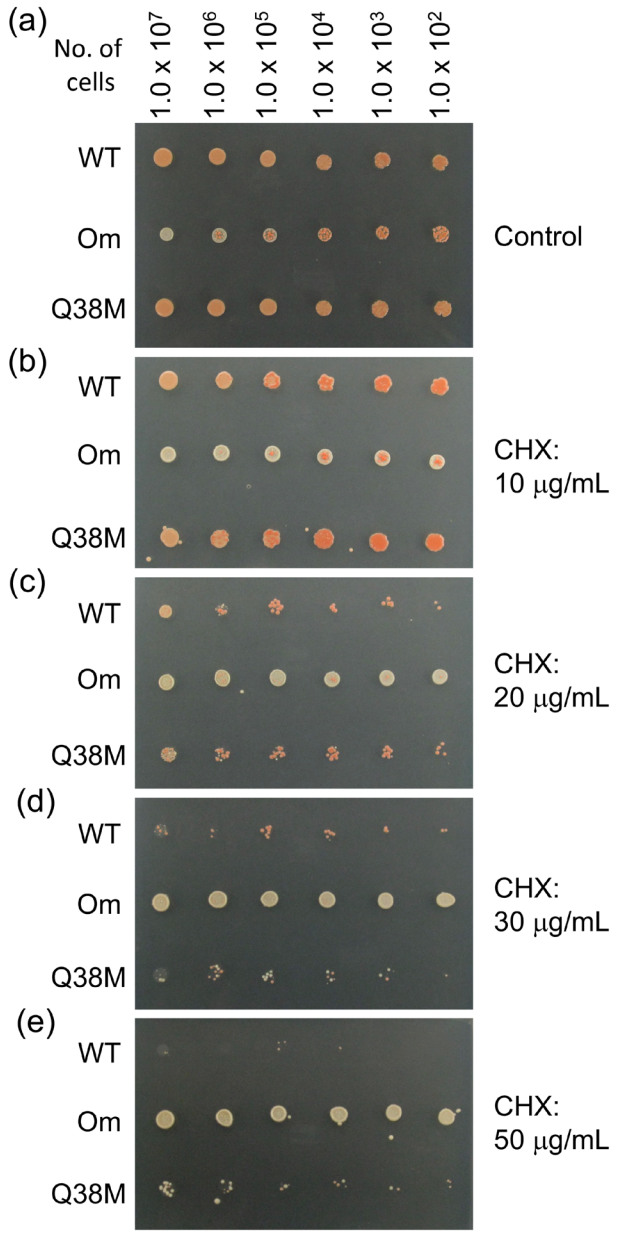


Fig. 7. Cycloheximide affects the development of RPL28-Q38M (Q38M), *O. minor* RPL27A (Om), and wild-type (WT) RPL28-producing *S. cerevisiae* strains. The *S. cerevisiae* cultures were progressively diluted with YPD before being used for the experiment.

algae,[27] protozoa,[28] and higher plants,[29] but not of bacteria.[25] Notably, cycloheximide prevents the production of proteins in both yeast and human cells.[30,31] Our research mainly focused on screening actinomycetes for compounds that can efficiently inhibit the seed germination of *O. minor* without significantly affecting the seed germination of *T. pratense*, the conidium germination of *A. oryzae*, or the growth of *E. coli*. However, our research showed that the substance that specifically inhibited *O. minor* seed germination was cycloheximide. It is important to note that cycloheximide suppresses *O. minor* seed germination at a dose that is 1/1,000 times lower than what is necessary to prevent *A. oryzae* conidia from germinating. *O. minor* RPL27A in *S. cerevisiae* was subjected to functional investigation, which revealed that it seems to be insensitive to cycloheximide. So, in contrast to how it affects *S. cerevisiae*, our work provides the first evidence for the different processes by which cycloheximide limits the germination of *O. minor* seeds.

Extremely tiny *Orobanche* species seeds fall into two categories depending on how they are shaped. *O. minor* and *Orobanche crenata* seeds are categorized as type II seeds, which may vary in size from 0.27–0.57×0.14–0.41 mm^2[2] and *O. crenata* seeds average 4.2 mg in weight per thousand.[32] This is much less than the *T. pratense* seed weight of 2 g in weight per thousand.[33] Therefore, in our screening procedure, a particular quantity of culture extracts could be more inhibitive to *O. minor* seed germination than to *T. pratense* seed germinations. We did not, however, expect that cycloheximide would be more successful in inhibiting *O. minor* seed germination than *A. oryzae* conidium germination (Fig. 5).

According to McGeachy, Meacham, and Ingolia (2019), the binding of cycloheximide to RPL28 is responsible for limiting yeast growth. We carried out studies (Fig. 7) to determine if the *S. cerevisiae* strains expressing *O. minor* RPL27A are susceptible to cycloheximide or resistant to it. The findings imply that *O. minor* RPL27A is not responsible for the lower IC_{50} for cycloheximide in *O. minor* compared to *A. oryzae* since strains expressing RPL27A are resistant to cycloheximide.

In yeast, pleiotropic drug resistance genes, which control the expression of multidrug-resistance transporter genes, transcription factors like PDR3,[34] and the 20S proteasomal genes in cycloheximide-resistant mutants have all been linked to gene modifications that result in cycloheximide resistance.[35] By increasing the transcription of PDR5, which encodes a potential membrane transporter, overexpression of PDR3 boosted cycloheximide resistance.[34] In a different instance, proteasome-dependent proteolysis mutations cause an imbalance between protein synthesis and breakdown, which inhibits growth.[36] According to Hanna *et al.*, it is not necessary for yeast growth to be inhibited for all proteins to be synthesized and broken down. According to Hannah *et al.*,[37] cycloheximide inhibits the buildup of certain proteins, notably ubiquitin, which results in a growth arrest mechanism.

Protein synthesis in plants is a labor-intensive metabolic process that consumes a substantial amount of the energy needed for plant development.[38] On the other hand, according to Scheurwater *et al.*,[39] 20 to 30% of the ATP created by root respiration is a result of protein breakdown. As a result, the disruption of the energy flow balance between synthesis and degradation caused by the restriction of protein synthesis may have an impact on plant development. Cycloheximide has been studied in *Arabidopsis*, where it has been discovered to stimulate the expression of many genes related to the abscisic acid (ABA) response, including hormone synthesis and response genes.[40] However, the effect of cycloheximide on the growth of Orobanchaceae is still unknown. According to Kermode, ABA is

known to inhibit seed germination and encourage seed dormancy.[41] The increase of enzymes involved in defense metabolism and cell wall formation is similarly accelerated by cycloheximide in *Pisum sativum*.[42] In *Arabidopsis thaliana*, it has been shown that the production of cell walls also promotes the defensive response and slows plant development.[43] The combination of those factors may clarify the processes by which cycloheximide inhibits seed germination in *O. minor*, while it is still important to confirm if similar effects are present in *Orobanche*. In addition, the cycloheximide absorption effectiveness and accumulation rate need attention, while the specifics are not yet known. Clarifying these issues could help us get a more thorough grasp of how cycloheximide works in Orobanchaceae.

Conclusions

Cycloheximide has been identified as an inhibitor for the germination of *O. minor* seeds, derived from culture extracts of *Streptomyces* sp. no. 226. It is noteworthy that cycloheximide effectively suppresses the germination of *O. minor* seeds at a dose that is 1/1,000 times lower than the required amount to hinder the *A. oryzae* conidial germination. The interaction between the 38th glutamine of RPL28 in *S. cerevisiae* and cycloheximide is essential for translational inhibition. However, it appears that the *O. minor* ortholog, RPL27A, does not play a critical role in the inhibition of *O. minor* seed germination by cycloheximide.

Acknowledgements

We thank Professor Yukihiro Sugimoto and Professor Masaki Kuse for supporting this study.

Funding

This work was supported by the JST/JICA SATREPS (JPMJSA1607 to S.T.).

Disclosure statement

No potential conflict of interest was supported by the authors.

Author's contribution

S.T. and T.K. conceived and designed the project. R.N., M.N., and R.I. conducted the experiments. K.K. conducted the part of chemical analysis. S.T wrote the paper. All authors read and approved the final manuscript.

Electronic supplementary materials

The online version of this article contains supplementary materials (Figs. S1, S2) which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

Data availability

Composition of media and primers used in this study were summarized in a supplementary file.

Supplementary material

Supplementary material is available at the Journal of Pesticide Science online.

References

1) M. Vurro: Are root parasitic broomrapes still a good target for bioherbicide control? *Pest Manag. Sci.* (2023). https://doi.org/10.1002/ps.7360
2) L. Plaza, I. Fernandez, R. Juan, J. Pastor and A. Pujadas: Micromorphological studies on seeds of *Orobanche* species from the Iberian Peninsula and the Balearic Islands, and their systematic significance. *Ann. Bot.* **94**, 167–178 (2004).
3) D. M. Joel, J. Hershenhorn, H. Eizenberg, R. Aly, G. Ejeta, P. J. Rich, J. K. Ransom, J. Sauerborn and D. Rubiales: Biology and management of weedy root parasites. *Hortic. Rev. (Am. Soc. Hortic. Sci.)* **33**, 267–349 (2007).
4) D. M. Joel, J. C. Steffens and D. E. Matthews: Germination of weedy root parasites. In "Seed development and germination," ed. by J. Kigel and G. Galili, Marcel Dekker, New York, pp. 567–597, 1995.
5) H. Eizenberg, R. Aly and Y. Cohen: Technologies for smart chemical control of broomrape (*Orobanche* spp. and *Phelipanche* spp.). *Weed Sci.* **60**, 316–323 (2017).
6) O. Klein and J. Kroshel: Biological control of *Orobanche* spp. with *Phytomyza orobanchia*, a review. *BioControl* **47**, 245–277 (2002).
7) H. Thomas, A. Heller, J. Sauerborn and D. Müller-Stöver: *Fusarium oxysporum* f. sp. orthoceras, a potential mycoherbicide, parasitizes seeds of *Orobanche cumana* (Sunflower broomrape): A cytological study. *Ann. Bot.* **83**, 453–458 (1999).
8) S. Barghouthi and M. Salman: Bacterial inhibition of *Orobanche aegyptiaca* and *Orobanche cernua* radical elongation. *Biocontrol Sci. Technol.* **20**, 423–435 (2010).
9) J. A. López-Ráez, T. Charnikhova, I. Fernández, H. Bouwmeester and M. J. Pozo: Arbuscular mycorrhizal symbiosis decreases strigolactone production in tomato. *J. Plant Physiol.* **168**, 294–297 (2011).
10) H. Adachi, S. Tani, S. Kanamasa, J. Sumitani and T. Kawaguchi: Development of a homologous transformation system for *Aspergillus aculeatus* based on the *sC* gene encoding ATP-sulfurylase. *Biosci. Biotechnol. Biochem.* **73**, 1197–1199 (2009).
11) S. Tani, N. Nishio, K. Kai, D. Hagiwara, Y. Ogata, M. Tojo, J. Sumitani, H. S. Judelson and T. Kawaguchi: Chemical genetic approach using β-rubromycin reveals that a RIO kinase-like protein is involved in morphological development in *Phytophthora infestans*. *Sci. Rep.* **10**, 22326 (2020).
12) T. Umeyama, Y. Tanabe, B. D. Aigle and S. Horinouchi: Expression of the *Streptomyces coelicolor* A3(2) *ptpA* gene encoding a phosphotyrosine protein phosphatase leads to overproduction of secondary metabolites in *S. lividans*. *FEMS Microbiol. Lett.* **144**, 177–184 (1996).
13) T. Wakabayashi, B. Joseph, S. Yasumoto, T. Akashi, T. Aoki, K. Harada, S. Muranaka, T. Bamba, E. Fukusaki, Y. Takeuchi, K. Yoneyama, T. Muranaka, Y. Sugimoto and A. Okazawa: Planteose as a storage carbohydrate required for early stage of germination of *Orobanche minor* and its metabolism as a possible target for selective control. *J. Exp. Bot.* **66**, 3085–3097 (2015).
14) U. Edwards, T. Rogall, H. Blöcker, M. Emde and E. C. Böttger: Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* **17**, 7843–7853 (1989).
15) M. J. Bibb: Understanding and manipulating antibiotic production in atinomycetes. *Biochem. Soc. Trans.* **41**, 1355–1364 (2013).
16) A. F. A. Moumbock, M. Gao, A. Qaseem, J. Li, P. A. Kirchner, B. Ndingkokhar, B. D. Bekono, C. V. Simoben, S. B. Babiaka, Y. I. Malange, F. Sauter, P. Zierep, F. Ntie-Kang and S. Gunther: StreptomeDB 3.0: An updated compendium of *Streptomycetes* natural products. *Nucleic Acids Res.* **49**(D1), D600–D604 (2021).
17) F. Carnevale Neto, T. N. Clark, N. P. Lopes and R. G. Linington: Evaluation of ion mobility spectrometry for improving constitutional

assignment in natural product mixtures. *J. Nat. Prod.* **85**, 519–529 (2022).
18) M. Blumauerová and J. Stárka: Reversal of antibiotic action of cycloheximide (actidione) by bivalent metal ions. *Nature* **183**, 261 (1959).
19) A. J. Whiffen: The production, assay, and antibiotic activity of actidione, an antibiotic from *Streptomyces griseus*. *J. Bacteriol.* **56**, 283–291 (1948).
20) L. Rajjou, K. Gallardo, I. Debeaujon, J. Vandekerckhove, C. Job and D. Job: The effect of α-amanitin on the *Arabidopsis* seed proteome highlights the distinct roles of stored and neosynthesized mRNAs during germination. *Plant Physiol.* **134**, 1598–1613 (2004).
21) Y. Tsuchiya, M. Yoshimura, Y. Sato, K. Kuwata, S. Toh, D. Holbrook-Smith, H. Zhang, P. McCourt, K. Itami, T. Kinoshita and S. Hagihara: Probing strigolactone receptors in *Striga hermonthica* with fluorescence. *Science* **349**, 864–868 (2015).
22) J. Jendrisak: The use of α-amanitin to inhibit *in vivo* RNA synthesis and germination in wheat embryos. *J. Biol. Chem.* **255**, 8529–8533 (1980).
23) M. N. Miranda, C. A. Masuda, A. Ferreira-Pereira, E. Carvajal, M. Ghislain and M. Montero-Lomeli: The serine/threonine protein phosphatase Sit4p activates multidrug resistance in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **10**, 674–686 (2010).
24) N. Garreau de Loubresse, I. Prokhorova, W. Holtkamp, M. V. Rodnina, G. Yusupova and M. Yusupov: Structural basis for the inhibition of the eukaryotic ribosome. *Nature* **513**, 517–522 (2014).
25) A. M. McGeachy, Z. A. Meacham and N. T. Ingolia: An accessible continuous-culture turbidostat for pooled analysis of complex libraries. *ACS Synth. Biol.* **8**, 844–856 (2019).
26) A. J. Whiffen, N. Bohonos and R. L. Emerson: The production of an antifungal antibiotic by *Streptomyces griseus*. *J. Bacteriol.* **52**, 610–611 (1946).
27) C. Palmer and T. E. Maloney: Preliminary screening for potential algicides. *Ohio J. Sci.* **55**, 1–8 (1955).
28) J. B. Loefer and T. S. Matney: Growth inhibition of free-living protozoa by actidione. *Physiol. Zool.* **25**, 272–276 (1952).
29) J. H. Ford, W. Klomparens and C. L. Hamner: Cycloheximide (actidione) and its agricultural uses. *Plant Dis. Rep.* **42**, 680–695 (1958).
30) D. Kerridge: The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in *Saccharomyces carlsbergensis*. *J. Gen. Microbiol.* **19**, 497–506 (1958).
31) M. R. Siegel and H. D. Sisler: Site of action of cycloheximide in cells of *Saccharomyces pastorianus*. I. effect of the antibiotic on cellular metabolism. *Biochim. Biophys. Acta* **87**, 70–82 (1964).
32) J. H. Grenz, A. M. Manschadi, P. DeVoil, H. Meinke and J. Sauerborn: Assessing strategies for *Orobanche* sp. control using a combined seedbank and competition model. *Agron. J.* **97**, 1551–1559 (2005).
33) M. Zielinska, P. Zapotoczny, I. Białobrzewski, K. Zuk-Golaszewska and M. Markowski: Engineering properties of red clover (*Trifolium pratense* L.) seeds. *Ind. Crops Prod.* **37**, 69–75 (2012).
34) D. J. Katzmann, P. E. Burnett, J. Golin, Y. Mahé and W. S. Moye-Rowley: Transcriptional control of the yeast PDR5 gene by the PDR3 gene product. *Mol. Cell. Biol.* **14**, 4653–4661 (1994).
35) U. M. Gerlinger, R. Gückel, M. Hoffmann, D. H. Wolf and W. Hilt: Yeast cycloheximide-resistant *crl* mutants are proteasome mutants defective in protein degradation. *Mol. Biol. Cell* **8**, 2487–2499 (1997).
36) A. M. Middleton, J. R. King, M. J. Bennett and M. R. Owen: Mathematical modelling of the Aux/IAA negative feedback loop. *Bull. Math. Biol.* **72**, 1383–1407 (2010).
37) J. Hanna, D. S. Leggett and D. Finley: Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. *Mol. Cell. Biol.* **23**, 9251–9261 (2003).
38) H. Ishihara, T. Obata, R. Sulpice, A. R. Fernie and M. Stitt: Quantifying protein synthesis and degradation in *Arabidopsis* by dynamic $^{13}CO_2$ labeling and analysis of enrichment in individual amino acids in their free pools and in protein. *Plant Physiol.* **168**, 74–93 (2015).
39) I. Scheurwater, M. Dünnebacke, R. Eising and H. Lambers: Respiratory costs and rate of protein turnover in the roots of a fast-growing (*Dactylis glomerata* L.) and a slow-growing (*Festuca ovina* L.) grass species. *J. Exp. Bot.* **51**, 1089–1097 (2000).
40) G. W. Bassel, P. Fung, T. F. Chow, J. A. Foong, N. J. Provart and S. R. Cutler: Elucidating the germination transcriptional program using small molecules. *Plant Physiol.* **147**, 143–155 (2008).
41) A. R. Kermode: Role of abscisic acid in seed dormancy. *J. Plant Growth Regul.* **24**, 319–344 (2005).
42) I. A. Tarchevsky, M. V. Ageeva, N. V. Petrova, A. N. Akulov and A. M. Egorova: Cycloheximide-induced phenolic burst in roots of *Pisum sativum* L. *Appl. Biochem. Microbiol.* **53**, 568–572 (2017).
43) C. Liu, H. Yu, A. Voxeur, X. Rao and R. A. Dixon: FERONIA and wall-associated kinases coordinate defense induced by lignin modification in plant cell walls. *Sci. Adv.* **9**, eadf7714 (2023).