Figures and figure supplements

An evolutionarily young defense metabolite influences the root growth of plants via the ancient TOR signaling pathway

Frederikke Gro Malinovsky et al
**Figure 1.** 3OHP reversibly inhibits root growth. (A) 7-d-old seedlings grown on MS medium supplemented with a concentration gradient of 3OHP. (B) Quantification of root lengths of 7-d-old. Results are averages ± SE (n = 3–7; p<0.001). (C) Accumulation of 3OHP in shoots/areal tissue of 10-d-old Col-0 wildtype seedlings grown on MS medium supplemented with 5 µM 3OHP. Results are least squared means ± SE over three independent experimental replicates with each experiment having an average eleven replicates of each condition (n = 31–33; ANOVA P_{Treat} < 0.001). (D) Accumulation of 3OHP in shoots of 10-d-old myb28 myb29 seedlings (aliphatic GSL-free) grown on MS medium supplemented with 5 µM 3OHP. Results are least squared means ± SE over two independent experimental replicates with each experiment having an average of four independent biological replicates of each condition (n = 8–14; ANOVA P_{Treat} < 0.001). (E) 14-d-old seedlings grown for 1 week with or without 3OHP as indicated. After one week of development, the plants were moved to the respective conditions showed in week 2.

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Figure 1—figure supplement 1. Root inhibition is affected by endogenous GSL levels. Results are least squared means ± SE over three independent experimental replicates with each experiment having an average of ten replicates per condition (n = 8–39). (A) Root growth for seedlings grown on MS medium supplemented with or without 5 µM 3OHP. Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v myb28myb29), Treatment (Control v 3OHP) and their interaction on root length. The ANOVA results from each day are presented in the table. (B) Root lengths in response to 3OHP (from A) displayed at each time point as relative to the untreated.

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Figure 2. Root growth is not inhibited by all aliphatic GSLs. (A) The aliphatic glucosinolate biosynthetic pathway, from the C3 3-methyl-sulphinyl-propyl (3MSP) to the secondary modified 3-hydroxy-propyl (3OHG) and 2-propenyl (allyl/sinigrin). (B–C) Root lengths of 7-d-old Col-0 wildtype seedlings grown on MS medium supplemented with a concentration gradient of the indicated aliphatic C3-GSL. The left most point in each plot shows the root length grown in the absence of the specific GSL treatment. Results are least squared means \( \pm \) SE over four independent experimental replicates with each experiment having an average of 21 replicates per condition (\( n_{3MSP} = 59–153; n_{Allyl} = 52–153 \)). Significance was determined via two-way ANOVA combining all experiments. (D) 7-d-old seedlings grown on MS medium with or without 50 \( \mu \)M of the indicated GSL. (E) The aliphatic glucosinolate biosynthetic pathway from the C4 4-methyl-sulphinyl-butyl (4MSB) to But-3-enyl. (F–G) Root lengths of 7-d-old Col-0 wildtype seedlings grown on MS medium supplemented with a concentration gradient of the indicated aliphatic C4-GSL. The left most point in each plot shows the root length grown in the absence of the specific GSL treatment. Least squared means \( \pm \) SE over four independent experimental replicates with each experiment having an average of 22 replicates condition (\( n_{4MSB} = 38–153; n_{But-3-enyl} = 68–164 \)). Significance was determined via two-way ANOVA combining all experiments. (H) 7-d-old seedlings grown on MS medium with or without 50 \( \mu \)M of the indicated GSL.

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Figure 2—figure supplement 1. Root lengths of 7 DAG Col-0 WT grown on MS media supplemented with 50 μM of the indicated GSL. Progoitrin = R enantiomer of 2-hydroxybut3-enyl GSL and Epiprogoitrin is the S enantiomer. Results were obtained in two fully independent experiments and tested with ANOVA. LSmeans are shown with letters showing treatments with statistical differences following a Tukey’s post-hoc t-test. The samples sizes are 97 seedlings for control, 152 for 3OHP, 160 for progoitrin and 94 for epiprogoitrin. LSmeans and SE are plotted.

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Figure 3. Conservation of 3OHP responsiveness suggests a evolutionally conserved target. (A) Stylized phylogeny showing the phylogenetic relationship of the selected plants from the Brassicales family, branch lengths are not drawn to scale. (B–F) plants from the Brassicales family, grown on Figure 3 continued on next page.
Figure 3 continued

MS medium supplemented with or without 5 µM 3OHP. (G) Stylized phylogeny showing the phylogenetic relationship of all the selected crop and model plants, branch lengths are not drawn to scale. (H–L) Root growth of plants from diverse eudicot lineages, grown on MS medium supplemented with or without 5 µM 3OHP. Results are least squared means ± SE for each species using the following number of experiments with the given biological replication. Camelina: three independent experimental replicates (n_{ctrl} = 6 and n_{3OHP} = 12). Rucola: three independent experimental replicates (n_{ctrl} = 17 and n_{3OHP} = 17). Cress: three independent experimental replicates (n_{ctrl} = 19 and n_{3OHP} = 18). Rape: seed four independent experimental replicates (n_{ctrl} = 14 and n_{3OHP} = 13). Broccoli: three independent experimental replicates (n_{ctrl} = 10 and n_{3OHP} = 13). Lotus: three independent experimental replicates (n_{ctrl} = 10 and n_{3OHP} = 10). Linseed: three independent experimental replicates (n_{ctrl} = 11 and n_{3OHP} = 11). Dill: three independent experimental replicates (n_{ctrl} = 14 and n_{3OHP} = 13). Oregano: four independent experimental replicates (n_{ctrl} = 40 and n_{3OHP} = 39). Tomato: three independent experimental replicates (n_{ctrl} = 11 and n_{3OHP} = 15). A significant effect of treatment on the various species was tested by two-way ANOVA combining all the experimental replicates in a single model with treatment as a fixed effect and experiment as a random effect.

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Figure 3—figure supplement 1. Yeast response to 3OHP suggests a conserved target throughout eukaryotes. Yeast growth in YPD media supplemented with none or increasing levels of 3OHP or Allyl. The hourly OD$_{600}$ increase is plotted against each concentration of either Allyl or 3OHP. The least squared means ±SE over four replicates are presented (n = 4). ANOVA was utilized to test for a significant effect of GLS treatment individually for each concentration of 3OHP and Allyl.

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**Figure 4.** 3OHP reduces root zone sizes. (A) Diagrammatic organization of a root tip; the meristem zone from the QC to the first cell elongation; the elongation zone ends when first root hair appears (Dolan and Davies, 2004). (B) Meristem size of 4-d-old Arabidopsis seedlings grown on MS medium with sucrose ±10 μM 3OHP. Results are least squared means ± SE over three independent experimental replicates with each experiment having an average of three replicates per condition (n_{ctrl} = 6; n_{3OHP}=9). Significance was tested via two-way ANOVA with treatment as a fixed effect and experiment as a random effect. (C) Confocal images of 4-d-old propidium iodide stained seedlings grown with and without 3OHP. Meristematic cells are marked with white asterisks, elongated cells with blue asterisks. (D) Appearance of first root hair, measured from the root tip on 4-d-old seedlings grown on MS medium with sucrose ±10 μM 3OHP. Results are least squared means ± SE over two independent experimental replicates with each experiment having an average of nine replicates per condition (n_{ctrl} = 17; n_{3OHP}=20). Significance was tested via two-way ANOVA with treatment as a fixed effect and experiment as a random effect. (E) Confocal images of 4-d-old propidium iodide stained seedlings grown with and without 3OHP. Protruding root hairs are marked with white/black asterisks. (F) 3OHP induced root hair deformations, confocal images of 4-d-old propidium iodide stained seedlings grown with 3OHP.

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Figure 5. TOR over-activation amplifies 3OHP response. (A) Root growth for low light grown seedlings. The seedlings were grown on MS medium without sucrose for 3 days, then transferred to the indicated media (Suc; sucrose). Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v TORox), Treatment (Control v Sucrose) and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (B) The root lengths grown photo-constrained and without sucrose (from A) displayed at each time point as relative to the respective sucrose activated roots. Results least squared means ± SE over three independent experimental replicates with each experiment having an average of nine replicates per condition (n = 26–30). Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v TORox), Treatment (Sucrose v Sucrose/3OHP) and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (C) Root growth for low light grown seedlings. (D) Photo-constrained root lengths in response to sucrose and 3OHP (from A) displayed at each time point as relative to the respective sucrose activated roots. Results are least squared means ± SE over two independent experimental replicates with each experiment having an average of six replicates per condition (n = 11–14). (E) Schematic model; over expression of the catalytic subunit TOR increases growth and the relative 3OHP response.

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Figure 5—figure supplement 1. Published TORox lines that did not display the TORox phenotype under our conditions. Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v specific TORox lines) on root length. All experiments were combined in the model and experiment treated as a random effect. There were no significant differences found. (A) Root growth for the published TORox line G166 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 μM 3OHP. Results are least squared means ± SE (n = 8–16). (B) Root growth for the published TORox line S7846 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 μM 3OHP. Results are least squared means ± SE ns across three biological repeats (n = 35–45). (C) Root growth for the published TORox line S7817 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 μM 3OHP. Results are least squared means ± SE (n = 10–24).
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Figure 5—figure supplement 2. RAPTOR1 haplo-insufficiency does not affect 3OHP response. (A) Root growth for heterozygous raptor1-2 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 mM 3OHP. Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v raptor1-2), Treatment (Control v 3OHP) and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (B) Root lengths in response to 3OHP (from A) displayed at each time point as relative to untreated. Results are least squared means ± SE over three independent experimental replicates with each experiment having an average of six replicates per condition (n = 16–19). (C) Root growth for heterozygous raptor1-2 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 mM 3OHP. Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v raptor1-1), Treatment (Control v 3OHP) and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (D) Root lengths in response to 3OHP (from C) displayed at each time point as relative to untreated. Results are least squared means ± SE over three independent experimental replicates with each experiment having an average of seven replicates per condition (n = 16–24). (E) Gene structure and T-DNA insertion sites for RAPTOR1.
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Figure 5—figure supplement 3. Loss of one of the two substrate-binding TORC-subunits affect 3OHP response. (A) Root growth for raptor2-1 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 μM 3OHP. Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v raptor2-1), Treatment (Control v 3OHP) and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (B) Root lengths in response to 3OHP (from A) displayed at each time point as relative to untreated. Results are least squared means ± SE over four independent experimental replicates with each experiment having an average of thirteen replicates per condition (n = 36–68). (C) Root growth for raptor2-2 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 μM 3OHP. Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v raptor2-1), Treatment (Control v 3OHP) and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (D) Root lengths in response to 3OHP (from C) displayed at each time point as relative to untreated. Results are least squared means ± SE over three independent experimental replicates with each experiment having an average of nineteen replicates per condition (n = 44–70). (E) Gene structure and T-DNA insertion sites for RAPTOR2. (F) Schematic model; loss of one of the substrate-binding subunits RAPTOR2 decreases growth, and the relative 3OHP response.

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Figure 6. 3OHP dampens sugar-mediated meristem activation. (A) Root growth for low light grown Col-0 wildtype seedlings. The seedlings were grown on MS medium without sucrose for 3 days, then transferred to the indicated media. Multi-factorial ANOVA was used to test the impact of Treatment on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (B) Schematic model; sucrose activates the TOR complex (TORC), leading to growth. (C) The root lengths (from A) displayed at each time point as relative to sucrose activated roots. Results are least squared means ± SE over five independent experimental replicates with each experiment having an average of eight replicates per condition (n_{+Suc} = 40; n_{-Suc} = 43). (D) Root growth for low light grown seedlings. The seedlings were grown on MS medium without sucrose for 3 days, then transferred to the indicated media. Multi-factorial ANOVA was used to test the impact of Treatment on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (E) The root lengths (from D) displayed at each time point as relative to sucrose activated roots (ctrl). Results are least squared means ± SE over two independent experimental replicates with each experiment having an average of seven replicates per condition (n = 12–16).

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Figure 7. Pharmacological interaction of 3OHPGSL and the TOR inhibitor, AZD. (A) Root lengths of 7-d-old Col-0 wildtype seedlings grown on MS medium with sucrose ± combinations of AZD and different concentrations of 3OHP. (B) Transition elongation to differentiation zone. (C) Images showing root tip morphology with 3OHP and AZD treatments. (D) Additional images illustrating root tip responses to different treatments. Figure 7 continued on next page.
Figure 7 continued

3OHP. Results are least squared means ± SE over three independent experimental replicates with each experiment having an average of nine replicates per condition (n = 18–58). Multi-factorial ANOVA was used to test the impact of the two treatments and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (B) Appearance of first root hair; measured from the root tip on 4–d-old seedlings grown on the indicated MS medium with sucrose. Results are least squared means ± SE over two independent experimental replicates with each experiment having an average of nine replicates per condition (n = 17–20). Multi-factorial ANOVA was used to test the impact of the two treatments and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (C) Confocal images of 4-d-old propidium iodide stained seedlings. The first protruding root hairs are marked with white/black asterisks on the left panel. Right panel shows zooms of first root hair, cell size is indicated. (D) Confocal images of 4-d-old propidium iodide stained seedlings.

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Figure 7—figure supplement 1. Pharmacological interactions between 3OHPGSL and diverse TOR inhibitors. (A) Root lengths of 7-d-old Col-0 wildtype seedlings grown on MS medium with sucrose ± the indicated mTOR or S6K inhibitors. (B) Imaging of root meristems under indicated conditions.
Figure 7—figure supplement 1 continued

Inhibitors. Results are averages ± SE (n = 8–41). (B) Confocal images of 4-d-old propidium iodide stained seedlings. Meristematic cells are marked with white asterisks, elongated cells with blue, and cells belonging to the differentiation zone are marked with purple asterisks. Arrows indicate approximate meristem sizes.

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Figure 8. Blocking autophagosome elongation amplifies the 3OHP response. (A) Root growth for atg5-1 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 µM 3OHP. Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v atg5-1), Treatment (Control v 3OHP) and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (B) Root lengths in response to 3OHP (from A) displayed at each time point as relative to untreated. Results are least squared means ± SE over two independent experimental replicates with each experiment having an average of 21 replicates per condition (n = 31–52). (C) Root growth for atg2-1 and wildtype Col-0 seedlings grown on MS medium supplemented with or without 5 µM 3OHP. Multi-factorial ANOVA was used to test the impact of Genotype (Col-0 v atg5-1), Treatment (Control v 3OHP) and their interaction on root length. All experiments were combined in the model and experiment treated as a random effect. The ANOVA results from each day are presented in the table. (D) Root lengths in response to 3OHP treatment (from C) displayed at each time point as relative to untreated. Results are least squared means ± SE over two independent experimental replicates with each experiment having an average of 26 replicates per condition (n = 36–66). (E) The TOR complex (TORC), is affected by several upstream input, leading to activation or repression of several downstream pathways. (F) Schematic model; Figure 8 continued on next page
sucrose activates TORC, leading to root growth. 3OHP represses root growth through interaction with TORC. Autophagy pathways via ATG5 negatively affect 3OHP response.
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